Critical Genes Regulated by FoxO Transcription Factors in Life Span Control

by

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Dedication

To my parents,

and everyone else who

gave me the opportunity

to do something worthwhile.

Acknowledgements

I have been given the wonderful opportunity to constantly learn and apply wisdom from others throughout my PhD. My advisor Patrick Hu ingrained in me the habit of knowing and articulating the advantages and disadvantages of every experimental method. This was critical during my PhD to decide what important findings in the literature needed clarification, how to clarify them, and how to properly support new discoveries that are worth caring about. More importantly, Patrick gave me the right combination of autonomy and high expectations to foster a highly rewarding period of intellectual growth, where I could continually learn from any person or resource.

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Preface

This dissertation is part of my experience at the University of Michigan's combined MD/PhD program. The exceptionally long training period for this career path necessitates a philosophy of long-term thinking. I was struck by learning about 100-year-olds who still work hard today to make the world a better place, employing their century of accumulated wisdom, skills, and experience to help others. This contrasts with the wonderful elderly patients I have met in the hospital, who have multiple co-morbidities and progressively require more and more assistance. Why can't everyone be like the first group? Aging research may yield incredible preventative medicine, and I was particularly motivated to write this thesis because the promise will only be fully realized in the long term.

This thesis is comprised of multiple convergent strategies to identify DAF-16/FoxO target genes that are critical for life span extension. Design and conception of most experiments was performed independently under the guidance of Dr. Patrick Hu. Many genetic crosses and replicates of most life span experiments were performed by Chunfang Guo. Bioinformatics for Chapters 3 and 4 was performed by Ana Rodrigues, Richard McEachin, and Manjusha Pande. For Chapter 4, Shohei Mitani and Sawako Yoshina (Tokyo Women's Medical University) generated the *daf-16/FoxO* isoform-specific mutants, and Christopher Hopkins and Knudra Transgenics generated the transgenes. Analysis of large quantities of life span data was made possible by Mallory Freeberg. Omar Itani proposed the original idea to use *daf-2* RNAi to screen mutants in Chapter 5. Screening of DAF-16/FoxO target genes in Chapter 5 was performed in collaboration with Breane Budaitis, Ian Waters, and Joe Kruempel.

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Chapter 1 Introduction

Aging: FoxO transcription factors as interventions

Aging is broadly understood as biological changes that occur with time and lead to progressive functional impairment, loss of homeostasis, and risk of mortality. Aging is a top risk factor for major causes of human morbidity and mortality, including cardiovascular disease, dementia, type 2 diabetes, osteoporosis, autoimmune disease, and cancer [1]. It increases susceptibility to disability and infection, and limits ability to perform activities of daily living. Modern technology, medicine, public health initiatives and lifestyle have increased life expectancy up to 30 years in many countries during the 20th century [2]. However, the continuing expansion of the elderly population has been accompanied by the expected burden of age-related disease.

Our understanding of aging has been revolutionized by the discovery that single gene mutations can dramatically extend the life span of the nematode *Caenorhabditis elegans* (up to 10-fold) [3]. It is now known that these findings translate to mammals and potentially humans. The fact that aging is under genetic control makes it possible to treat or prevent age-related disease by manipulating gene function pharmacologically. As proof of principle, drugs such as rapamycin extend life span and slow age-related changes in mice [4,5]. Importantly, many interventions that extend longevity in model organisms delay age-related disease and slow functional decline [6].

FoxO transcription factors have emerged as major candidates to understand the causes, processes and consequences of aging. Identification of specific FoxO activities that affect aging may yield treatments to prevent and ameliorate disease, as well as improve quality of life for an aging population.

FoxO transcription factors

FoxO transcription factors (TFs) comprise a subclass of the forkhead superfamily, which regulates gene expression, is defined by a ~100 amino-acid winged helix DNA binding domain, and is conserved in all metazoans [7]. Humans and other mammals possess four FoxO transcription factors encoded by separate genes: FoxO1, FoxO3, FoxO4, and FoxO6 [8]. The model organisms *Drosophila melanogaster* and *C. elegans* each possess single FoxO orthologs, termed dFoxO and DAF-16/FoxO respectively [9].

FoxO transcription factors are homeostatic regulators [10], responding to numerous signals concerning nutrient and energy availability, stress, and growth requirements. FoxO proteins integrate these signals to coordinate many gene expression programs for cellular survival, stress resistance, metabolism, maintenance, and proliferation. These allow organisms to grow, develop, and reproduce in a manner appropriate to the organism's circumstances [10].

The functions of FoxO TFs are highly varied and context-dependent. For example, FoxO can promote either cell survival or cell death depending on the precise conditions of growth factor signaling [11]. The pleiotropy and complexity of FoxO TFs makes them attractive targets for manipulating complex biological processes to treat disease, but this requires a fundamental understanding of how FoxO controls distinct and sometimes opposing genetic programs.

Role of FoxO and insulin/IGF signaling (IIS) in longevity control

FoxO transcription factors are negatively regulated in a conserved manner by insulin and insulin-like growth factor (IGF) signaling (IIS) via a phosphoinositide 3-kinase and protein kinase B (PI3K/Akt) phosphorylation cascade [12]. IIS control of FoxO and longevity was initially discovered by work in the nematode *C. elegans*, and is now a major subfield of mammalian insulin and IGF biology (Figure 1.1).

A single mutation in the *C. elegans daf-2* gene doubles life span and slows age-related decline, effects that are completely reversed by a single mutation in another gene, *daf-16* [13]. Cloning revealed that *daf-2* is homologous to mammalian insulin and IGF receptors [14], and *daf-16* is homologous to mammalian FoxO transcription factors [15,16]. These *daf-2/IGFR* and *daf-16/FoxO* mutations reduce or eliminate gene function, and therefore reduced insulin/IGF signaling (IIS) extends life span dependent on FoxO.

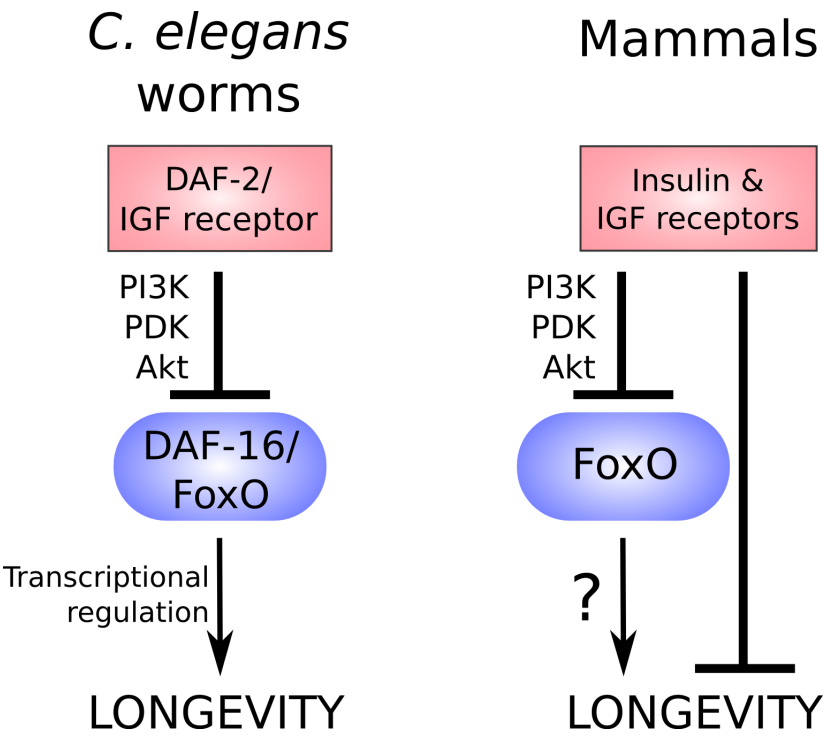
Reduced IIS influences life span in a conserved manner across animal species. Mutations in the *Drosophila* insulin-like receptor pathway extend life span up to 85% [17,18]. Approximately a dozen studies have examined the longevity of mice with mutations affecting the IGF-1 or insulin signaling pathways, or the growth hormone signaling pathway that regulates IGF-1 production [19]. Consistent with findings in invertebrates, most of these studies showed that reduced IIS extends life span. There is also some evidence from human candidate gene association studies that the IIS pathway influences longevity [19]. In particular, mutations that impair IGF receptor function are associated with extreme longevity in Ashkenazi Jewish populations [20]. The precise mechanisms by which IIS influences mammalian longevity have yet to be elucidated, but dysregulated IIS has been linked to type 2 diabetes, cardiovascular disease, cancer, and dementia [6].

FoxO plays a crucial and conserved role in life span extension due to IIS reduction. Similar to *C. elegans* DAF-16/FoxO, the *Drosophila* FoxO ortholog is required for the longevity of IIS mutants [21]. DAF-16/FoxO mediates the majority of gene expression changes resulting from *daf-2/IGFR* mutation, and specific genes regulated by DAF-16/FoxO influence life span [22,23]. Accordingly, overexpression of FoxO extends life span in both worms and flies [24,25]. Therefore, DAF-16/FoxO is not simply a permissive factor, but indeed plays an active and causative role in life span extension.

FoxO is implicated in human longevity, given that IIS influences mammalian aging, mechanisms of FoxO regulation by IIS are highly conserved, and FoxO mediates IIS mutant longevity in invertebrates. However, a causative role for FoxO in mammalian longevity has not been strictly proven. At present, the strongest evidence for involvement

of FoxO comes from candidate gene association studies, where human FoxO polymorphisms have been linked to longevity in multiple and diverse human cohorts [26–29]. Some studies also found associations with improved cardiovascular health, lower incidence of cancer, and improved insulin sensitivity. Regardless, FoxO possesses diverse functions in mammals. Uncovering the mechanisms by which FoxO promotes longevity in model organisms should narrow the search for candidate mechanisms that may be active in humans.

Figure 1.1: Insulin and insulin-like signaling (IIS) controls FoxO transcription factors and longevity in a conserved manner.



Role of FoxO in mammalian biology and age-related disease

The initial discovery in *C. elegans* that FoxO transcription factors are regulated by IIS [15,16] has motivated intensive study of FoxO's conserved role in mammalian IIS and numerous other biological processes [30]. Animal models and human clinical studies implicate dysregulation of FoxO transcription factors in age-related pathologies, including osteoporosis, cancer, type 2 diabetes, and more general loss of tissue integrity [31].

Deletion of FoxO1, FoxO3, and FoxO4 together (FoxO1/3/4) in adulthood increases mouse osteoblast apoptosis resulting in loss of bone mass, implying FoxO activity can protect against osteoporosis [32]. Loss of FoxO1/3/4 in mice also causes widespread formation of metastatic cancer, especially lymphomas and hemangiomas [33]. Furthermore, FoxO acts as a tumor suppressor in human breast cancer, renal cell carcinoma, stomach cancer, and others [34–37]. Despite these desirable influences on osteoporosis and cancer, a few studies have identified potentially detrimental roles for FoxO in these diseases [38,39], suggesting that manipulation of FoxO activity must be done in a targeted manner.

FoxO activation also appears to be pathogenic in the context of diabetes. FoxO1 haploinsufficiency in the liver reverses the diabetes-like phenotype caused by *InsR* (insulin receptor) mutation and high-fat diet [40,41], and restores deficient pancreatic beta cell proliferation in *Irs2* (insulin-receptor substrate 2) mutants [42]. Thus, FoxO1 can contribute to metabolic and cellular derangements of diabetes.

Mouse studies suggest that FoxO transcription factors may protect against age-related disease by promoting tissue homeostasis [43], which normally declines with age resulting in inappropriate cell proliferation and reduced regeneration [44]. FoxO1/3/4 loss in the hematopoietic system causes depletion of hematopoietic stem cells and over-proliferation of immune cells [45]. A similar effect on neural stem cells and brain size is observed upon deletion of FoxO1/3/4 in the central nervous system [32]. FoxO3 appears to be particularly important for tissue homeostasis, as whole-body knockout of FoxO3 alone

causes early infertility due to premature ovarian failure, widespread organ inflammation secondary to immune cell abnormalities, and depletion of neural stem cells [46–48].

In summary, both protective and pathogenic effects of FoxO have also been described in age-related phenotypes. Even if FoxO does not influence longevity *per se* in humans, FoxO transcription factors are excellent candidates for intervening in age-related disease. However, the complexity of FoxO biology demonstrates the need to dissect specific outputs of FoxO transcription factors so that therapies can selectively promote beneficial activities of FoxO.

C. elegans: model organism for FoxO control of longevity

The initial discovery of FoxO's role in longevity control and its regulation by IIS was greatly facilitated by advantages of the *C. elegans* model system (Figure 1.2). In particular, it is easy to manipulate multiple genes simultaneously and measure life span, and these advantages help to further dissect the mechanism of life span extension by FoxO.

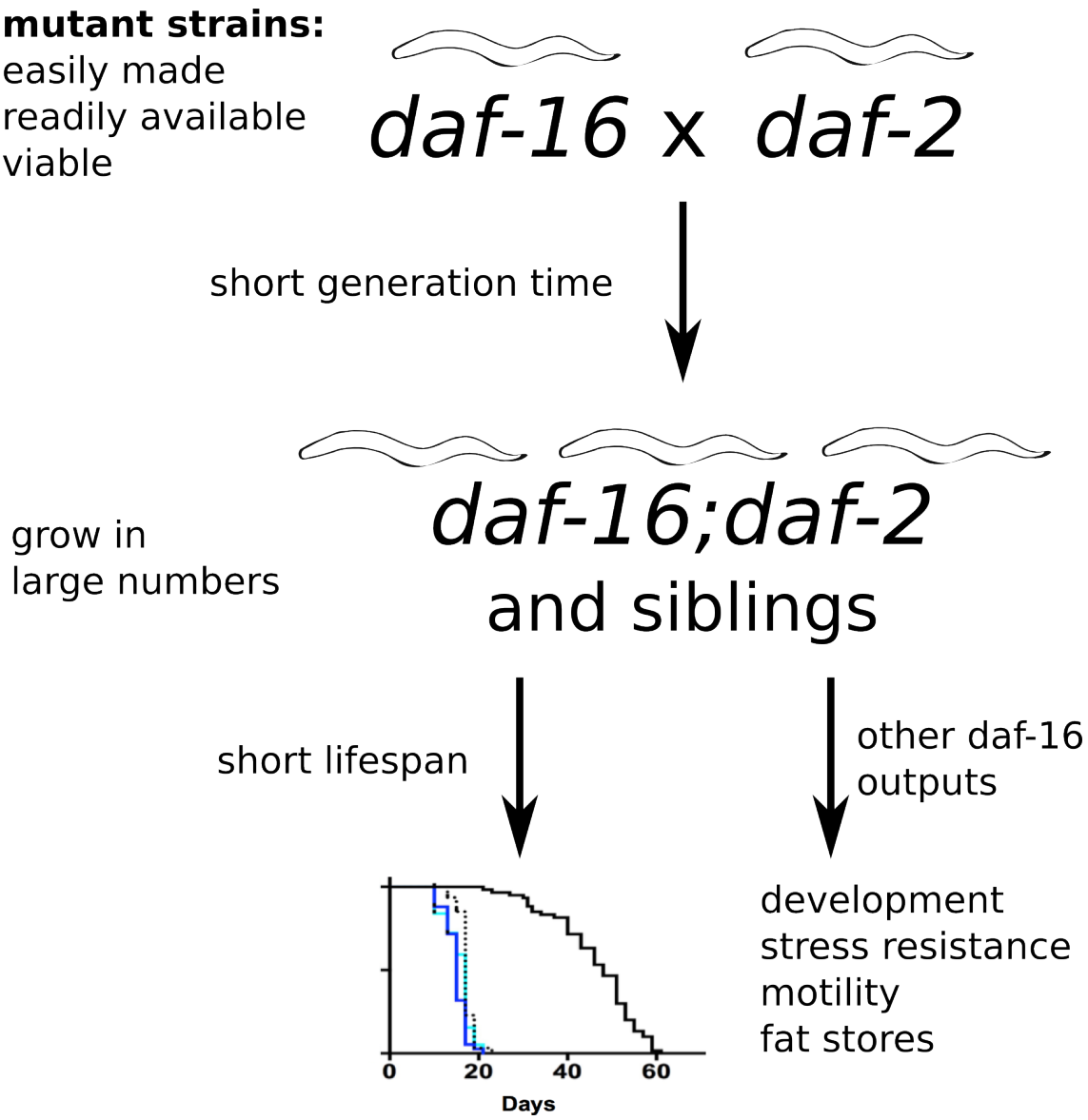
Mutating genes in *C. elegans* to investigate function is easy, rapid and cheap, facilitating screening and analysis of large numbers of genes. The *C. elegans* genome harbors an estimated 20,470 protein-coding genes, and many variants (alleles) of nearly all those genes have already been isolated from mutagenesis screens and are readily available to researchers. Alternatively, gene function can be reduced by RNA interference (RNAi) simply by feeding RNAi to animals [49].

Rapid breeding of *C. elegans* worms harboring multiple mutations is enabled by powerful genetic tools and short generation time. For assays, large numbers of animals can be easily grown in a synchronized and uniform fashion. Wild-type *C. elegans* (the N2 Bristol strain) live approximately 2-3 weeks, while *daf-2/IGFR* mutants live 6-9 weeks. Therefore, many life span experiments can be conducted in a short period of time. In contrast, many mouse longevity studies report the results of a single cohort of mice due to prohibitive cost and length (2-4 years) of experiments.

The *C. elegans* model system is also particularly advantageous for study of FoxO transcription factors. A single IIS input from *daf-2/IGFR* exists in *C. elegans*, simplifying pathway analysis. Mutant strains of *daf-16*/*FoxO* are viable, while knockout of FoxO1 in mice results in embryonic lethality due to failure of vascular development [50]. DAF-16/FoxO activity influences many other easily assayable traits, including development, stress resistance, motility, and fat stores. Finally, a well-annotated genome and transcriptome facilitates study of FoxO's role as a transcription factor.

C. elegans is an excellent system for identifying and functionally testing specific genes that influence longevity. 35% of *C. elegans* genes have human homologs [51] and are similar enough that many human genes can be introduced and functionally replace their *C. elegans* counterparts. Thus, genes found to influence longevity in *C. elegans* are likely to shed light on their counterparts in humans.

Figure 1.2. Advantages of the C. elegans model system, specifically in the fields of FoxO biology and longevity control.
We can apply this to study of any other gene in longevity control using mutant alleles of that gene in place of *daf-16*.

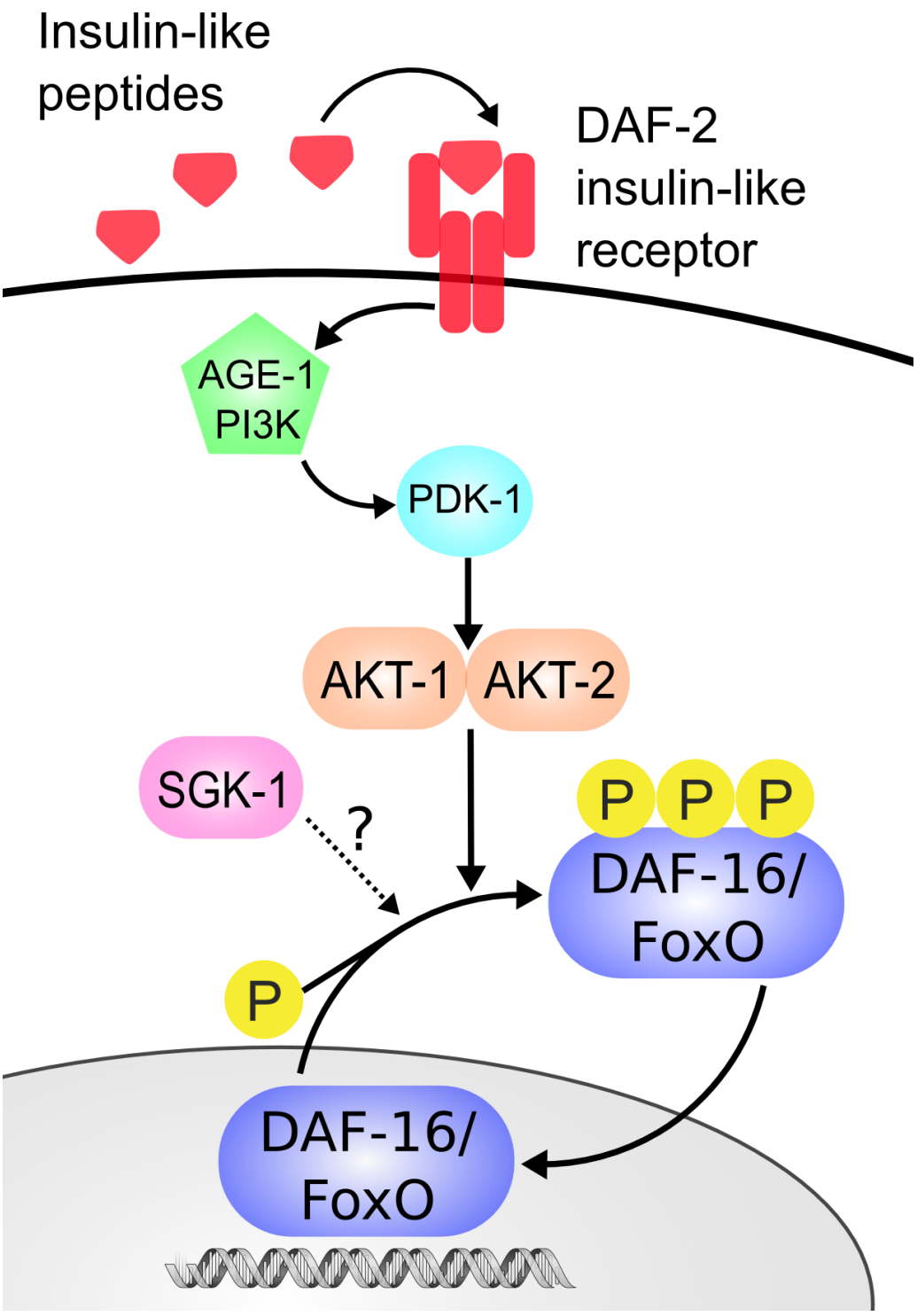


IIS regulation of DAF-16/FoxO in *C. elegans*

The mechanisms by which IIS regulates the FoxO ortholog DAF-16 in *C. elegans* is remarkably similar to those in mammals, with all major components of the IIS pathway functionally and molecularly conserved. Binding of insulin-like peptides to the *C. elegans* insulin-like receptor DAF-2 [14] activates a signaling pathway comprised of the phosphatidylinositol-3-kinase AGE-1 [52], synthesis of phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), the phosphoinositol-dependent kinase PDK-1 [53], and serine-threonine kinases AKT-1 and AKT-2 [54]. These AKT kinases phosphorylate three conserved RxRxxS/T consensus motifs on DAF-16/FoxO. This promotes cytoplasmic sequestration of DAF-16/FoxO [55] enhanced by binding to 14-3-3 proteins [56,57], thus preventing DAF-16/FoxO from regulating transcription of its target genes. Loss-of-function mutations in components of this pathway promote longevity in a DAF-16/FoxO-dependent manner. Thus, DAF-2 insulin-like signaling inhibits the transcriptional activity of DAF-16/FoxO to control longevity (Figure 1.3).

SGK-1 (serum/glucocorticoid regulated kinase) is highly related to AKT kinases and is predicted to inhibit DAF-16/FoxO due to similar substrate specificity (RxRxxS/T motifs). Indeed, mammalian Sgk inhibits FoxO transcriptional activity in cell culture [58], and inactivation of *sgk-1* by RNA interference (RNAi) has been reported to promote DAF-16/FoxO nuclear localization and life span extension in *C. elegans* [59]. However, several recent reports utilizing *sgk-1* mutations [60–62] have called into question the paradigm that SGK-1 inhibits DAF-16/FoxO. It remains an open question as to how SGK-1 influences DAF-16/FoxO and longevity. See Chapter 2 for greater detail.

Figure 1.3. The *C. elegans* insulin-like signaling pathway inhibits DAF-16/FoxO.



Multiple DAF-16/FoxO isoforms with divergent functions

While mammals possess four FoxO isoforms encoded by distinct loci, a single *C. elegans daf-16/FoxO* genomic locus encodes multiple protein isoforms that share C-termini but differ in their transcriptional start sites and N-termini. There are three functional isoforms: DAF-16A, DAF-16B, and DAF-16F [60,63]. See Chapter 4 for greater detail.

DAF-16A and DAF-16F share 428 carboxy-terminal amino acids and are the most similar to human FoxO1, FoxO3, and FoxO4. The forkhead DNA binding domain is highly conserved (76% identity with FoxO1) and accordingly DAF-16A binds the same DNA motif as mammalian FoxO *in vitro* [64]. Key regulatory elements are also conserved, including 14-3-3 binding sites and RxRxxS/T motifs phosphorylated in response to IIS. DAF-16B is more distantly related and differs in a portion of the DNA binding domain. In place of the N-terminal RxRxxT, DAF-16F has a unique QxRxxS motif that is present in FoxO3 and FoxO4 but not FoxO1, suggesting that regulation and function of specific DAF-16/FoxO isoforms may parallel that of specific mammalian FoxO isoforms.

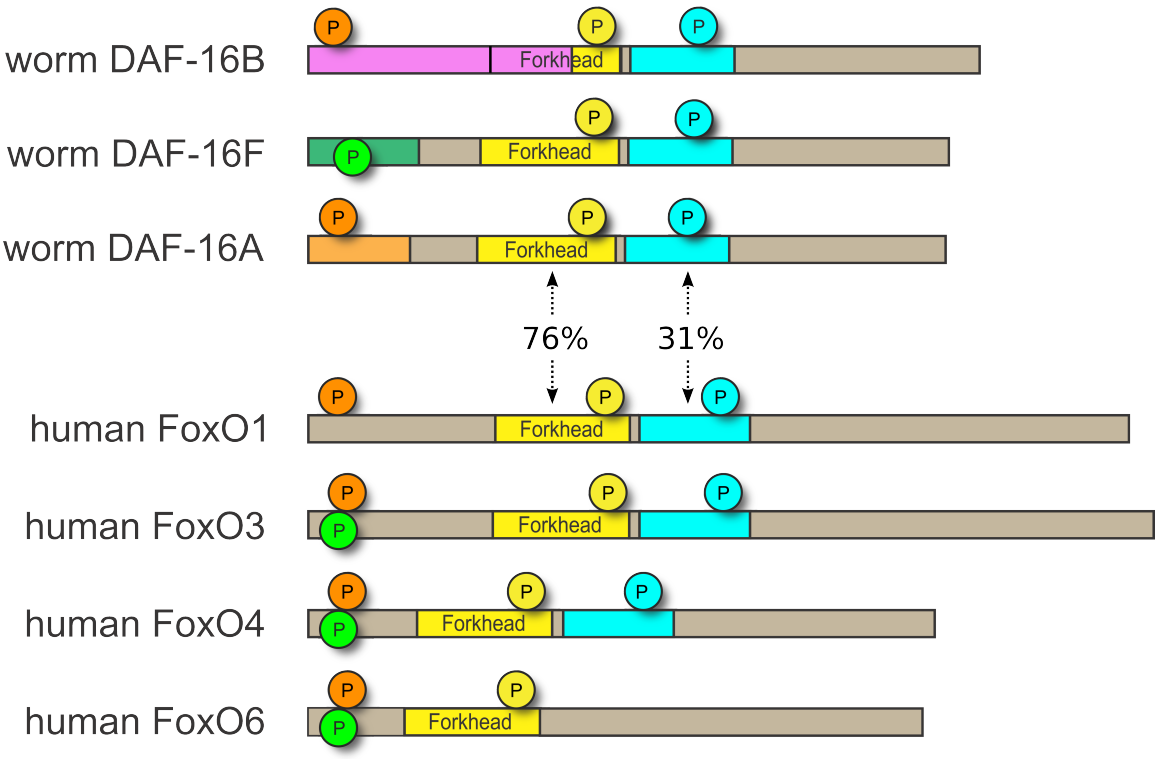
Mammalian FoxO TFs display strongly overlapping expression patterns among tissues, yet show dramatically distinct phenotypes when knocked out individually [31]. However, consistent with significant sequence homology, they also have redundant functions. For example, loss of FoxO1, FoxO3, and FoxO4 in adulthood causes formation of widespread cancer, deletion of one or two FoxO isoforms causes very mild tumorigenic phenotypes [33]. The divergent but overlapping functions of FoxO isoforms in mammals raise the strong possibility that conserved DAF-16/FoxO isoforms also have a mixture of unique and shared functions.

Most data on the distinct functions of DAF-16 isoforms is based on transgenes that over-express the isoforms in animals lacking DAF-16 activity. Experiments indicate distinct tissue expression, regulation by distinct kinases, and differential contributions to longevity, target gene regulation, development, and stress resistance [60,65]. In particular, rescue of either DAF-16A or DAF-16F in animals lacking DAF-16 restores

daf-2/IGFR longevity. DAF-16B does not appear to contribute to longevity, and DAF-16F may play a stronger role than DAF-16A. However, the use of *daf-16* isoform over-expression and lack of isoform-specific mutants limits the interpretation of existing data.

Thus, *C. elegans* isoforms possess distinct but probably overlapping functions. Only two isoforms, DAF-16A and DAF-16F, are capable of promoting longevity [60,66]. Elucidating the endogenous contributions of DAF-16A and DAF-16F may suggest differential contributions of mammalian FoxO to control of longevity and age-related disease.

Figure 1.4: FoxO isoforms in *C. elegans* (worm) and *Homo sapiens* (human).
Pink, green, and orange indicate unique N-termini of DAF-16/FoxO isoforms. "P" indicates phosphorylation sites critical for determination of subcellular localization. Yellow indicates the Forkhead DNA binding domain, the region of greatest homology between *C. elegans* DAF-16 and mammalian FoxO. This domain is 76% identical between DAF-16A and FoxO1. For comparison, the forkhead domain of FoxO1 is 86%, 83%, and 88% identical those of FoxO3, FoxO4, and FoxO6, respectively. Blue indicates the region of 2nd greatest homology, being 31% identical between DAF-16A and FoxO1. For comparison, this region in FoxO1 is 65% and 51% identical to those in FoxO3 and FoxO4, respectively. See text and Chapter 4 for greater detail.



Mechanisms of transcriptional regulation by DAF-16/FoxO

Mammalian FoxO proteins and *C. elegans* DAF-16 bind highly similar sequences to control transcription of target genes. *In vitro*, FoxO1, FoxO3, FoxO4, and DAF-16 all bind the same core consensus sequence TTGTTTAC, named the DAF-16-binding element (DBE) [64], confirmed by crystal structures of FoxO1 and FoxO3 bound to the DBE [67]. Single nucleotide mutations in the core TGTT sequence are sufficient to abolish binding, and the number of promoter DBEs correlates with FoxO1-mediated transcription in cell culture reporter assays [64]. However, distinct FoxO proteins vary in their preferences for 5' flanking sequences of the DBE [64]. A global analysis demonstrated that genes are most responsive to DBEs located within 300bp upstream of the transcriptional start site [23]. However, DAF-16/FoxO probably regulates genes through binding to other sequences as well, including those within introns or coding regions [68,69]. DAF-16/FoxO also regulates a large subset of genes indirectly [70] by mechanisms that may be conserved in mammals [71].

Other transcription factors participate in the regulation of both direct and indirect DAF-16/FoxO targets. Some target genes are co-regulated by HSF-1 [72] and SKN-1 [65,73], which also contribute to the longevity of *daf-2/IGFR* mutants. The DAE (DAF-16-Associated Element; CTTATCA) is a sequence enriched in the promoters of DAF-16/FoxO-regulated genes and is the reverse complement of the mammalian GATA site [74]. Tissue-specific GATA factors bind the DAE and contribute to tissue-specific expression of DAF-16/FoxO target genes and life span control [68,75,76]. The transcription factor PQM-1 also regulates gene expression dependent on the DAE [23]. DAF-16/FoxO antagonizes PQM-1 nuclear localization and indirectly regulates genes through PQM-1 inhibition [23].

Indirect regulation by DAF-16/FoxO is most striking when acting at a distance across tissues. Increasing DAF-16/FoxO activity in one tissue alters gene expression and aging-related phenotypes in other tissues, presumably through systemic signals [77,68]. Intriguingly, DAF-16/FoxO activity limited to the intestine can retard aging of the entire organism, and does not require DAF-16/FoxO activity in responding tissues [68,77].

In summary, DAF-16/FoxO controls gene expression through numerous mechanisms, exerting far-reaching effects on *C. elegans* biology. This complexity poses significant challenge to understanding the mechanisms by which DAF-16/FoxO promotes longevity.

Known DAF-16/FoxO target genes

Several studies have collectively discovered thousands of potential gene regulatory events mediated directly or indirectly by DAF-16/FoxO in the context of reduced IIS (Figure 1.5; [22,23,78]). The most common method is genome-wide RNA transcript profiling, for example by printed DNA microarrays, or serial analysis of gene expression (SAGE) [22,79]. These studies search for RNA transcripts whose abundance increases or decreases under conditions where DAF-16 is on (*e.g.* following *daf-2* mutation or RNAi treatment) compared to conditions where DAF-16 is mostly or fully inactive (*e.g.* wild-type animals or *daf-16;daf-2* double mutants). This method does not distinguish between direct and indirect regulatory events.

Other methods such as chromatin immunoprecipitation (ChIP) and DNA adenine methyltransferase identification (DamID) have identified genes directly bound by DAF-16/FoxO, though many of the binding events do not appear to mediate transcriptional regulation [69,70,80]. Proteomics studies have also identified a number of genes whose protein levels are altered by DAF-16/FoxO activity [78]. Some protein targets are not changed in RNA abundance, and these may be indirectly regulated via other DAF-16/FoxO transcriptional targets.

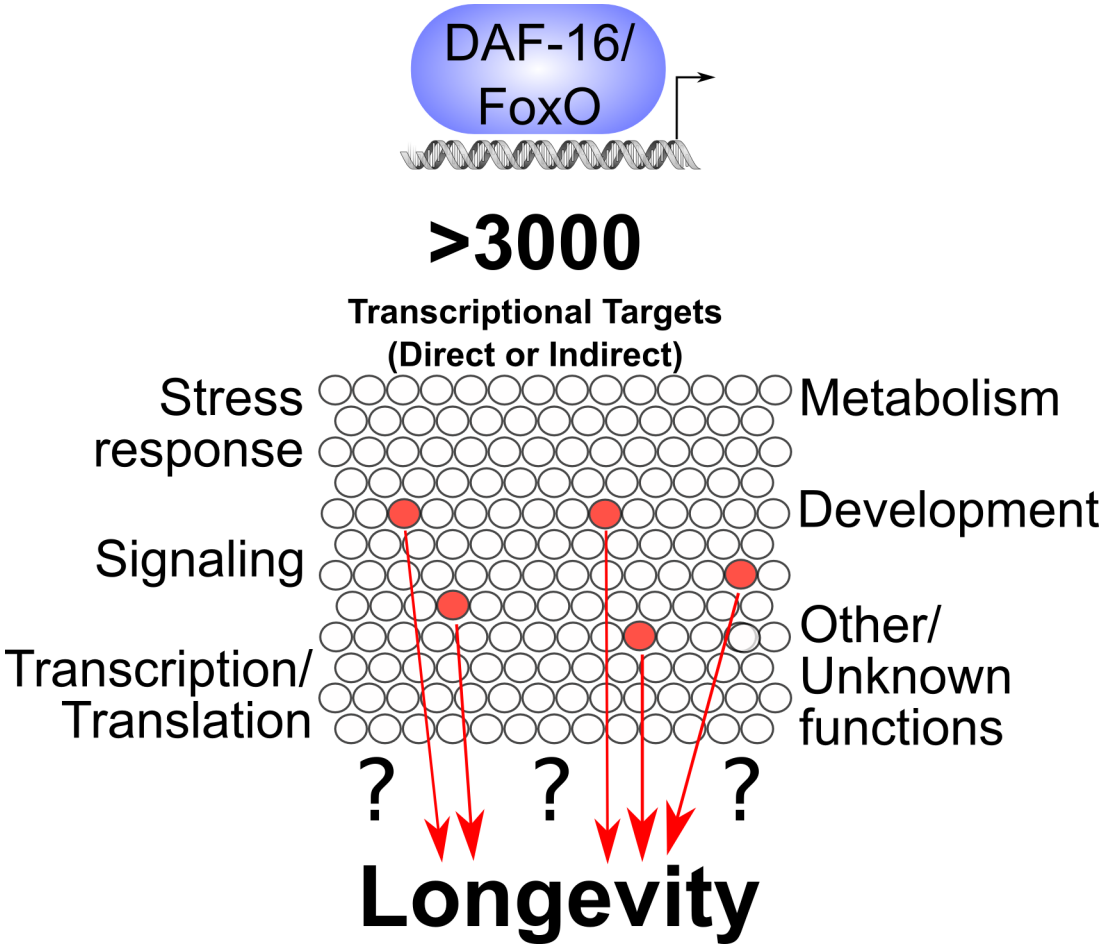
DAF-16/FoxO target gene function has primarily been determined by homology and GO term annotation [22,79]. Many of the genes are involved in response to stresses including extreme temperature, osmotic stress, free radicals, toxins, physical stress, and infection. Others are involved in metabolism or transport of nucleic acids, lipids, protein, carbohydrates, and other molecules. Growth, reproduction, development, and structural genes are also affected. Apoptosis and cell cycle DAF-16/FoxO targets influence germline proliferation [81]. Direct targets of DAF-16/FoxO are enriched for signaling,

transcription, and translation genes that could potentially influence the expression of indirect targets [69,70]. Notably, DAF-16/FoxO regulates many genes of unknown function. FoxO regulates many of the same gene classes in mammals, especially those involved in oxidative stress resistance, immunity, apoptosis, metabolism, and signaling [10,82].

However, some of the existing data on DAF-16/FoxO targets is seemingly conflicting. An analysis by McElwee and colleagues suggests that out of ~3000 genes identified by at least one of three microarray studies, only ~650 are shared by two or three of the studies [83]. Differences in laboratory conditions, experimental conditions such as animal age and use of sterile strains, technology platforms, and bioinformatics may all confound the analysis. The data has been partially reconciled using a microarray meta-analysis to combine the results of all published microarray comparisons between DAF-16-on and DAF-16-off conditions [23]. This study found 1,633 genes upregulated by DAF-16/FoxO and 1,733 genes downregulated by DAF-16/FoxO, using a 5% false discovery rate. Interestingly, ~50% of the top 100 DAF-16/FoxO targets were not found by individual microarray studies [23], suggesting that statistical power and methodology may also explain incompleteness and differences between studies. Systematic large-scale qPCR validation of target genes would be helpful to identify *bona fide* DAF-16/FoxO targets, but has not yet been performed.

In summary, identification of DAF-16/FoxO target genes remains an ongoing effort that has already shed light on how DAF-16/FoxO influences many aspects on animal biology. However, a more vexing problem remains: given the thousands of genes potentially regulated by DAF-16/FoxO, how do we utilize existing and future data to determine the mechanism by which DAF-16/FoxO promotes longevity? This question must be addressed before experimentally testing which conserved FoxO targets might influence longevity and age-related disease in humans.

Figure 1.5: DAF-16/FoxO transcriptionally regulates numerous genes, posing a significant challenge to understanding the mechanism of longevity control.



An open question: How does DAF-16/FoxO promote longevity?

A major hypothesis is that DAF-16/FoxO extends longevity through numerous gene expression changes, each with minor but cumulative effects on longevity. This hypothesis is intuitively appealing, as aging involves many different kinds of biological changes, and there are many potential causes of death for an organism that are exacerbated by aging. Therefore, it seems highly unlikely that DAF-16/FoxO could promote longevity through a single gene or process.

However, it is possible that some genes may be far more important than others for longevity assurance and delay of age-related phenotypes. This possibility has not been experimentally tested due to the large number of genes regulated by DAF-16/FoxO. Even the hypothesis that a single target gene is responsible for DAF-16/FoxO-mediated longevity is difficult to strictly disprove, as this would require testing every single gene individually, and currently an exhaustive search is not experimentally feasible. If a small set of DAF-16/FoxO target genes with major effects on life span exist, then those are excellent candidates for understanding and intervening in the aging process.

Role of DAF-16/FoxO target genes in life span control

The most thorough analysis to date of the roles of DAF-16/FoxO target genes in promoting *daf-2/IGFR* longevity was performed by Murphy and colleagues [74]. They performed microarray analysis across many conditions to identify 514 high-confidence DAF-16/FoxO target genes. The authors then utilized RNAi to knock down 39 DAF-16/FoxO-upregulated genes (termed Class 1) and 19 downregulated genes (termed Class 2). Knockdown of nearly all the Class 1 genes modestly reduced *daf-2/IGFR* life span, implying that they normally function to promote longevity. In contrast, knockdown of most Class 2 genes modestly increased wild-type life span, implying they normally function to limit longevity. No single gene recapitulated the effect of either *daf-16* or *daf-2* RNAi. Life span was altered by genes involved in stress responses, signaling, and metabolism, as well as genes with no known function. Other studies have expanded the list of target genes that influence life span, though the concordance between direction of

regulation by DAF-16/FoxO and effect on life span was not as strong as in the study by Murphy and colleagues [22,84,85]. Nevertheless, these findings are consistent with the hypothesis that DAF-16/FoxO target genes collectively and cumulatively influence aging and longevity.

Because using mutant alleles of a gene to study gene function is far more time-consuming than RNAi treatment, very few DAF-16/FoxO target genes have been tested by mutation. Therefore, it is unknown if mutation analysis would support the hypothesis that many DAF-16/FoxO target genes cumulatively contribute to the longevity of *daf-2/IGFR* mutants. RNAi is prone to both false positives due to off-target effects, as well as false negatives due to incomplete knockdown [86]. A study of morpholinos, a system analogous to RNAi in zebrafish, showed that 80% of morpholino-induced phenotypes were not recapitulated by mutation [87]. It is conceivable that many of the small changes in life span upon RNAi knockdown of DAF-16/FoxO target genes are due to off-target effects, and the lack of any genes that recapitulated the effect of DAF-16/FoxO could be attributable to incomplete knockdown.

The potential value of testing mutants is illustrated by the Class 1 gene *sod-3*, a manganese superoxide dismutase localizing to mitochondria [88]. RNAi knockdown of *sod-3* shortens life span of *daf-2/IGFR* mutants [74], but mutation of *sod-3* was separately reported to have no effect [89]. It is unclear if this discrepancy is due to off-target effects of RNAi, or if the role of *sod-3* is more complex. Therefore, it is important to verify RNAi life span phenotypes using mutation.

Two recent studies used mutations in target genes to develop the idea that DAF-16/FoxO acts as a "regulator of regulators" [78]. *aakg-4* and *mdl-1* are genes directly bound and upregulated by DAF-16/FoxO [70,90,91]. *aakg-4* is involved in signaling and *mdl-1* is a transcription factor. *aakg-4* and *mdl-1* mutations both reduce *daf-2* but not wild-type longevity, and cause a number of gene expression changes when mutated. This suggests that even if numerous genes contribute to longevity, a few DAF-16/FoxO target genes may be responsible for activating the rest, and therefore play a disproportionate role in

longevity control. However, as comparable studies have not rigorously tested other categories of target genes, the relative contribution of targets involved in signaling and transcription compared to other processes is not clear. For our purposes, these studies demonstrate that a logical strategy to determine which DAF-16/FoxO target genes are most likely to influence life span is valuable for selecting high-priority candidate genes for detailed mechanistic studies.

In summary, the functional significance of specific DAF-16/FoxO target genes in life span control has not been tested in a rigorous fashion. Almost none have been tested using genetic mutants, and most have not been tested by RNAi except for a subset of the top candidates. Therefore, it is plausible that a small number of genes play a disproportionate effect in longevity, and we need a logical strategy to identify and test these genes.

A strategy to identify and test high-priority DAF-16/FoxO targets

The motivating question of this dissertation is: What is the mechanism by which DAF-16/FoxO promotes longevity? The fact that DAF-16/FoxO influences the expression of thousands of genes [23] is a major barrier to experimentally investigating this question. Although multiple studies utilizing microarray approaches have identified a number of DAF-16/FoxO target genes that may influence life span [22,92], there is surprisingly low agreement among these studies [22,23,83], making it difficult to prioritize candidate genes for functional validation. Furthermore, more recently developed whole transcriptome profiling (RNA-seq) shows greater sensitivity than microarray-based approaches [93,94], and thus it is likely that potentially critical subsets of DAF-16/FoxO targets remain unidentified. Taken together, the current list of genes is experimentally intractable for rigorous functional validation by traditional genetic approaches (*e.g.* genetic mutants), even in spite of the advantages of the *C. elegans* model system compared to vertebrate models (Figure 1.2). A new strategy is required to narrow this list and determine the mechanisms of life span extension mediated by DAF-16/FoxO.

The overall strategy of this dissertation is to use genetic approaches to isolate and selectively activate distinct DAF-16/FoxO transcriptional programs. If only specific programs promote longevity, then the genes that comprise those programs are high-priority candidates. Given the thousands of genes regulated by DAF-16/FoxO, the search for specific DAF-16 target genes that promote longevity can be likened to searching for needles in a haystack. Selective activation of DAF-16/FoxO programs is akin to separating this haystack into many different haystacks. These haystacks are then small enough for rigorous functional testing.

Chapter 2 is concerned with two highly related kinases reported to have very similar effects on DAF-16/FoxO activity, but which have opposite effects on longevity, potentially representing distinct DAF-16/FoxO programs. Chapter 3 identifies a set of genes associated with longevity across many contexts (the "high-priority" haystack). Chapter 4 investigates the role of distinct DAF-16/FoxO isoforms in life span control and identifies genes specifically regulated by those isoforms, effectively separating target genes into multiple groups. Finally, Chapter 5 rigorously tests the groups of genes found in previous chapters, and identifies key genes involved in longevity.

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Chapter 2 Effects of *C. elegans sgk-1* mutations on life span, stress resistance, and DAF-16/FoxO regulation[1]

Abstract

The AGC family serine-threonine kinases Akt and Sgk are similar in primary amino acid sequence and *in vitro* substrate specificity, and both kinases are thought to directly phosphorylate and inhibit FoxO transcription factors. In the nematode *Caenorhabditis elegans*, it is well established that AKT-1 controls dauer arrest and life span by regulating the subcellular localization of the FoxO transcription factor DAF-16. SGK-1 is thought to act similarly to AKT-1 in life span control by phosphorylating and inhibiting the nuclear translocation of DAF-16/FoxO. Using *sgk-1* null and gain-of-function mutants, we now provide multiple lines of evidence indicating that AKT-1 and SGK-1 influence *C. elegans* life span, stress resistance, and DAF-16/FoxO activity in fundamentally different ways. Whereas AKT-1 shortens life span, SGK-1 promotes longevity in a DAF-16/FoxO-dependent manner. In contrast to AKT-1, which reduces resistance to multiple stresses, SGK-1 promotes resistance to oxidative stress and ultraviolet radiation but inhibits thermotolerance. Analysis of several DAF-16/FoxO target genes that are repressed by AKT-1 reveals that SGK-1 represses a subset of these genes while having little influence on the expression of others. Accordingly, unlike AKT-1, which promotes the cytoplasmic sequestration of DAF-16/FoxO, SGK-1 does not influence DAF-16/FoxO subcellular localization. Thus, in spite of their similar *in vitro* substrate specificities, Akt and Sgk

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influence longevity, stress resistance, and FoxO activity through distinct mechanisms *in vivo*. Our findings highlight the need for a re-evaluation of current paradigms of FoxO regulation by Sgk.

Introduction

Akt/Protein Kinase B (PKB) and Sgk are two highly related members of the AGC family of serine-threonine kinases that act in cellular signaling pathways to modulate survival, growth, proliferation, metabolism, and other processes [1]. Akt/PKB has evolutionarily conserved functions in the control of development, growth, metabolism, cell survival, and longevity, and dysregulation of Akt/PKB contributes to the pathogenesis of common human diseases such as cancer and Type 2 diabetes [2].

The mechanism of Akt/PKB activation is well established. In response to growth factors, Akt/PKB is activated in a phosphoinositide 3-kinase (PI3K)-dependent manner by phosphorylation at two critical regulatory sites: T308 within its kinase domain, and S473 within a C-terminal hydrophobic motif [3]. The 3-phosphoinositide-dependent kinase PDK1 phosphorylates Akt/PKB at T308 [4,5], and members of the PI3K-related kinase (PIKK) family such as TOR complex 2 phosphorylate Akt/PKB at S473 [6–8].

Activated Akt/PKB phosphorylates several substrates *in vivo* at sites that lie within RxRxxS/T motifs [9,10]. Among these substrates are the FoxO family of transcription factors that control development, metabolism, growth, and aging [11]. Akt/PKB-dependent phosphorylation of FoxO at three conserved RxRxxS/T motifs inhibits FoxO activity by promoting its export from the nucleus and sequestration in the cytoplasm [12]. FoxO is a critical substrate of Akt/PKB *in vivo*, as its disinhibition in mice with reduced hepatic Akt/PKB signaling impairs metabolic homeostasis [13], and a null mutation in *daf-16*, which encodes the sole FoxO transcription factor in the nematode *Caenorhabditis elegans*, suppresses the dauer-constitutive and life span extension phenotypes of animals

with reduced Akt/PKB activity [14,15]. Thus, Akt/PKB has an evolutionarily conserved function as a direct inhibitor of FoxO transcription factors.

The serum- and glucocorticoid-regulated kinase gene *sgk* encodes a serine-threonine kinase highly homologous to Akt/PKB that was first identified as a gene whose transcription is induced acutely by serum and glucocorticoids in a rat mammary tumor cell line [16]. Like Akt/PKB activation, Sgk activation by growth factors is PI3K-dependent and involves the phosphorylation of a site in the kinase domain (T256) by PDK1 [17,18] and a C-terminal site within a hydrophobic motif by TOR complex 2 [19]. Furthermore, Sgk also phosphorylates sites that lie within RxRxxS/T motifs [20]. In spite of these similarities, some Akt/PKB substrates are poor substrates for Sgk *in vitro* and vice versa [21,22]; this is probably due at least in part to amino acids in the vicinity of the phosphoacceptor residue that confer substrate specificity [23]. The distinct substrate specificities of Akt/PKB and Sgk are reflected in the observation that, although both mammalian Akt/PKB and Sgk can promote the phosphorylation of the FoxO3 transcription factor in cultured cells at sites within all three conserved RxRxxS/T motifs, they do so with distinct efficiencies within each motif [24].

In mammalian cell culture, Sgk inhibits FoxO3 activity [24,25], and in *C. elegans*, SGK-1 is thought to limit life span by inhibiting DAF-16/FoxO activity [26]. Taken together with the known role of Akt/PKB in FoxO regulation, these studies have established a paradigm whereby Akt/PKB and Sgk are thought to act via similar mechanisms to inhibit FoxO activity [1,27,28].

We and others recently reported that, in contrast to the life span extension phenotype observed after RNAi knockdown of *sgk-1* [26], *sgk-1* null mutations shorten *C. elegans* life span [15,29,30]. This phenotype is the opposite of that observed for *akt-1* null mutations [15,29,30] and is inconsistent with prevailing models implicating Sgk as a FoxO inhibitor. In light of these results, we have performed a detailed phenotypic analysis of *sgk-1* null and gain-of-function mutants. Our results indicate that in *C. elegans*, Akt/PKB and Sgk influence life span, stress resistance, and FoxO transcription

factor activity through distinct mechanisms. These surprising findings call into question current paradigms of FoxO regulation by Sgk and reveal that the interaction of Sgk and FoxO transcription factors may be more complex than previously appreciated.

Results

Effects of *sgk-1* mutations on life span

Three alleles of *sgk-1* facilitate analysis of gene function (Figure 2.1A). We and others have shown that the *sgk-1(mg455)* mutation shortens life span [30,29]. The *mg455* allele is a nonsense mutation that is predicted to result in truncation of SGK-1 within its kinase domain [29]; therefore, this is likely to be a null mutation. A third group has shown that the *sgk-1(ok538)* deletion mutation, which is predicted to remove half of the SGK-1 kinase domain and is also probably a null mutation [26], also reduces life span [15]. We confirmed these results by measuring the life spans of both *sgk-1(ok538)* and *sgk-1(mg455)* null mutants in the same assay (Figure 2.1B and Supplemental Table 2.1). *sgk-1(ok538)* (heretofore referred to as "null #1") and *sgk-1(mg455)* (heretofore referred to as "null #2") each shorten mean life span by at least 27.5% and median life span by at least 19.0% and 33.3% respectively ($P<0.0001$ by the log-rank test). The observation that two outcrossed strains harboring independently isolated *sgk-1* null mutations both have short life spans compared to wild-type animals strongly suggests that these short life span phenotypes are a consequence of reduced SGK-1 activity. These results contrast with the reported life span extension induced by *sgk-1* RNAi [26] and are consistent with a model whereby SGK-1 promotes longevity.

One possible explanation for the discrepancy between the life spans of animals harboring *sgk-1* loss-of-function mutations and animals subjected to *sgk-1* RNAi is that strong loss-of-function mutations could cause developmental abnormalities that shorten adult life span by reducing general fitness; such abnormalities can be avoided by initiating RNAi during late larval or early adult stages. To address this possibility, we assayed the life spans of animals harboring the *sgk-1(ft15)* gain-of-function mutation.

sgk-1(ft15) emerged from a genetic screen for suppressors of the developmental delay phenotype of animals harboring a loss-of-function mutation in *lpo-6/rict-1*, which encodes the *C. elegans* ortholog of the TOR complex 2 component Rictor [31,29]. *sgk-1(ft15)* suppresses both the developmental delay and small body size phenotypes of *lpo-6/rict-1* loss-of-function mutants, and this suppression is abrogated by *sgk-1* RNAi [31]. Taken together with the observations that *lpo-6/rict-1* and *sgk-1* act in the same genetic pathway [31,29] and mammalian TOR complex 2 activates Sgk by promoting its phosphorylation [19], these data strongly suggest that *sgk-1(ft15)* is a gain-of-function allele.

We reasoned that, if *sgk-1* null mutants are short-lived because SGK-1 plays a role in promoting longevity, then animals harboring *sgk-1(ft15)* (heretofore referred to as "*sgk-1(gf)*") should live longer than animals with wild-type *sgk-1*. However, if *sgk-1* null mutants are short-lived because they are sick, then *sgk-1(gf)* animals would not be expected to live long. *sgk-1(gf)* animals consistently lived ~ 15-20% longer than non-sibling wild-type animals (Supplemental Table 2.1B). When siblings harboring wild-type *sgk-1* were used as controls, *sgk-1(gf)* animals exhibited a more modest but statistically significant extension in median and mean life span in eight of ten experimental trials (Figure 2.1B-E and Supplemental Table 2.1A and C). In Figure 1B, *sgk-1(gf)* increased mean and median life span by 17.5% and 9.5% respectively, compared to wild-type siblings (P=0.0008). This life span extension was suppressed by a null mutation in *daf-16/FoxO* (Figure 2.1C and Supplemental Table 2.1A).

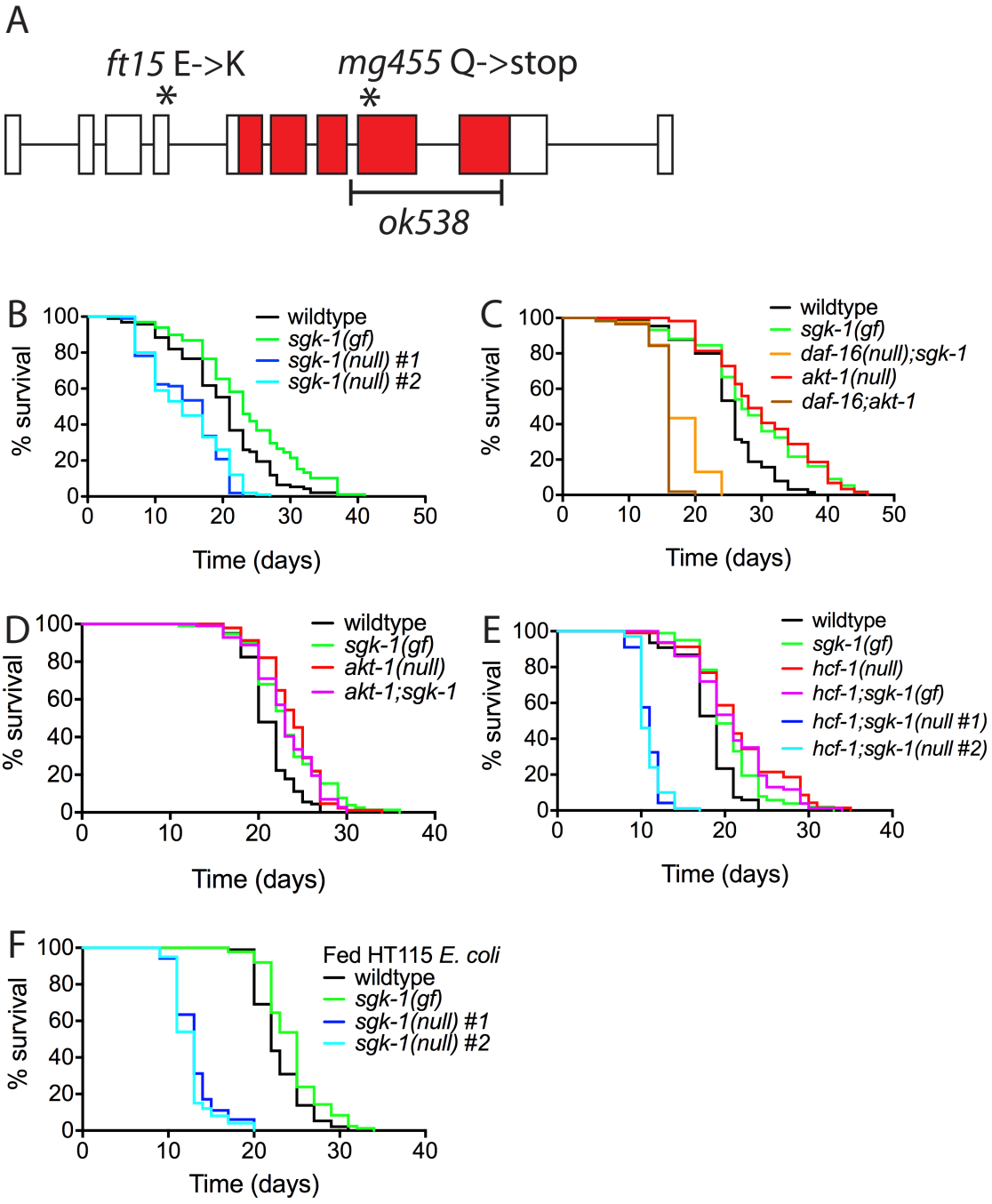
In *C. elegans*, DAF-16/FoxO activity is regulated through at least two mechanisms. Phosphorylation of DAF-16/FoxO by kinases such as AKT-1 inhibits DAF-16/FoxO by promoting its export from the nucleus [15,30,32–34]. Other regulatory proteins such as HCF-1 and EAK-7 inhibit DAF-16/FoxO activity without influencing its subcellular localization [30,35]. To determine whether SGK-1 acts specifically in either of these pathways to promote longevity, we tested the effect of *sgk-1* mutations on the life spans of *akt-1* and *hcf-1* null mutants. We previously reported that SGK-1 is required for the longevity of *akt-1* mutants [30]. *sgk-1(gf)* did not extend the life span of *akt-1(null)*

animals (Figure 2.1D and Supplemental Table 2.1A). Similarly, in *hcf-1(null)* animals, *sgk-1* was required for life span extension, but *sgk-1(gf)* did not further increase life span (Figure 2.1E and Supplemental Table 2.1A). Based on these data, whether SGK-1 acts specifically with AKT-1 or HCF-1 to influence life span is not clear. It is possible that DAF-16/FoxO activation by SGK-1 is attenuated in backgrounds such as *akt-1(null)* and *hcf-1(null)* in which DAF-16/FoxO is already activated.

As the *E. coli* HB101-derived HT115 strain used in experiments demonstrating that *sgk-1* RNAi extends life span [26] differs from the OP50 strain used in our experiments (Figure 2.1B-E), we sought to determine the influence of E. coli strain differences on the life spans of *sgk-1* mutants. Therefore, we assayed the life spans of *sgk-1(null)* and *sgk-1(gf)* mutants grown on HT115. As observed in experiments using OP50 as a food source, *sgk-1(null)* shortened and *sgk-1(gf)* extended life span in animals feeding on either HT115 or HB101 (Figure 2.1F and Supplemental Table 2.1C). Therefore, the pro-longevity activity of SGK-1 is not significantly influenced by differences between *E. coli* OP50 and HT115/HB101.

Taken together, these results suggest that, in contrast to existing paradigms of FoxO inhibition by Sgk [24,26], SGK-1 promotes *C. elegans* longevity in a DAF-16/FoxO-dependent manner.

Figure 2.1: Effects of *sgk-1* mutations on life span.
(A) Schematic of the *sgk-1* genomic locus (not to scale). Locations of the *ft15* missense gain-of-function, *ok538* deletion, and *mg455* nonsense mutations are shown. Exons encoding the kinase domain are colored red. (B) Life spans of *sgk-1* mutants *ft15* (gf), *ok538* (null #1), and *mg455* (null #2). (C) Effect of the *daf-16(mu86)* null mutation on the life spans of *sgk-1(gf)* animals. (D) Effect of *sgk-1(gf)* on the life span of *akt-1(mg306)* null mutant animals. (E) Effect of *sgk-1(gf)* on the life span of *hcf-1(pk924)* null mutant animals. (F) Life spans of *sgk-1* mutants on HT115 *E. coli*. Raw data and statistics are presented in Supplemental Table 2.1.



Effects of *sgk-1* mutations on dauer arrest

Since DAF-16/FoxO promotes developmental arrest in the dauer larval stage in animals with reduced DAF-2 insulin-like signaling [36,37], we tested the effect of *sgk-1(null)* and *sgk-1(gf)* on dauer arrest. In agreement with a previous report [26], neither *sgk-1(null)* nor *sgk-1(gf)* had significant effects on dauer arrest at 27°C (Table 2.1A). Although a significant percentage of *sgk-1(null)* animals arrested during larval development (Table 2.1A), analysis of these animals using Nomarski microscopy revealed no evidence of dauer alae or pharyngeal constriction (Supplemental Figure 2.3), indicating that these animals were non-dauer larvae. In contrast and as previously reported [38], an *akt-1* null mutation had a strongly penetrant DAF-16/FoxO-dependent dauer-constitutive phenotype under the same assay conditions. Neither *sgk-1(null)* nor *sgk-1(gf)* significantly influenced the dauer-constitutive phenotype of *daf-2(e1368)* (Table 2.1B). Therefore, SGK-1 does not function in dauer regulation.

Table 2.1. Effects of *sgk-1* mutations on dauer arrest.

(A) Dauer formation of *sgk-1* and *akt-1* mutants at 27°C. (B) Effect of *sgk-1* mutations on *daf-2(e1368)* dauer formation at 25°C. Siblings were used in each experiment.

Table 1A

Effects of *sgk-1* and *akt-1* mutations on dauer formation at 27°C									
	Trial 1		**Trial 2**		**Trial 3**		**Average(SD)**		**N**
Genotype	**%dauer**	**%non-dauer larvae**	**%dauer**	**%non-dauer larvae**	**%dauer**	**%non-dauer larvae**	**%dauer**	**%non-dauer larvae**	
Wildtype	0.0	0.0	0.0	0.0	0.0	2.3	0 (0)	0.8 (1.3)	991
akt-1 (null)	97.7	0.6	94.5	5.0	89.1	10.9	93.8 (4.3)	5.5 (5.2)	705
daf-16(null);akt-1	0.0	1.8	0.0	9.3	0.0	15.2	0 (0)	8.8 (6.7)	811
sgk-1(gf)	0.0	0.0	0.0	0.0	0.0	10.3	0 (0)	3.4 (5.9)	968
daf-16;sgk-1(gf)	0.0	0.3	0.0	1.9	0.0	23.6	0 (0)	8.6 (13.0)	893
sgk-1(null) #1	0.0	26.7	0.0	52.8	0.0	90.0	0 (0)	56.5 (31.8)	1144
sgk-1(null) #2	0.0	67.5	0.0	46.5	0.0	91.8	0 (0)	68.6 (22.6)	1087

Table 1B

Effects of *sgk-1* mutations on *daf-2(e1368)* dauer formation at 25°C					
Genotype	**Trial 1 % dauer**	**Trial 2 % dauer**	**Trial 3 % dauer**	**Average (SD)**	**N**
wildtype	0.0	0.0	0.0	0 (0)	768
sgk-1(gf)	0.0	0.0	0.0	0 (0)	1007
daf-2(e1368)	81.2	90.7	46.8	72.9 (23.1)	882
daf-2;sgk-1(gf)	83.1	86.8	50.3	73.1 (20.1)	596
wildtype	0.0	0.0	0.0	0 (0)	1018
sgk-1(null) #1	0.0	0.0	0.0	0 (0)	1498
daf-2(e1368)	87.0	90.8	87.1	88.3 (2.2)	1128
daf-2;sgk-1(null) #1	89.8	95.6	94.3	93.2 (3.0)	1016
wildtype	0.0	0.0	0.0	0 (0)	861
sgk-1(null) #2	0.0	0.0	0.0	0 (0)	1508
daf-2(e1368)	93.5	90.6	83.2	89.1 (5.3)	1064
daf-2;sgk-1(null) #2	93.2	89.5	88.9	90.5 (2.3)	958

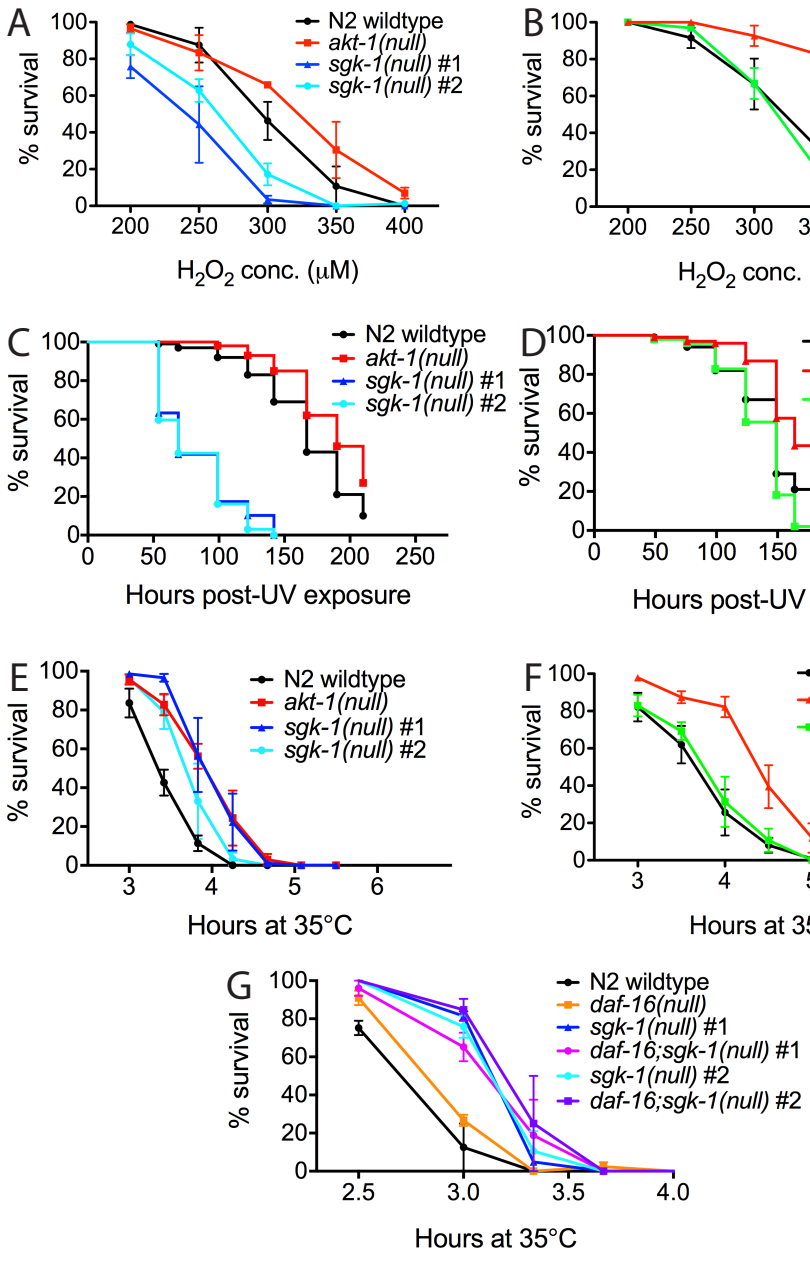
Effects of *sgk-1* mutations on stress resistance

In light of our observations on the effects of *sgk-1* mutations on life span (Figure 2.1), we tested *sgk-1(null)* and *sgk-1(gf)* for their sensitivity to oxidative stress, ultraviolet radiation (UVR), and heat. *akt-1* null mutants were slightly more resistant to hydrogen peroxide than wild-type animals, although this difference was only statistically significant in one of four assays (Figure 2.2A-B and Supplemental Table 2.2). *akt-1* null mutants were significantly more resistant to UVR and heat than wild-type animals (Figure 2.2C-F, Supplemental Table 2.3, Supplemental Table 2.4). In contrast, both *sgk-1* null mutants were more sensitive to hydrogen peroxide (statistically significant in 2 of 3 trials for each mutant) and UVR (statistically significant in 3 of 3 trials) than wild-type animals (Figure 2.2A and C, Supplemental Table 2.2, Supplemental Table 2.3), consistent with their short life spans. *sgk-1(gf)* did not significantly influence sensitivity to any of the three stressors tested (Figure 2.2B, D, and F).

Both *sgk-1* null mutations enhanced thermotolerance to at least the same extent that an *akt-1* null mutation did (Figure 2.2E and Supplemental Table 2.4). This result is consistent with a previous report examining thermotolerance of the *sgk-1(ok538)* null mutant (Hertweck *et al.* 2004). Taken together with our observation that the *sgk-1(gf)* mutation extends life span, this enhanced thermotolerance phenotype of *sgk-1* null mutants strengthens the argument that the short-lived phenotype of *sgk-1* null mutants is not simply a consequence of frailty secondary to developmental abnormalities. In contrast to AKT-1, which promotes general sensitivity to environmental stress, SGK-1 is protective against oxidative stress and UVR but enhances sensitivity to heat.

As the thermotolerance of *sgk-1(ok538)* is thought to require DAF-16/FoxO [26], we tested the effect of a *daf-16* null mutation on the thermotolerance of both *sgk-1* null mutants. Surprisingly, *daf-16* null mutation did not significantly influence the thermotolerance of either *sgk-1* null mutant (Figure 2.2G and Supplemental Table 2.4). Therefore, our results suggest that SGK-1 promotes sensitivity to heat in a DAF-16/FoxO-independent manner.

Figure 2.2. Effects of *sgk-1* mutations on stress resistance.
(A-F) Stress resistance assays exposing animals to hydrogen peroxide (A-B), UV radiation (C-D), and heat (E-F). Assays were performed on *sgk-1(ok538)* (null #1), *sgk-1(mg455)* (null #2) (A, C, E), and *sgk-1(ft15) (gf)* (B, D, F). (G) Effect of *daf-16(mu86)* null mutation on the thermotolerance of *sgk-1* null mutants. Raw data and statistics are presented in Supplemental Table 2.2, Supplemental Table 2.3, and Supplemental Table 2.4.



Effects of *sgk-1* mutations on DAF-16A::GFP subcellular localization

As our life span data are consistent with a model in which SGK-1 promotes longevity by activating DAF-16/FoxO, we sought to determine the influence of *sgk-1* mutations on the subcellular localization of DAF-16/FoxO. Sgk promotes the nuclear export and cytoplasmic sequestration of FoxO in mammalian cells [24]; however, based on conflicting reports in the literature [15,26], the role of *C. elegans* SGK-1 in regulating DAF-16/FoxO localization remains unclear. We constructed *sgk-1(null)* and *sgk-1(gf)* strains expressing a functional DAF-16A::GFP fusion protein as the sole source of DAF-16/FoxO in the animal and determined DAF-16A::GFP subcellular localization in young adult animals raised in the same conditions used for life span assays (Figure 2.3A). Under these conditions, *akt-1* null mutation promoted the translocation of DAF-16A::GFP from the cytoplasm to the nucleus, as previously shown [30,33,34]. Neither the *sgk-1(ok538)* null mutation nor *sgk-1(gf)* had a significant influence on the nucleocytoplasmic distribution of DAF-16A::GFP. These data suggest that, unlike AKT-1, SGK-1 does not control DAF-16/FoxO activity *in vivo* by regulating its subcellular localization.

Effects of *sgk-1* mutations on DAF-16/FoxO target gene expression

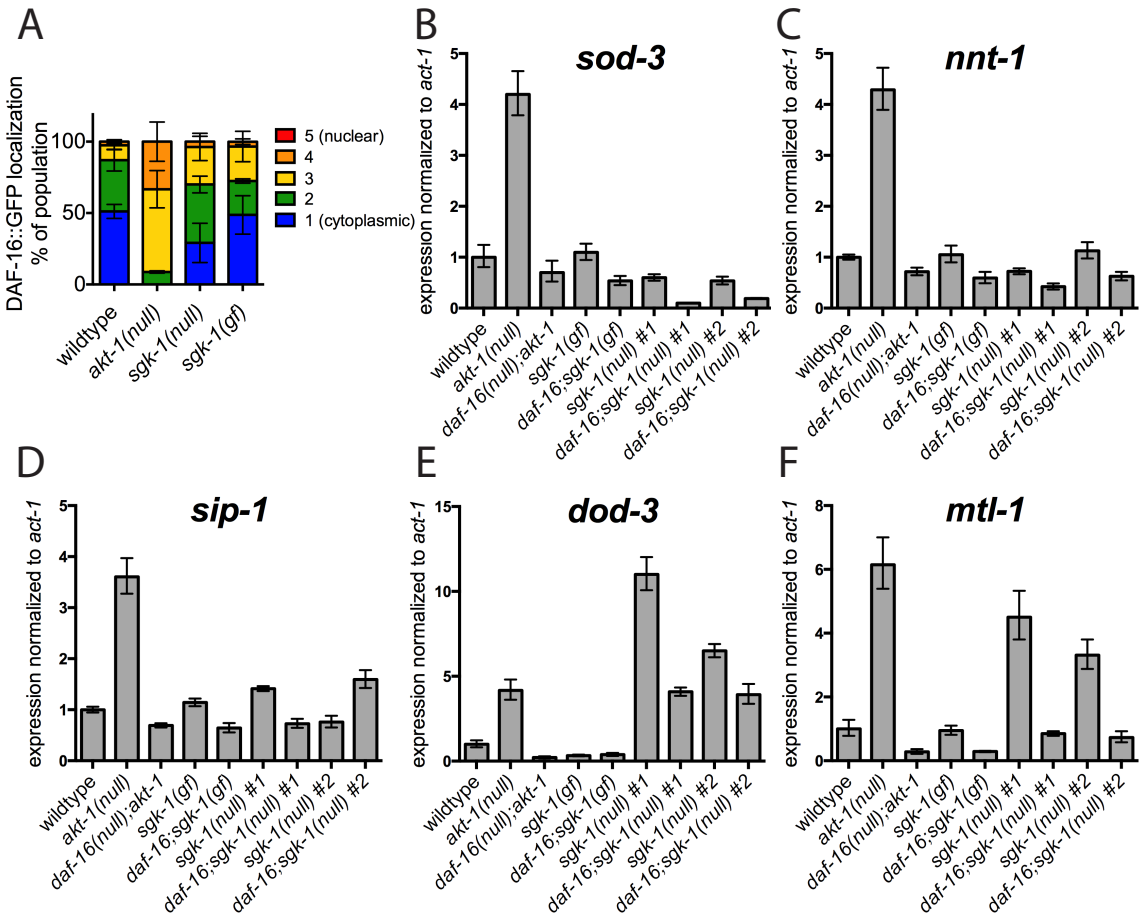
The dependence of *sgk-1(gf)* life span extension on *daf-16/FoxO* (Figure 2.1C) suggests that SGK-1 may increase life span by activating DAF-16/FoxO, even in the absence of a significant effect on DAF-16A::GFP localization (Figure 2.3A). Therefore, we quantified the expression of five DAF-16/FoxO target genes [15,30,34,39,40] in young adult *sgk-1(null)* and *sgk-1(gf)* animals.

As expected for *bona fide* DAF-16/FoxO target genes, the expression of all five of these genes is increased in a DAF-16/FoxO-dependent manner in the context of *akt-1* null mutation (Figure 2.3B-F and Supplemental Table 2.6) [30,34]. In contrast, *sgk-1* mutations had varying influences on the expression of these five genes. *sod-3* expression was not influenced by *sgk-1(gf)* but was reduced in *sgk-1* null mutants Figure 2.3B and Supplemental Table 2.6). Thus, null mutations in *sgk-1* and *akt-1* have opposite effects on *sod-3* expression. Neither *sgk-1* null mutation nor *sgk-1(gf)* reproducibly influenced the

expression of *nnt-1* and *sip-1* (Figure 2.3C-D and Supplemental Table 2.6). Expression of both *dod-3* and *mtl-1* was elevated in a DAF-16/FoxO-dependent manner in the context of *sgk-1* null mutation in five of six trials (Figure 2.3E-F and Supplemental Table 2.6), suggesting that SGK-1 and AKT-1 may act similarly to regulate these two DAF-16/FoxO target genes.

In aggregate, these data indicate that AKT-1 and SGK-1 control DAF-16/FoxO target gene expression through distinct mechanisms. The heterogeneity of the influence of *sgk-1* mutations on DAF-16/FoxO target gene expression suggests that the molecular basis for SGK-1 regulation of DAF-16/FoxO activity is significantly more complex than has been appreciated.

Figure 2.3. Effects of *sgk-1* mutations on DAF-16A::GFP subcellular localization and DAF-16/FoxO target gene expression (next page).
(A) Subcellular localization of DAF-16A::GFP in *akt-1* and *sgk-1* mutants. Nuclear localization is increased by *akt-1(mg306)* null mutation (two-way ANOVA, F=14.47, P<0.0001), but not by *sgk-1(ok538)* null mutation (F=1.825, P=0.1733) or by *sgk-1(ft15)* gain-of-function mutation (F=0.869, P=0.5037). Error bars represent SEM for 3 cohorts of 20-30 animals per genotype imaged separately. All animals also harbored the *daf-16(mu86)* null allele, so no endogenous DAF-16/FoxO is present. Representative images are shown in Supplemental Figure 2.2. Raw data and statistics are presented in Table S5. (B-F) Representative experiments measuring *sod-3* (B), *nnt-1* (C), *sip-1* (D*), dod-3* (E), and *mtl-1* (F) transcript levels using quantitative RT-PCR on total RNA isolated from young adult animals. Values are normalized to expression levels in wild-type animals. Columns represent mean +/- SEM of three technical replicates. Raw data and statistics for biological replicates are summarized in Supplemental Table 2.6. (G) Summary of statistically significant gene expression changes (P<0.05; Supplemental Table 2.6; unpaired two-tailed t-test with Welch's correction) in *akt-1* and *sgk-1* mutants and their dependence on DAF-16/FoxO. The asterisk indicates that *dod-3* expression was increased significantly in eight of twelve trials. The number sign indicates that *daf-16(null)* significantly reduced expression of *dod-3* and *mtl-1* in *sgk-1(null)* mutants in five of six trials.



G **Summary: Effects of *akt-1* and *sgk-1* mutations on DAF-16/FoxO activity**

	akt-1(null)	dependent on DAF-16?	*sgk-1(null)*	dependent on DAF-16?	*sgk-1(gf)*
sod-3	increase	yes	decrease	n/a	no change
nnt-1	increase	yes	no change	n/a	no change
sip-1	increase	yes	no change	n/a	no change
dod-3	increase	yes	increase*	no	decrease
mtl-1	increase	yes	increase	partial #	no change

Discussion

Akt/PKB inhibits FoxO transcription factors via a well-established and evolutionarily conserved mechanism involving phosphorylation of FoxO at three sites that lie within conserved RxRxxS/T motifs [2,10]. Based on both its similarity in primary structure [16] and substrate specificity [20] to Akt/PKB as well as data from mammalian cell culture [24,25] and *C. elegans* [26], Sgk is also thought to inhibit FoxO by promoting its phosphorylation at RxRxxS/T motifs. Our data challenge this model of FoxO regulation by Sgk and support the notion that in *C. elegans,* Akt/PKB and Sgk regulate FoxO activity in fundamentally different ways.

Our conclusions are at odds with those of the only study in the literature that has focused on Sgk action in *C. elegans* life span control and FoxO regulation [26]. This study showed that *sgk-1* RNAi extends life span in a DAF-16/FoxO-dependent manner. One possible explanation for this discrepancy is that the *E. coli* strain used for RNAi (the HB101-related strain HT115) is different from the standard strain used for growth and maintenance of *C. elegans* (the *E. coli* B-related OP50) that we used in our experiments. Indeed, wild-type *C. elegans* grown on HT115 live nearly 20% longer than wild-type animals grown on OP50 [41]. However, we have shown that *sgk-1(null)* and *sgk-1(gf)* animals are respectively short-lived and long-lived when cultured on *E. coli* OP50, HT115, or HB101 (Figure 2.1 and Supplemental Table 2.1), indicating that the life span phenotypes of *sgk-1(null)* and *sgk-1(gf)* are not significantly influenced by differences between OP50 and HT115/HB101 *per se*.

We did confirm the previously reported finding that *sgk-1* null mutant animals are thermotolerant compared to wild-type animals [26]. This suggests that *sgk-1* null mutant animals are not short-lived due to general frailty or sickness, as such animals would be expected to be generally hypersensitive to environmental stresses. Intriguingly, *daf-16/FoxO* was not required for thermotolerance in *sgk-1(null)* animals, suggesting that, although AKT-1 and SGK-1 both promote thermosensitivity, they likely do so through distinct mechanisms. Our results dissociate thermotolerance from longevity and suggest

that divergent molecular pathways act downstream of SGK-1 to influence life span and responses to increased ambient temperature.

Our results also contrast with a detailed analysis of mammalian FoxO3 regulation demonstrating that both Sgk and Akt/PKB can inhibit FoxO3 activity in cell culture by promoting the phosphorylation of all three conserved sites that lie within RxRxxS/T motifs [24]. This discrepancy may be due in part to differences in experimental context; these experiments were performed in cell culture, where growth factors are frequently added in excess of physiologic concentrations and overexpressed proteins may exhibit activities that are not discernible when they are expressed at endogenous levels. The effect of Sgk knockdown or deletion on the activity of endogenous FoxO transcription factors has not been investigated in mammals. Although it is conceivable that Sgk regulates FoxO activity through distinct mechanisms in mammals and *C. elegans*, this is unlikely in light of the conservation of mechanisms of FoxO regulation by insulin-like growth factor signaling pathways [42].

Although the increased life span phenotypes caused by *akt-1* null mutation and the *sgk-1(gf)* both require *daf-16/FoxO* (Figure 2.1C and Supplemental Table 2.1), the expression of DAF-16/FoxO target genes was influenced by these two mutations in starkly discordant ways (Figure 2.3B-F). Whereas the expression of five DAF-16/FoxO target genes is induced in a DAF-16/FoxO-dependent manner in *akt-1* null mutants, *sgk-1(null)* and *sgk-1(gf)* mutations had distinct and varying influences on the expression of specific DAF-16/FoxO target genes. This difference is likely a reflection of underlying differences in the molecular basis for DAF-16/FoxO regulation by AKT-1 and SGK-1.

These observations suggest that the underlying mechanisms of life span control by AKT-1 and SGK-1 are fundamentally different. In contrast to AKT-1, which inhibits DAF-16/FoxO by promoting its nuclear export and cytoplasmic retention [26,30,33,34], SGK-1 may promote longevity by regulating other proteins that functionally and/or physically interact with DAF-16/FoxO, such as SKN-1, HSF-1, or HCF-1 [35,43,44]. In this regard, DAF-16/FoxO may play a permissive role in life span control by SGK-1 without being

directly regulated by SGK-1. Alternatively, SGK-1 may directly regulate DAF-16/FoxO activity in a small number of cells, which in turn could control life span by influencing other cells in a DAF-16/FoxO-independent manner.

In summary, we have shown that the AGC kinase family members Akt/PKB and Sgk control *C. elegans* life span and stress resistance in fundamentally different ways, and they likely influence FoxO transcription factor activity through distinct mechanisms *in vivo*. Our findings challenge existing paradigms of FoxO regulation by Sgk and should engender a reassessment of the role of Sgk in FoxO transcription factor regulation.

Materials and Methods

Strains and reagents

The following strains were used: N2 Bristol (wild-type), *sgk-1(ft15)* [31], *akt-1(mg306)* [38], *sgk-1(ok538)* [26], *sgk-1(mg455)* [29], *daf-16(mu86)* [45], *hcf-1(pk924)* [35] and TJ356 (*zIs356*) [46]. Since *sgk-1(ft15)* was isolated after mutagenesis of animals harboring the linked *akt-2(tm812)* mutation [31], we confirmed the absence of *akt-2(tm812)* prior to further analysis. Throughout the manuscript, *sgk-1(ft15)* is referred to as "*sgk-1(gf)*," *akt-1(mg306)* as "*akt-1(null)*," *sgk-1(ok538)* as "*sgk-1(null)* #1," and *sgk-1(mg455)* as "*sgk-1(null)* #2." *sgk-1* mutant strains were outcrossed with N2 at least seven times prior to phenotypic analysis. Wild-type siblings of *sgk-1*(*ft15*) from the seventh outcross with N2 Bristol were used as controls for phenotypic comparison to *sgk-1(ft15)*. This sibling is labeled "wild-type" in all figures, in contrast to "N2 wild-type." Double and triple mutants were generated using standard genetic techniques. For maintenance and all assays, animals were grown in Percival I-30NL or I-36NL incubators (Percival Scientific, Inc., Perry, IA).

Life span assays

Life span assays were performed at 20°C as described [30,34]. Briefly, animals were treated with alkaline hypochlorite and grown for at least three generations at 15°C. A synchronized egglay was then performed to yield animals for the life span assay. These were grown at 20°C until the L4 larval stage, at which time they were picked to separate plates and grown until they were Day 2 adults. They were then transferred to NGM plates (10-15 animals per plate) containing 25 μg/mL (100 μM) 5-fluoro-2'-deoxyuridine (FUDR; Sigma-Aldrich, St. Louis, MO, USA) and 10 μg/mL nystatin (Sigma-Aldrich) and seeded with 20X concentrated OP50. Animals were incubated at 20°C and scored every 1-2 days. Animals that were not moving, did not respond to prodding, and did not exhibit pharyngeal pumping were scored as dead and removed. Animals that died due to desiccation on the side of the plate, a compromise in vulval integrity, or bagging were

censored. Statistical significance was assessed using the standard chi-square-based log-rank test in GraphPad Prism (GraphPad Software; La Jolla, CA, USA).

Dauer assays

Dauer assays were performed at 25° or 27°C as previously described [38]. Briefly, animals were synchronized in a 4-6 hour egglay and grown at 25° or 27°C on NGM plates. Dauers were scored when wild-type animals were gravid adults and *daf-2(e1368)* or *akt-1(mg306)* mutant animals were arrested as dauers (approximately 60-84 hours after egglay). *sgk-1* null mutant animals were plated twelve hours prior to other strains to compensate for developmental delay. Plates were observed for two additional days after initial scoring to account for possible dauer arrest in animals with severe developmental delay.

Stress resistance assays

Animals were grown at 20°C for 48 hours after a 4-6 hour egglay until most animals were L4 larvae. *sgk-1(null)* animals were grown starting 12 hours earlier than other strains for L4 synchronization due to developmental delay [26,29,31]. Young adults, L3 larvae, and males were removed by suction. Cohorts were sufficiently large to allow for thermotolerance, oxidative stress, and UV assays to be performed in parallel. All assays were performed in triplicate.

For oxidative stress assays, L4 larvae were transferred to fresh seeded NGM plates, grown for an additional 18 hours, washed two or three times with M9 buffer, and diluted to a concentration of ~ 50 animals/mL of M9. 0.5 mL of animals was dispensed to Eppendorf tubes and rocked for ~ 20 minutes to allow animals to digest *E. coli*. Four tubes were used per genotype per concentration of H_2O_2. 0.5 mL of H_2O_2 dissolved in M9 was then added to each tube to the final concentration, followed by rocking for 2 hours protected from light. The H_2O_2 solution was then removed and the animals were washed with M9. Animals were then pipetted back onto fresh NGM plates and scored after an 18-hour recovery period at 20°C. Two-way ANOVA was conducted using

GraphPad Prism, with survival of animals on each plate as the dependent variable and H_2O_2 dose and genotype as independent variables.

UV stress assays were performed as described [47]. Briefly, animals were transferred to plates containing 25 μg/mL FUDR on Day 1 of adulthood. After four days, they were transferred to plates lacking bacteria and irradiated with 1200 J/m^2 UV-C using a Stratalinker 2400 UV crosslinker (Stratagene, La Jolla, CA). They were then transferred onto NGM plates with food and scored daily for survival. Statistical significance was assessed using the standard chi-square-based log-rank test.

Thermotolerance assays were performed essentially as described [15]. Briefly, L4 larvae were transferred to fresh seeded NGM plates (~20 per plate), and then grown for an additional 18 hours prior to shifting them to an incubator set at 35°C. Four plates were used per genotype per time point. At each time point, plates to be scored were removed and incubated further for 18 hours at 20°C, after which living and dead animals were scored. Two-way ANOVA was conducted using GraphPad Prism, with survival of animals on each plate as the dependent variable and time at 35°C and genotype as independent variables.

DAF-16A::GFP localization assays

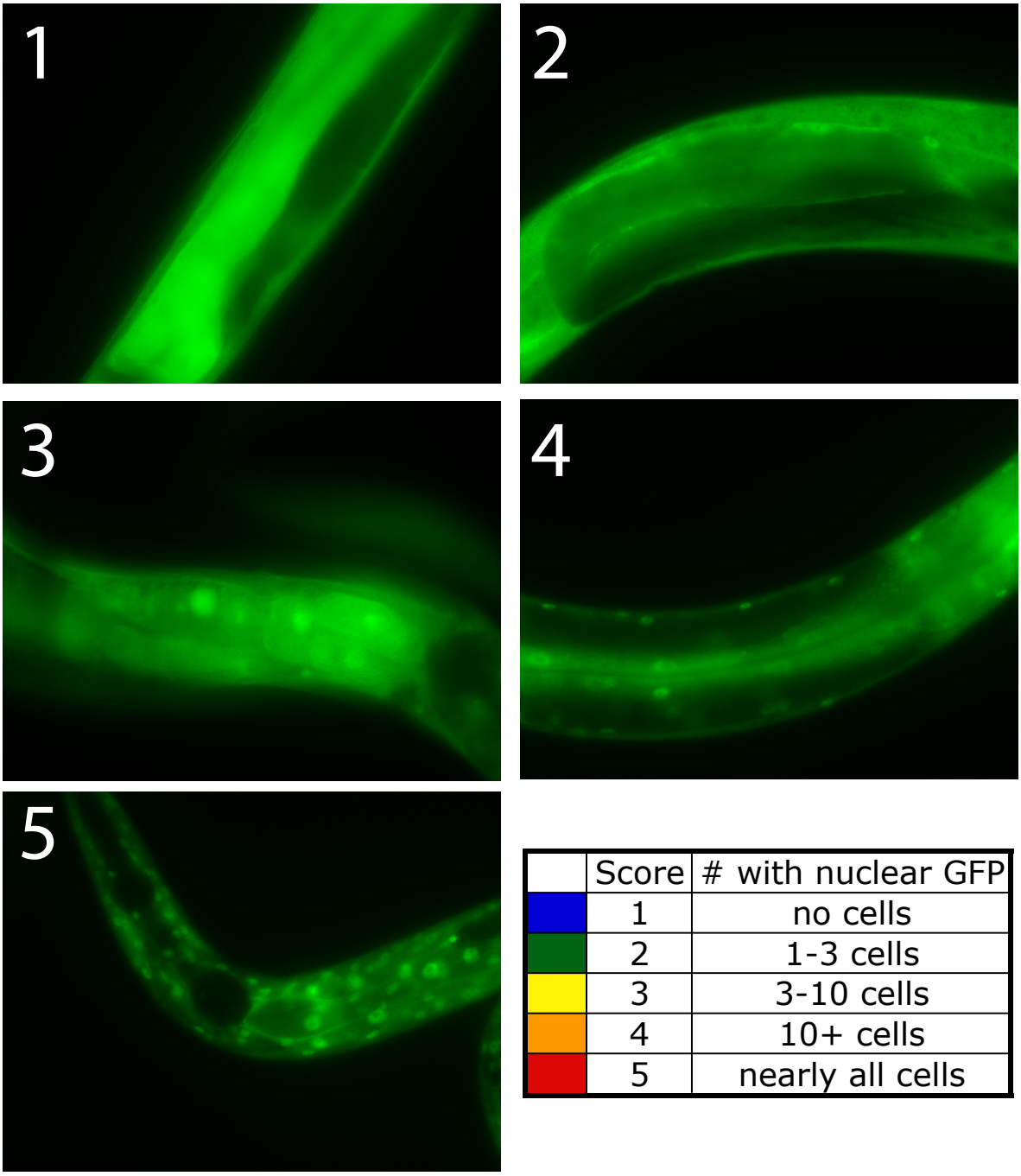
Animals were mounted onto slides in M9 with 10 mM sodium azide. Approximately ten young adults were picked to each slide, and the anterior segment of each animal was imaged within five minutes after mounting. Images were scored according to the criteria shown in Figure S1. Both imaging and scoring were performed in a blinded fashion. Two-way ANOVA was used to assess statistical significance in GraphPad Prism.

Quantitative RT-PCR

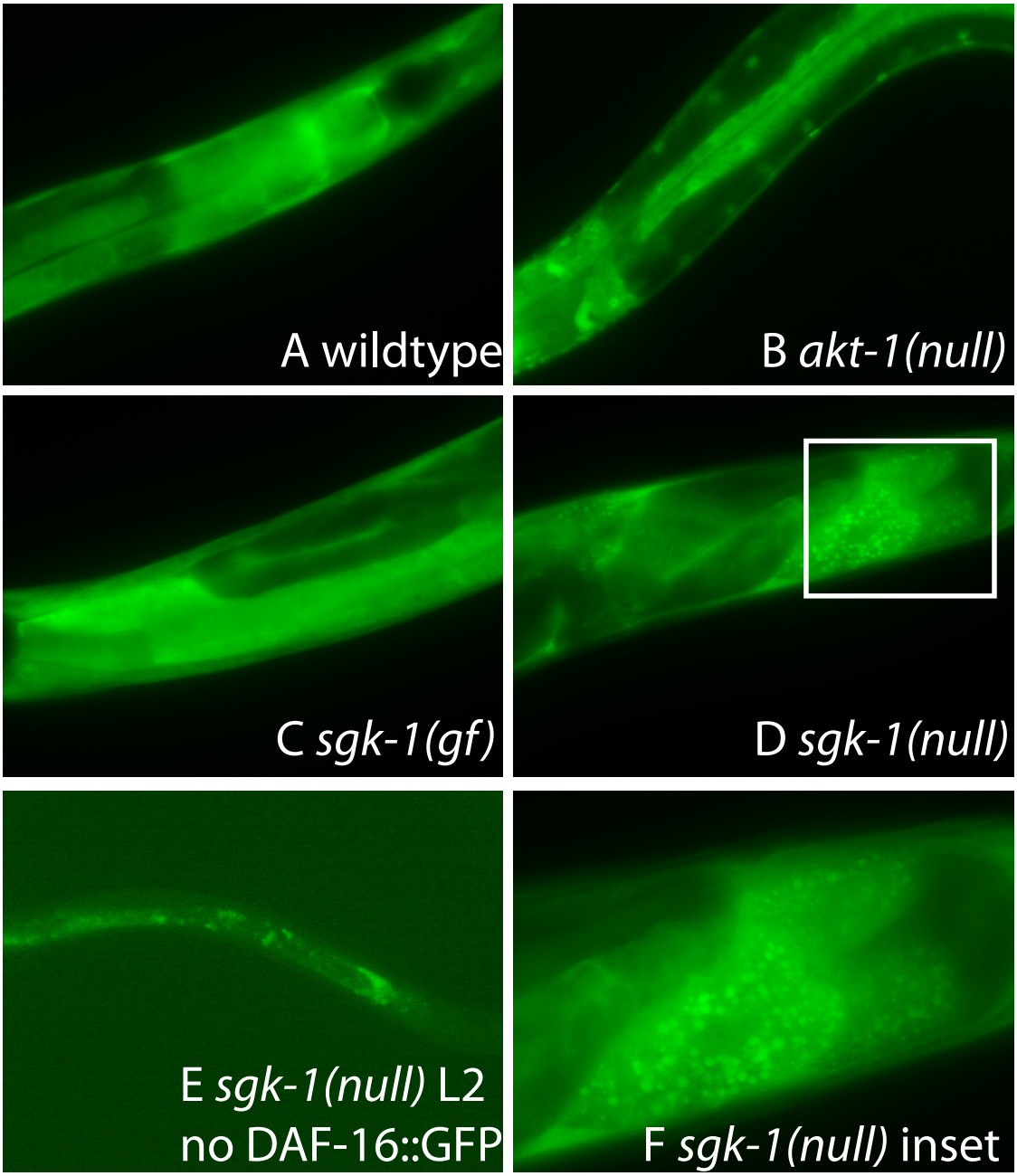
Animals from a 4.5 hour egglay were grown at 20°C for 48 hours until most animals were L4 larvae. *sgk-1(null)* animals were grown starting 12 hours earlier than other strains for L4 synchronization due to developmental delay [26,29,31]. Young adults and

L3 larvae were removed by suction, and the remaining animals were grown for an additional 12 hours. Total RNA was isolated from 600-1000 young adults per strain per biological replicate using TRIzol (Invitrogen; Carlsbad, CA, USA) and purified using an RNeasy Kit (QIAGEN Inc.; Valencia, CA, USA). cDNA was synthesized using a Superscript III Reverse Transcriptase Kit (Invitrogen). SYBR Green (Applied Biosystems, Warrington, UK) Real Time PCR was then performed using primers corresponding to the DAF-16/FoxO target genes *sod-3, nnt-1, sip-1, dod-3,* and *mtl-1*. *act-1* was used as an internal control. Quantitative PCR primer sequences are listed in Supplemental Table 2.7. Statistical analysis was performed in GraphPad Prism by unpaired two-tailed t-test with Welch's correction.

Supplemental Information

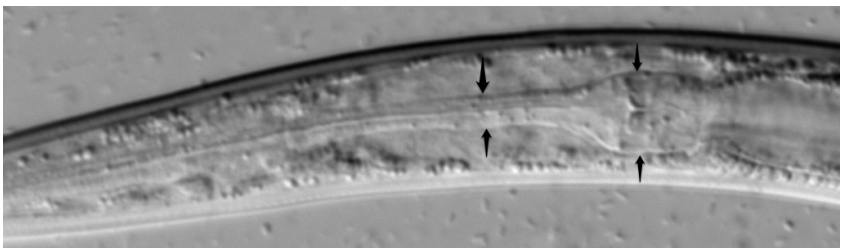


Supplemental Figure 2.1. Criteria for blind scoring of DAF-16::GFP subcellular localization.
daf-16(mu86) mutant animals carrying high-copy DAF-16::GFP (TJ356) and *sgk-1* or *akt-1* alleles (see Supplemental Figure 2.2) were grown at 20°C and picked to slides as young adults. The anterior of each animal was imaged and scored blindly and classified into one of five groups by the criteria shown.

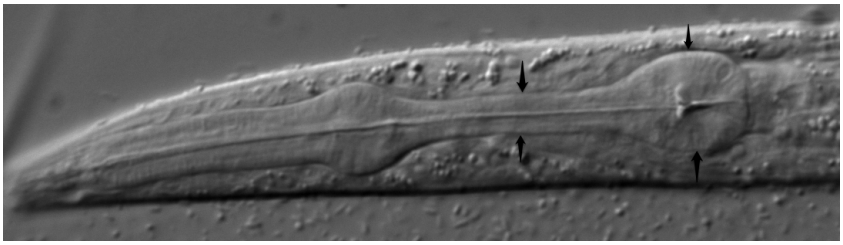


Supplemental Figure 2.2. Example photographs of wildtype, *sgk-1(null)*, *sgk-1(gf)*, and *akt-1(null)* animals expressing DAF-16::GFP.
daf-16(mu86);DAF-16::GFP (TJ356) were built with wildtype (A), *akt-1(mg306)* null (B), *sgk-1(ft15)* gain-of-function (C), *sgk-1(ok538)* null (D) alleles, and imaged and scored as described in Fig. S1. *sgk-1(ok538)* null animals showed autofluorescent granules (inset) in their intestines that were too small to be nuclei and were present in non-GFP animals, as early as the L2 larval stage (E), and thus were not scored as nuclei in DAF-16::GFP localization assays (F).

daf-2(e1368)
dauer



sgk-1(mg455)
arrested larva



Supplemental Figure 2.3. Example photograph of the pharynx of an arrested *sgk-1(null)* larva at 27°C (lower panel).
For comparison, a remodeled pharynx of a *daf-2(e1368)* dauer is shown in the upper panel.

relevant figures	genotype	deaths (censored)	mean survival (days)	SD	median survival (days)	P value (Log-rank)	P value compared to	% change mean survival	% change median survival	P value (Log-rank)	P value compared to	% change mean survival	% change median survival
Figure 1B	wildtype sib of *ft15*	93(3)	20.0	7.0	21	n/a	n/a	n/a	n/a				
replicate 1	*sgk-1(ft15)*	98(1)	23.5	7.8	23	0.0008	wildtype sib of *ft15*	+17.5	+9.5				
***shown figure**	*sgk-1(ok538)*	101(0)	14.5	5.4	17	<0.0001	wildtype sib of *ft15*	-27.5	-19.0				
	sgk-1(mg455)	100(1)	14.5	5.9	14	<0.0001	wildtype sib of *ft15*	-27.5	-33.3	0.35	*sgk-1(ok538)*	0	-17.6
Figure 1B	wildtype sib of ft15	64(2)	25.1	5.9	26	n/a	n/a	n/a	n/a				
replicate 2	*sgk-1(ft15)*	56(4)	28.5	9.0	27	0.0005	wildtype sib of *ft15*	+13.5	+3.8				
Figure 1C	*sgk-1(ok538)*	63(0)	12.3	3.6	12	<0.0001	wildtype sib of *ft15*	-51.0	-53.8				
replicate 1	*sgk-1(mg455)*	65(0)	12.1	1.4	12	<0.0001	wildtype sib of *ft15*	-51.2	-53.8	0.8091	*sgk-1(ok538)*	-1.6	0
***shown figure**	*daf-16(mu86);sgk-1(ft15)*	45(2)	17.6	2.7	16	<0.0001	*sgk-1(ft15)*						
	akt-1(mg306)	59(2)	30.0	7.6	28	<0.0001	wildtype sib of *ft15*	+19.5	+7.7				
	daf-16;akt-1	54(4)	15.3	2.1	16	<0.0001	*akt-1(mg306)*			0.0001	*daf-16;sgk-1(ft15)*	-13.0	0
Figure 1B	wildtype sib of *ft15*	97(2)	20.7	5.5	21	n/a	n/a	n/a	n/a				
replicate 3	*sgk-1(ft15)*	91(8)	21.5	5.1	21	0.5082	wildtype sib of *ft15*	+3.9	0				
Figure 1C	*sgk-1(ok538)*	98(0)	10.9	4.0	10	<0.0001	wildtype sib of *ft15*	-47.3	-52.4				
replicate 2	*sgk-1(mg455)*	100(0)	11.1	3.6	11	<0.0001	wildtype sib of *ft15*	-46.4	-47.6	0.7079	*sgk-1(ok538)*	+1.8	+10.0
	daf-16(mu86);sgk-1(ft15)	96(5)	13.1	1.5	13	<0.0001	*sgk-1(ft15)*						
	akt-1(mg306)	61(4)	32.2	5.8	34	<0.0001	wildtype sib of *ft15*	+55.5	+61.9				
	daf-16;akt-1	95(4)	13.5	1.5	14	<0.0001	*akt-1(mg306)*			0.5116	*daf-16;sgk-1(ft15)*	+3.1	+7.7
Figure 1C	wildtype sib of *ft15*	61(39)	17.2	2.8	18	n/a	n/a	n/a	n/a				
replicate 3	N2 wildtype	65(35)	16.6	2.8	17	0.857	wildtype sib of *ft15*	-3.5	-5.6				
	sgk-1(ft15)	55(45)	15.9	3.7	15	0.0659	wildtype sib of *ft15*	-7.6	-16.6				
	daf-16(mu86)	61(43)	13.3	1.9	13	<0.0001	wildtype sib of *ft15*	-22.7	-28.2				
	daf-16(mu86);sgk-1(ft15)	77(24)	13.3	1.6	13	<0.0001	*sgk-1(ft15)*	-16.3	-13.3	0.217	*daf-16(mu86)*	0	0
Figure 1D	wildtype	91(10)	21.1	2.6	20	n/a	n/a	n/a	n/a				
all are siblings	*sgk-1(ft15)*	79(21)	23.1	4.2	23	<0.0001	wildtype	+9.5	+15.0				
replicate 1	*akt-1(mg306)*	87(13)	23.7	3.2	24	<0.0001	wildtype	+12.3	+20.0				
***shown figure**	*akt-1;sgk-1*	74(26)	22.8	3.7	23	0.4614	*sgk-1(ft15)*	-1.3	0	0.3489	*akt-1(mg306)*	-1.3	-4.2
Figure 1D	wildtype	82(18)	20.3	3.1	21	n/a	n/a	n/a	n/a				
all are siblings	*sgk-1(ft15)*	79(21)	21.9	3.1	22	0.0031	wildtype	+7.9	+4.8				
replicate 2	*akt-1(mg306)*	85(15)	24.1	2.8	24	<0.0001	wildtype	+18.7	+14.3				
	akt-1;sgk-1	73(27)	24.5	5.0	24	<0.0001	*sgk-1(ft15)*	+11.9	+9.1	0.0375	*akt-1(mg306)*	+1.7	0
Figure 1E	wildtype	70(8)	18.0	3.1	19								
first four are	*sgk-1(ft15)*	56(53)	20.1	3.7	19	<0.0001	wildtype	+11.7	0				
siblings	*hcf-1(pk924)*	73(27)	21.3	5.4	21	<0.0001	wildtype	+18.3	+10.5				
replicate 1	*hcf-1;sgk-1(ft15)*	81(19)	20.5	5.0	21	0.413	*sgk-1(ft15)*	+2.0	+10.5	0.4130	*hcf-1(pk924)*	-3.8	0
***shown figure**	*hcf-1;sgk-1(ok538)*	96(4)	10.8	1.5	11	<0.0001	wildtype	-40.0	-42.1	<0.0001	*hcf-1(pk924)*	-49.3	-47.6
	hcf-1;sgk-1(mg455)	100(0)	10.9	1.4	10	<0.0001	wildtype	-39.4	-47.4	<0.0001	*hcf-1(pk924)*	-48.8	-52.4
Figure 1E	wildtype	91(10)	18.7	3.0	18	n/a	n/a	n/a	n/a				
first four are	*sgk-1(ft15)*	67(28)	19.8	5.9	19	0.0309	wildtype	+5.9	+5.6				
siblings	*hcf-1(pk924)*	79(21)	20.6	5.8	21	<0.0001	wildtype	+10.2	+16.7				
replicate 2	*hcf-1;sgk-1(ft15)*	75(24)	22.0	5.5	22	0.0642	*sgk-1(ft15)*	+11.1	+15.8	0.2348	*hcf-1(pk924)*	+6.8	+4.8
	hcf-1;sgk-1(ok538)	100(0)	11.2	1.8	11	<0.0001	wildtype	-40.1	-38.9	<0.0001	*hcf-1(pk924)*	-45.6	-47.6
	hcf-1;sgk-1(mg455)	93(7)	11.5	1.6	11	<0.0001	wildtype	-38.5	-38.9	<0.0001	*hcf-1(pk924)*	-44.2	-47.6

Table S1B. Additional life span data using N2 wildtype

Figure 1B	N2 wildtype	69(31)	14.3	1.5	14.5	n/a	n/a	n/a	n/a				
	sgk-1(ft15)	71(29)	17.8	3.8	17	<0.0001	N2 wildtype	+24.5	+17.2				
Figure 1B	N2 wildtype	77(23)	16.6	2.3	16.5	n/a	n/a	n/a	n/a				
	sgk-1(ft15)	96(4)	21.4	3.7	20	<0.0001	N2 wildtype	+28.9	+21.2				
Figure 1B	N2 wildtype	63(37)	14.3	2.0	14.5	n/a	n/a	n/a	n/a				
	sgk-1(ft15)	73(29)	17.0	3.6	17	<0.0001	N2 wildtype	+18.9	+17.2				
Figure 1B	N2 wildtype	70(30)	14.9	1.8	14.5	n/a	n/a	n/a	n/a				
Figure 1C	*sgk-1(ft15)*	84(16)	18.4	4.7	17.5	<0.0001	N2 wildtype	+23.5	+20.7				
	daf-16(mu86)	93(8)	14.2	1.8	14.5	0.0313	N2 wildtype	-4.7	0				
	daf-16;sgk-1	84(16)	13.6	2.0	13.5	0.0002	N2 wildtype	-8.7	-6.9	0.0584	*daf-16(mu86)*	-4.2	-6.9

Table S1C. Additional life span data on different *E. coli* strains

Figure 1B	wildtype sib of *ft15*	94(8)	22.8	2.8	22	n/a	n/a	n/a	n/a				
replicate 4	*sgk-1(ft15)*	84(20)	24.4	3.3	25	0.0005	wildtype sib of *ft15*	+7.0	+13.6				
HT115	*sgk-1(ok538)*	101(3)	13.0	2.5	13	<0.0001	wildtype sib of *ft15*	-43.0	-40.9				
bacteria	*sgk-1(mg455)*	100(0)	12.5	2.2	13	<0.0001	wildtype sib of *ft15*	-45.2	-40.9	0.0999	*sgk-1(ok538)*	-3.8	0
Figure 1B	N2 wildtype	69(31)	18.1	2.9	18.5	n/a	n/a	n/a	n/a				
HB101	*sgk-1(ft15)*	90(10)	21.5	3.1	21.5	<0.0001	N2 wildtype	+18.8	+16.2				
bacteria	*sgk-1(ok538)*	100(0)	13.9	2.4	13.5	<0.0001	N2 wildtype	-23.2	-27.0				
	sgk-1(mg455)	100(0)	15.1	3.0	14.5	<0.0001	N2 wildtype	-16.6	-21.6	0.0028	*sgk-1(ok538)*	+8.6	+7.4

Supplemental Table 2.1. Life span raw data and statistics

Life span data and statistics for (A) each replicate of Figure 2.1, (B) replicates using N2 wild-type as a control, and (C) replicates feeding HB101 or HT115 bacteria. All assays were conducted at 20°C. We used GraphPad Prism (GraphPad Software; La Jolla, CA, USA) to perform statistical analysis, including the log-rank (Mantel-Cox) test. Data used in Figures 1B-1E are labeled as "* shown figure."

relevant figures	genotype	# animals	mean lethal H2O2 conc. (μM * 2 hours)	SD	median lethal H2O2 conc. (μM * 2 hours)	P value (two-way ANOVA)	P value compared to	% change mean lethal H2O2 conc.	% change median lethal H2O2 conc.	P value (two-way ANOVA)	P value compared to	% change mean lethal H2O2 conc.	% change median lethal H2O2 conc.
Figure 2A	N2 wildtype	188	322	44	300	n/a	n/a	n/a	n/a				
replicate 1	*sgk-1(ok538)*	274	262	42	250	0.0002	N2 wildtype	-18.6	-16.7				
***shown figure**	*sgk-1(mg455)*	266	285	47	300	0.0012	N2 wildtype	-11.5	0	0.0689	*sgk-1(ok538)*	+8.8	+20.0
	akt-1(mg306)	328	345	62	350	0.1299	N2 wildtype	+7.1	+16.7				
Figure 2A	N2 wildtype	656	315	65	300	n/a	n/a	n/a	n/a				
replicate 2	*sgk-1(ok538)*	685	276	69	250	0.0911	N2 wildtype	-12.4	-16.7				
	sgk-1(mg455)	610	260	77	250	0.0250	N2 wildtype	-17.5	-16.7	0.4749	*sgk-1(ok538)*	-5.8	0
	akt-1(mg306)	678	327	64	300	0.3666	N2 wildtype	+3.8	0				
Figure 2A	N2 wildtype	476	304	57	300	n/a	n/a	n/a	n/a				
replicate 3	*sgk-1(ok538)*	932	242	39	250	<0.0001	N2 wildtype	-20.4	-16.7				
	sgk-1(mg455)	738	313	65	300	0.4804	N2 wildtype	+3.0	0	<0.0001	*sgk-1(ok538)*	+29.3	+20.0
	akt-1(mg306)	496	317	64	300	0.3462	N2 wildtype	+4.3	0				
Figure 2B	wildtype sib of *ft15*	319	348	53	350	n/a	n/a	n/a	n/a				
replicate 1	*sgk-1(ft15)*	306	343	42	350	0.6429	wildtype sib of *ft15*	-1.4	0				
***shown figure**	*akt-1(mg306)*	304	>400	43	>400	<0.0001	wildtype sib of *ft15*	> +14.9	> +14.3				
Figure 2B	wildtype sib of *ft15*	398	335	51	350	n/a	n/a	n/a	n/a				
replicate 2	*sgk-1(ft15)*	516	321	62	350	0.0401	wildtype sib of *ft15*	-4.2	0				
Figure 2B	wildtype sib of *ft15*	596	353	74	350	n/a	n/a	n/a	n/a				
replicate 3	*sgk-1(ft15)*	688	335	64	350	0.1173	wildtype sib of *ft15*	-5.1	0				

Supplemental Table 2.2. H2O2 survival data and statistics

Young adult animals were subjected to H2O2 treatment in M9 for 2 hours in Eppendorf tubes, allowed to recover for 18 hours, and then scored for survival. Animal counts listed for each genotype are split among 5 treatments ranging from 200 to 400 μM and between 4 tubes per genotype per treatment. Survival for each tube was calculated, allowing mean and median lethal dose to be calculated. Pairing by H2O2 concentration was used to conduct a Student's t-test in GraphPad Prism. Data used in Figures 2A and 2D are labeled as '*.'

relevant figures	genotype	# animals	mean survival (hours)	SD	median survival (hours)	P value (Log-rank)	P value compared to	% change mean survival	% change median survival	P value (Log-rank)	P value compared to	% change mean survival	% change median survival
Figure 2C	N2 wildtype	100	162.3	35.5	167	n/a	n/a	n/a	n/a				
replicate 1	*sgk-1(mg455)*	99	80.0	27.3	69	<0.0001	N2 wildtype	-50.7	-41.3				
***shown figure**	*sgk-1(ok538)*	98	82.1	29.8	69	<0.0001	N2 wildtype	-49.4	-41.3	0.3953	*sgk-1(mg455)*	-2.6	0
	akt-1(mg306)	100	175.5	29.5	190	<0.0001	N2 wildtype	+8.1	+13.8				
Figure 2C	N2 wildtype	65	135.2	26.7	144	n/a	n/a	n/a	n/a				
replicate 2	*sgk-1(mg455)*	34	112.8	25.4	118	0.0044	N2 wildtype	-16.6	-18.1				
	sgk-1(ok538)	69	94.4	34.8	95	<0.0001	N2 wildtype	-30.2	-34.0	0.0019	*sgk-1(mg455)*	-16.3	-34.0
Figure 2C	N2 wildtype	87	121.1	21.4	122	n/a	n/a	n/a	n/a				
replicate 3	*sgk-1(mg455)*	97	89.8	23.9	96	<0.0001	N2 wildtype	-25.8	-21.3				
Figure 2D	*sgk-1(ok538)*	100	82.2	21.4	66	<0.0001	N2 wildtype	-32.1	-45.9	0.0023	*sgk-1(mg455)*	-8.5	-31.2
replicate 1	*sgk-1(ft15)*	100	115.6	22.1	122	0.8032	N2 wildtype	-4.5	0				
	akt-1(mg306)	73	131.9	15.0	>142	<0.0001	N2 wildtype	+8.9	> 16.4				
Figure 2D	wildtype sib of *ft15*	100	145.5	35.1	149	n/a	n/a	n/a	n/a				
replicate 2	*sgk-1(ft15)*	99	134.9	26.1	149	0.0057	wildtype sib of *ft15*	-7.3	0				
***shown figure**	akt-1(mg306)	99	170.0	37.3	164	<0.0001	wildtype sib of *ft15*	+16.8	+10.1				
Figure 2D	wildtype sib of *ft15*	60	174.7	37.3	196	n/a	n/a	n/a	n/a				
replicate 3	*sgk-1(ft15)*	51	160.9	40.2	176	0.0163	wildtype sib of *ft15*	-7.9	-10.2				
	akt-1(mg306)	59	192.8	26.0	196	0.0008	wildtype sib of *ft15*	+10.4	0				

Supplemental Table 2.3. UV survival data and statistics

Synchronized animals were maintained on FUDR plates until they were 4 days old, transferred to plates without food and exposed to ultraviolet radiation (1200 J/m2 UV-C) using a Stratalinker UV Crosslinker. Survival was scored daily for each animal from time of UV exposure until death, similar to life span assays. We used GraphPad Prism to perform statistical analysis, including the log-rank (Mantel-Cox) test. Data used in Figures 2B and 2E are labeled as '*.'

relevant figures	genotype	# animals	mean lethal time (hours at 35°C)	SD	median lethal time (hours at 35°C)	P value (two-way ANOVA)	P value compared to	% change mean lethal time	% change median lethal time	P value (two-way ANOVA)	P value compared to	% change mean lethal time	% change median lethal time
Figure 2E	N2 wildtype	414	3.57	0.37	3.42	n/a	n/a	n/a	n/a				
replicate 1	*sgk-1(ok538)*	504	4.09	0.37	4.25	<0.0001	N2 wildtype	+14.6	+24.3				
***shown figure**	*sgk-1(mg455)*	551	4.15	0.36	3.83	0.0040	N2 wildtype	+16.2	+7.3	0.0561	*sgk-1(ok538)*	+1.5	-9.9
	akt-1(mg306)	432	3.88	0.48	4.25	<0.0001	N2 wildtype	+8.7	+24.3				
Figure 2E	N2 wildtype	485	3.94	0.69	3.5	n/a	n/a	n/a	n/a				
replicate 2	*sgk-1(ok538)*	445	5.15	0.70	5.17	<0.0001	N2 wildtype	+30.7	+47.7				
	sgk-1(mg455)	424	5.15	0.67	5.17	<0.0001	N2 wildtype	+30.7	+47.7	0.3041	*sgk-1(ok538)*	0	0
	akt-1(mg306)	439	4.79	0.80	4.83	<0.0001	N2 wildtype	+21.6	+38.0				
Figure 2E	N2 wildtype	649	3.39	0.38	3.83	n/a	n/a	n/a	n/a				
replicate 3	*sgk-1(ok538)*	558	3.92	0.43	4.25	0.0046	N2 wildtype	+15.6	+11.0				
	sgk-1(mg455)	634	3.73	0.49	4.25	0.0045	N2 wildtype	+10.0	+11.0	0.6014	*sgk-1(ok538)*	-4.8	0
	akt-1(mg306)	603	3.72	0.50	3.83	0.0125	N2 wildtype	+9.7	0				
Figure 2F	wildtype sib of *ft15*	567	3.89	0.59	4	n/a	n/a	n/a	n/a				
replicate 1	*sgk-1(ft15)*	671	3.97	0.60	4	0.5288	wildtype sib of *ft15*	+2.1	0				
***shown figure**	*akt-1(mg306)*	645	4.62	0.62	4.5	<0.0001	wildtype sib of *ft15*	+18.8	+12.5				
Figure 2F	wildtype sib of *ft15*	407	4.21	0.55	4	n/a	n/a	n/a	n/a				
replicate 2	*sgk-1(ft15)*	490	4.34	0.67	4.67	0.1980	wildtype sib of *ft15*	+3.1	+20				
	akt-1(mg306)	460	4.42	0.66	4.67	0.1309	wildtype sib of *ft15*	+5.0	+20				
Figure 2F	wildtype sib of *ft15*	634	4.14	0.44	4.25	n/a	n/a	n/a	n/a				
replicate 3	*sgk-1(ft15)*	520	4.26	0.50	4.25	0.2377	wildtype sib of *ft15*	+2.9	0				
	akt-1(mg306)	563	4.45	0.44	4.25	0.0157	wildtype sib of *ft15*	+7.5	0				
Figure 2G	N2 wildtype	190	2.92	0.26	3	n/a	n/a	n/a	n/a				
replicate 1	*daf-16(mu86)*	197	3.04	0.22	3	0.0635	N2 wildtype	+4.1	0				
***shown figure**	*sgk-1(ok538)*	170	3.30	0.18	3.33	<0.0001	N2 wildtype	+13.0	+11.1				
Figure 2E	*daf-16;sgk-1(ok538)*	156	3.26	0.29	3.33	0.7658	*sgk-1(ok538)*	-1.2	0	0.0185	*daf-16(mu86)*	+7.2	+11.1
	sgk-1(mg455)	164	3.29	0.19	3.33	0.0005	N2 wildtype	+12.7	+11.1				
	daf-16;sgk-1(mg455)	155	3.37	0.21	3.33	0.4355	*sgk-1(mg455)*	+2.4	0	0.0078	*daf-16(mu86)*	+10.9	+11.1
Figure 2G	N2 wildtype	228	3.92	0.49	4.25	n/a	n/a	n/a	n/a				
replicate 2	*daf-16(mu86)*	304	4.12	0.46	4.25	0.0087	N2 wildtype	+5.1	0				
Figure 2E	*sgk-1(ok538)*	216	4.49	0.15	4.5	<0.0001	N2 wildtype	+14.5	+5.9				
	daf-16;sgk-1(ok538)	218	4.48	0.14	4.5	0.8540	*sgk-1(ok538)*	-0.2	0	0.0022	*daf-16(mu86)*	+8.7	+5.9
Figure 2G	N2 wildtype	254	3.67	0.46	3.67	n/a	n/a	n/a	n/a				
replicate 3	*daf-16(mu86)*	240	3.87	0.49	4	0.0204	N2 wildtype	+5.4	+9.0				
Figure 2E	*sgk-1(ok538)*	245	4.04	0.35	4.25	0.1259	N2 wildtype	+10.1	+15.8				
	daf-16;sgk-1(ok538)	209	4.31	0.27	4.25	0.1571	*sgk-1(ok538)*	+6.7	0	0.0027	*daf-16(mu86)*	+11.4	+6.3
Figure 2G	N2 wildtype	260	3.39	0.35	3.33	n/a	n/a	n/a	n/a				
replicate 4	*daf-16(mu86)*	284	3.45	0.29	3.33	0.6399	N2 wildtype	+1.8	0				
Figure 2E	*sgk-1(mg455)*	204	3.84	0.25	3.83	0.0011	N2 wildtype	+13.3	+15.0				
	daf-16;sgk-1(mg455)	202	3.73	0.33	3.83	0.4184	*sgk-1(mg455)*	-2.9	0	0.0427	*daf-16(mu86)*	+8.1	+15.0
Figure 2G	N2 wildtype	274	3.52	0.27	3.33	n/a	n/a	n/a	n/a				
replicate 5	*daf-16(mu86)*	246	3.56	0.24	3.58	0.6990	N2 wildtype	+1.1	+7.5				
Figure 2E	*sgk-1(mg455)*	224	3.83	0.06	3.58	0.0015	N2 wildtype	+8.8	+7.5				
	daf-16;sgk-1(mg455)	229	3.70	0.24	3.83	0.1781	*sgk-1(mg455)*	-3.4	+7.0	0.2681	*daf-16(mu86)*	+3.9	+7.0

Supplemental Table 2.4. Thermotolerance data and statistics

Young adult animals were subjected to 35°C for 3 to 6 hours on NGM plates, allowed to recover for 18 hours, and then scored for survival. Animal counts listed for each genotype are split among 6 or 7 time points and between 4 plates per genotype per time point. Survival for each plate was calculated, allowing mean and median lethal time exposure to be calculated. Pairing by time point was used to conduct a Student's t-test in GraphPad Prism. Data used in Figure 2.2 are labeled as '*.'

Supplemental Table 2.5. DAF-16A::GFP subcellular localization data.

daf-16(mu86) mutant animals carrying a DAF-16A::GFP (TJ356, Henderson and Johnson, 2001) and *sgk-1* or *akt-1* alleles were grown, imaged, and scored as described in Figure S2.

Localization Score	wildtype	*akt-1(mg306)*	*sgk-1(ok538)*	*sgk-1(ft15)*
1	38	0	23	40
2	27	6	30	20
3	8	39	19	21
4	2	25	3	3
5	0	0	0	0
N	75	70	75	84
P-value vs wildtype	n/a	<0.0001	0.1733	0.5037

Supplemental Table 2.6. qPCR data and statistics

Five DAF-16/FoxO targets were each measured in six independent biological replicates, although not all genotypes were included in every replicate. All animals listed in the same column were grown and harvested at the same time under identical conditions. Three technical replicate measurements were performed per target/genotype/biological replicate and compared to three technical replicate measurements of *act-1*. All data is normalized to wildtype within the same cohort. An unpaired two-tailed t-test with Welch's correction ($P < 0.05$) was performed to make relevant comparisons, and statistically significant changes are bolded. Note that *sgk-1* null mutant changes in *dod-3* and *mtl-1* gene expression did not always reach statistical significance due to high variability.

	Expression normalized to *act-1* compared to wildtype											
	Independent cohorts						Statistical Analysis					
	1	2	3	4	5	6	Mean	SD	P value (unpaired t-test)	P value compared to	Fold change	# cohorts showing trend
sod-3												
wildtype	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	n/a	n/a	n/a	n/a
akt-1(mg306)	2.00	4.20	n/a	2.41	4.23	2.62	3.09	1.05	**0.0112**	**wildtype**	**3.09**	**5 of 5**
daf-16(mu86);akt-1(mg306)	0.52	0.70	n/a	0.54	0.72	0.37	0.57	0.14	**0.0054**	***akt-1(mg306)***	**0.18**	**5 of 5**
sgk-1(ft15)	0.57	1.04	1.09	0.69	1.02	0.51	0.82	0.26	0.1506	wildtype	0.82	3 of 6
daf-16;sgk-1(ft15)	0.44	0.52	0.54	0.43	0.49	0.22	0.44	0.11	**0.0140**	***sgk-1(ft15)***	**0.53**	**6 of 6**
sgk-1(ok538)	0.25	0.41	0.60	0.67	0.27	0.33	0.42	0.18	**0.0005**	**wildtype**	**0.42**	**6 of 6**
daf-16;sgk-1(ok538)	0.20	0.16	0.10	n/a	n/a	n/a	0.15	0.05	**0.0126**	***sgk-1(ok538)***	**0.37**	**3 of 3**
sgk-1(mg455)	0.48	0.43	0.54	0.45	0.48	0.28	0.44	0.09	**<0.0001**	**wildtype**	**0.44**	**6 of 6**
daf-16;sgk-1(mg455)	0.26	0.16	0.19	n/a	n/a	n/a	0.20	0.05	**0.0016**	***sgk-1(mg455)***	**0.45**	**3 of 3**
nnt-1												
wildtype	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	n/a	n/a	n/a	n/a
akt-1(mg306)	4.29	1.95	n/a	1.01	3.89	3.20	2.87	1.37	**0.0378**	**wildtype**	**2.87**	**4 of 5**
daf-16(mu86);akt-1(mg306)	0.72	1.33	n/a	0.26	0.80	0.45	0.71	0.41	**0.0216**	***akt-1(mg306)***	**0.25**	**5 of 5**
sgk-1(ft15)	1.05	1.09	0.80	0.55	1.08	0.47	0.84	0.28	0.2172	wildtype	0.84	2 of 6
daf-16;sgk-1(ft15)	0.59	0.84	0.56	0.50	0.69	0.36	0.59	0.16	0.0945	*sgk-1(ft15)*	0.70	6 of 6
sgk-1(ok538)	0.72	1.60	0.72	0.70	1.27	0.82	0.97	0.38	0.8676	wildtype	0.97	n/a
daf-16;sgk-1(ok538)	0.42	0.59	0.34	n/a	n/a	n/a	0.45	0.13	**0.0192**	***sgk-1(ok538)***	**0.46**	**3 of 3**
sgk-1(mg455)	1.13	1.16	0.57	0.74	1.24	0.91	0.96	0.26	0.7127	wildtype	0.96	n/a
daf-16;sgk-1(mg455)	0.62	0.39	0.24	n/a	n/a	n/a	0.42	0.19	**0.0143**	***sgk-1(mg455)***	**0.44**	**3 of 3**
sip-1												
wildtype	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	n/a	n/a	n/a	n/a
akt-1(mg306)	3.61	3.48	n/a	1.82	2.08	2.13	2.62	0.85	**0.0129**	**wildtype**	**2.62**	**5 of 5**
daf-16(mu86);akt-1(mg306)	0.69	0.29	n/a	0.34	0.44	0.52	0.46	0.16	**0.0040**	***akt-1(mg306)***	**0.17**	**5 of 5**
sgk-1(ft15)	1.14	1.15	1.42	1.21	0.91	0.97	1.13	0.18	0.1369	wildtype	1.13	2 of 6
daf-16;sgk-1(ft15)	0.64	0.73	0.75	0.61	0.52	0.51	0.63	0.10	**0.0004**	***sgk-1(ft15)***	**0.55**	**6 of 6**
sgk-1(ok538)	1.41	3.36	2.04	1.16	1.06	1.05	1.68	0.90	0.1239	wildtype	1.68	3 of 6
daf-16;sgk-1(ok538)	0.73	0.99	0.62	n/a	n/a	n/a	0.78	0.19	0.0590	*sgk-1(ok538)*	0.46	3 of 3
sgk-1(mg455)	0.76	2.00	2.69	1.54	1.03	1.25	1.54	0.71	0.1174	wildtype	1.54	4 of 6
daf-16;sgk-1(mg455)	1.59	1.48	0.80	n/a	n/a	n/a	1.29	0.43	0.5260	*sgk-1(mg455)*	0.84	2 of 3
dod-3												
wildtype	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	n/a	n/a	n/a	n/a
akt-1(mg306)	8.06	4.17	n/a	2.38	5.50	5.58	5.14	2.09	**0.0114**	**wildtype**	**5.14**	**5 of 5**
daf-16(mu86);akt-1(mg306)	0.78	0.22	n/a	0.50	0.55	0.92	0.59	0.27	**0.0078**	***akt-1(mg306)***	**0.12**	**5 of 5**
sgk-1(ft15)	0.43	0.33	0.52	0.66	0.54	0.30	0.46	0.14	**0.0002**	**wildtype**	**0.46**	**6 of 6**
daf-16;sgk-1(ft15)	0.28	0.38	0.50	0.34	0.59	0.36	0.41	0.11	0.4645	*sgk-1(ft15)*	0.88	2 of 6
sgk-1(ok538)	3.43	11.00	4.26	1.01	2.81	0.76	3.88	3.75	0.1186	wildtype	3.88	4 of 6
daf-16;sgk-1(ok538)	1.21	4.08	1.29	n/a	n/a	n/a	2.20	1.63	0.3798	*sgk-1(ok538)*	0.57	3 of 3
sgk-1(mg455)	0.86	6.50	3.89	0.90	3.86	1.77	2.96	2.20	0.0809	wildtype	2.96	4 of 6
daf-16;sgk-1(mg455)	1.05	3.92	1.13	n/a	n/a	n/a	2.03	1.63	0.5050	*sgk-1(mg455)*	0.69	2 of 3
mtl-1												
wildtype	1.00	1.00	1.00	1.00	1.00	1.00	1.00	0.00	n/a	n/a	n/a	n/a
akt-1(mg306)	6.73	6.15	n/a	2.33	2.68	3.03	4.18	2.08	**0.0269**	**wildtype**	**4.18**	**5 of 5**
daf-16(mu86);akt-1(mg306)	0.41	0.28	n/a	0.25	0.18	0.21	0.27	0.09	**0.0136**	***akt-1(mg306)***	**0.06**	**5 of 5**
sgk-1(ft15)	0.51	0.95	0.81	1.05	1.15	0.63	0.85	0.25	0.1968	wildtype	0.85	3 of 6
daf-16;sgk-1(ft15)	0.17	0.29	0.23	0.18	0.26	0.09	0.20	0.07	**0.0009**	***sgk-1(ft15)***	**0.24**	**6 of 6**
sgk-1(ok538)	1.31	4.50	2.83	5.82	2.95	0.85	3.04	1.88	**0.0450**	**wildtype**	**3.04**	**5 of 6**
daf-16;sgk-1(ok538)	1.75	0.85	0.39	n/a	n/a	n/a	1.00	0.69	**0.0511**	***sgk-1(ok538)***	**0.33**	**2 of 3**
sgk-1(mg455)	2.14	3.31	4.76	5.78	1.79	2.27	3.34	1.61	**0.0162**	**wildtype**	**3.34**	**6 of 6**
daf-16;sgk-1(mg455)	0.37	0.73	0.95	n/a	n/a	n/a	0.68	0.29	**0.0089**	***sgk-1(mg455)***	**0.20**	**3 of 3**

Target	Forward primer (5' to 3')	Reverse primer (5' to 3')	Reference
act-1	CCAGGAATTGCTGATCGTATGCAGAA	TGGAGAGGGAAGCGAGGATAGA	Alam *et al.* 2010
sod-3	TATTAAGCGCGACTTCGGTTCCCT	CGTGCTCCCAAACGTCAATTCCAA	Alam *et al.* 2010
nnt-1	CAGTAGAAACTGCTGACATGCTTC	GAGCGATGGGATATTGTGCCTGAG	Ghazi *et al.* 2009
sip-1	AAGAGATCGTTCACTCGCCAG	AGCCAAGTCGACGTCCTTTG	Kwon *et al.* 2010
dod-3	AAAAAGCCATGTTCCCGAAT	GCTGCGAAAAGCAAGAAAAT	Alam *et al.* 2010
mtl-1	ATGGCTTGCAAGTGTGACTG	CACATTTGTCTCCGCACTTG	Alam *et al.* 2010
lipl-4	CAAAACAAGACCTGGAAGAAACG	GCTTCCCTGAACGACTTGAGA	Shen *et al.* 2012

Supplemental Table 2.7. qPCR primers.

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Chapter 3 Identification of DAF-16/FoxO target genes highly associated with longevity

Abstract

FoxO transcription factors modulate age-related disease in mammals and promote longevity in invertebrates. In *C. elegans*, reduced DAF-2 insulin-like signaling and germline ablation both extend life span by activating the FoxO ortholog DAF-16, but their mechanisms of life span extension downstream of DAF-16/FoxO have not been directly compared. Combining whole transcriptome profiling (RNA-seq) with a unique genetic filter, we find that half of the longevity-associated DAF-16/FoxO target genes regulated by the germline are shared with reduced IIS. We further identify a core set of 46 genes regulated by DAF-16/FoxO highly correlated with longevity in multiple contexts. This set is tractable for rigorous functional validation, and we used qPCR to validate genes not previously identified in microarray-based studies. As the majority of these genes likely have functions conserved in humans, understanding the biological function of these genes may help develop strategies to promote healthy aging.

Introduction

FoxO transcription factors promote longevity in invertebrates [1–3] and modulate phenotypes related to age-related disease in mammals [4–9]. FoxO may be involved in human life span control, as FoxO1 and FoxO3 polymorphisms are associated with longevity in multiple cohorts of human centenarians [10–12]. Thus, identifying conserved genes regulated by FoxO to control life span promises to elucidate mechanisms of aging that can be targeted to treat and prevent age-related disease. However, a major challenge in identifying and studying FoxO target genes that influence longevity is that thousands

of genes are regulated by FoxO, many of which may be unrelated to aging and longevity [13,14].

The role of FoxO in life span control is best understood in *C. elegans*, where the FoxO ortholog DAF-16 promotes longevity in the contexts of reduced IIS (insulin and insulin-like signaling) and germline ablation [1,15]. DAF-16/FoxO activation by reduced IIS is well-understood and conserved in mammals. Reduced activation of the insulin-like growth factor receptor DAF-2/IGFR turns off a conserved PI3K/Akt cascade, resulting in hypophosphorylation and nuclear localization of DAF-16/FoxO. In this context, both DAF-16 and mammalian FoxO proteins regulate gene expression by binding to a conserved DNA motif (TTGTTTAC) [16]. Indeed, classes of target genes are shared between DAF-16 and mammalian FoxO, strongly suggesting that understanding DAF-16/FoxO-mediated life span extension will shed light on human aging [13,17,18].

Less is known about the mechanism of DAF-16 activation by germline ablation, but it is at least partially divergent from activation by IIS reduction. Germline ablation extends the life span of *daf-2* mutants [15,19], but this interaction is complicated by the fact that *daf-2* alleles are hypomorphic rather than null. Specific molecular components such as KRI-1 and TCER-1 are required for DAF-16/FoxO to extend life span in germline-ablated animals but are dispensable for *daf-2/IGFR* mutant longevity [20–22].

It is clear that IIS and signals from the germline converge on DAF-16/FoxO, but it is not known if DAF-16/FoxO regulates the same set of target genes to extend life span upon reduction of those signals. An *ad hoc* comparison between two rigorous microarray studies of DAF-16/FoxO targets in reduced IIS and germline ablation only identifies ~30 shared targets out of ~600. However, two separate studies of *daf-2* mutants only found ~160 out of ~1700 genes in common [23–25]. Technical differences in laboratory conditions, technological platforms, and bioinformatic analysis pipelines complicate direct comparisons between studies. Therefore, direct comparison should ideally be performed using identical conditions and procedures.

Recently, new efforts have begun to identify DAF-16/FoxO targets by sequencing-based whole transcriptome profiling (RNA-seq). RNA-seq is superior to microarray-based approaches for detecting expression changes in high- and low-abundance transcripts, unannotated transcripts, and non-coding RNAs [26,27]. RNA-seq displays greater sensitivity, specificity and quantification than tiling arrays in direct comparisons in *C. elegans* [28]. Thus, employing RNA-seq increases the chances of identifying novel *bona fide* targets of DAF-16/FoxO.

We reasoned that if there do exist similar mechanisms downstream of DAF-16/FoxO in both *daf-2/IGFR* mutants and animals lacking germlines, these would be strongly associated with longevity and therefore high-priority candidates for functional testing. Here, we use RNA-seq profiling technology to perform the first head-to-head comparison of insulin-like signaling and germline ablation. We further demonstrate that in both these contexts, the list of longevity-associated genes can be filtered using alleles of *daf-16* with distinct effects on gene expression.

Results

Design of genetic filter to identify DAF-16 targets associated with longevity

The *daf-16* genomic locus encodes multiple isoforms that share C-termini but differ in their N-termini [29,30]. All published profiling experiments to identify DAF-16 target genes have utilized either mutations or RNAi constructs that affect all *daf-16* isoforms. We reasoned that the large list of DAF-16 targets could be filtered if *daf-16* isoforms controlled different targets.

The roles of endogenous DAF-16 isoforms are unclear due to lack of isoform-specific mutants. However, according to previous reports utilizing animals harboring multiple copies of DAF-16 isoform-specific transgenes, DAF-16A and F are both capable of promoting longevity in the context of *daf-2* mutation, while DAF-16B can only promote longevity if over-expressed at very high copy number [29–31]. More detailed analysis will be required to understand the individual contributions of endogenous DAF-16A and

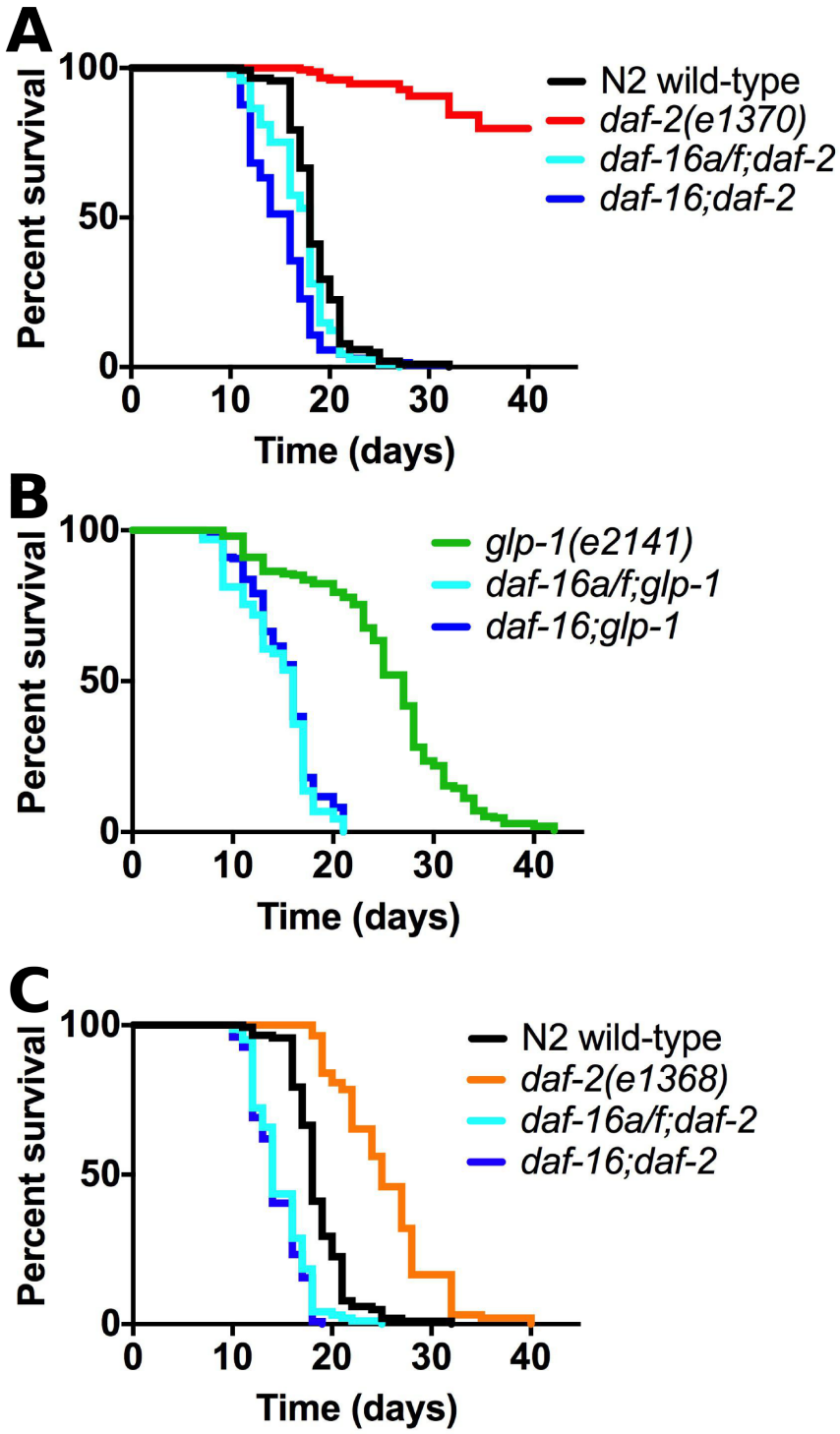
DAF-16F. Importantly, DAF-16B is reported to have unique functions in pharyngeal remodeling and neurite outgrowth [32,33] and therefore is likely to have unique targets. As DAF-16B does not appear to contribute to life span extension, exclusion of its targets could be useful for filtering.

Furthermore, we noted that the *mg54* allele is predicted to introduce an early stop codon specifically into *daf-16a* and *daf-16f*, but not *daf-16b*. *mg54* will henceforth be referred to as *daf-16a/f*. Therefore, if DAF-16B does not promote longevity, then *daf-16a/f* mutation should have the same effect on longevity as *daf-16* null mutation. Indeed, it was reported that *daf-16a/f* suppresses longevity to the same extent as *daf-16* null mutation (*mgDf47* allele) in the context of the *daf-2(e1370)* Class 2 mutation [33]. We confirmed this result (Figure 3.1A) using the *daf-16(mu86)* null allele (henceforth referred to as "*daf-16*" or "*daf-16* null" for our purposes). We further measured the longevity of *daf-16a/f;daf-2(e1368)* and *daf-16;daf-2(e1368)* in the same assay and found they had identical life spans (Figure 3.1B). Finally, we found the same result in the context of germline ablation, utilizing the temperature-sensitive *glp-1(e2141)* mutation (Figure 3.1C). Therefore, endogenous DAF-16B, in the absence of DAF-16A and F, is not sufficient for longevity in the contexts of *daf-2* mutation or germline ablation.

Since *daf-16a/f* and *daf-16* null mutation both suppress longevity to the same extent (Figure 3.1), DAF-16B targets are far less likely to contribute to life span extension. We reasoned that using the *daf-16(mg54)* allele could filter out irrelevant DAF-16B targets.

Figure 3.1. *daf-16a/f* and null *daf-16* mutation suppress longevity to the same extent in (A) *daf-2(e1370)* animals, (B) *daf-2(e1368)* animals, and (C) germline-ablated animals.

Data from 5 pooled cohorts are shown. Animals used for these life span cohorts were derived from populations analyzed by whole-transcriptome profiling in Figure 3.2.



daf-16a/f and *daf-16* null mutation cause distinct changes in global gene expression

To our knowledge, *daf-16a/f* mutants have not been previously profiled. To determine if the difference between *daf-16* null and *daf-16a/f* mutation can be used as a genetic filter, we performed whole transcriptome profiling on adult *daf-2(e1370)*, *daf-2(e1368)*, and *glp-1(e2141)* animals with wild-type *daf-16*, *daf-16a/f*, or *daf-16* null mutations. For each strain, 5 cohorts were utilized for experimental replicates, and we measured the life spans of a subset of each cohort to confirm that they had the expected phenotypes (Figure 3.1). We performed pair-wise comparisons based on fold-change and false discovery rate criteria (see Materials and Methods for precise criteria).

We will use terminology introduced by Murphy and colleagues [24] to indicate direction of gene expression change. The term "Class 1" denotes genes upregulated by DAF-16 and therefore are reduced upon *daf-16* mutation. "Class 2" denotes genes downregulated by DAF-16 and increased upon *daf-16* mutation.

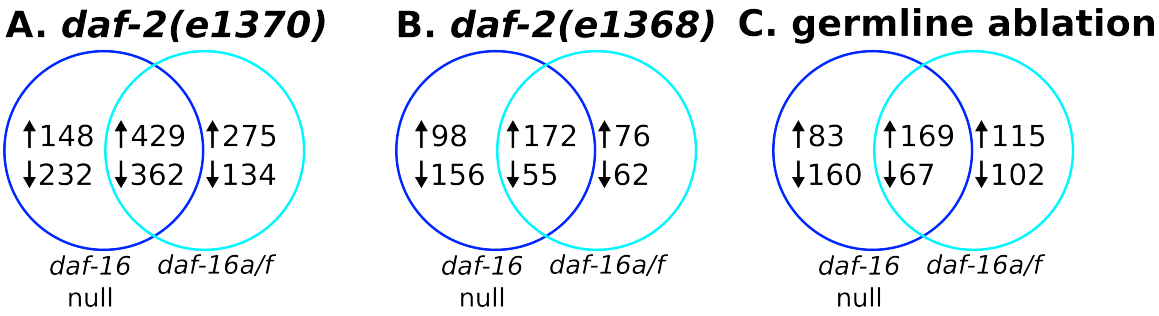
We first examined comparisons between *daf-2(e1368)* and either *daf-16;daf-2* or *daf-16a/f;daf-2* (Figure 3.2). *daf-16* null mutation yielded 270 Class 1 transcripts and 211 Class 2 transcripts. *daf-16a/f* mutation yielded 248 Class 1 transcripts and 117 Class 2 transcripts. We noticed the disparity in the number of Class 2 transcripts, and examined the overlap in Class 2 transcripts. Indeed, only 20% of the Class 2 transcripts (55/273) found in either comparison are shared, while the overlap for Class 1 transcripts is 50% (172/346). Thus, *daf-16* null mutation affects Class 2 transcripts more than *daf-16a/f* mutation, suggesting that DAF-16B may be disproportionately responsible for Class 2 transcript expression changes.

We confirmed that *daf-16a/f* and null *daf-16* mutation have distinct effects in two other contexts: (1) *daf-2(e1370)* mutation which is stronger than *daf-2(e1368)*, and (2) germline ablation (Figure 3.2). In *daf-2(e1370)* mutants, *daf-16* null mutation affected 577 Class 1 transcripts and 594 Class 2 transcripts, while *daf-16a/f* mutation affected 704 Class 1 transcripts and 496 Class 2 transcripts. The overlap was 50% for both classes (Class 1: 429/852; Class 2: 362/728). In *glp-1* germlineless animals, *daf-16a/f* again

altered the expression of more Class 1 targets compared to Class 2 (284 vs. 169), while *daf-16* null mutation affected approximately equal numbers of Class 1 and 2 targets (252 vs. 227). 46% (169/367) of Class 1 transcripts were shared between *daf-16a/f* and *daf-16* null, while only 20% (67/329) of Class 2 transcripts were shared. Thus, DAF-16B also appears to be disproportionately responsible for Class 2 transcript expression changes in *daf-2(e1370)* mutants and germline-ablated animals.

In summary, the *daf-16a/f* mutation is useful for identifying targets associated with longevity. At the very least, it serves as an additional *daf-16* allele, allowing us to control for genetic background, as well as technical and biological noise. However, the data are consistent with DAF-16B activity remaining intact in *daf-16a/f* mutants. As DAF-16B does not contribute to life span extension, we are able to enrich for targets that are required for longevity.

Figure 3.2. *daf-16a/f* and null *daf-16* mutation have distinct effects on global gene expression, especially on DAF-16-downregulated Class 2 genes.



Identification of common DAF-16 targets regulated by IIS and germline ablation

We sought to compare gene expression changes mediated by DAF-16A/F in the contexts of reduced IIS and germline ablation.

We defined two separate sets of DAF-16A/F targets that are regulated by DAF-2 insulin-like signaling (Figure 3.3A, B): (1) in the context of *daf-2(e1370)* tyrosine kinase domain mutation, and (2) in the context of *daf-2(e1368)* ligand-binding domain mutation. *daf-2(e1370)* is often considered a stronger loss-of-function allele than *daf-2(e1368)* due because of its larger magnitude of life span extension, higher penetrance of dauer arrest, and additional phenotypes [30,34]. Thus, to define two sets of DAF-2-regulated transcripts, we identified transcripts whose expression is altered in either *daf-2(e1368)* or *daf-2(e1370)* compared to wild-type. We then applied the genetic filter previously described to identify those transcripts changed in the opposite direction by both null *daf-16* and *daf-16a/f* mutation. Consistent with stronger loss-of-function, *daf-2(e1370)* affected most of the same DAF-16A/F targets *daf-2(e1368)* did (110 out of 150), as well as almost 600 additional targets (Figure 3.3D).

For the DAF-16A/F targets regulated by germline ablation (Figure 3.3C), a wild-type strain was not included due to potential confounding caused by germline transcripts in wild-type animals. Therefore, we simply applied our genetic filter to define the 236 transcripts changed by both null *daf-16* and *daf-16a/f* mutation. Interestingly, this set does not overlap much with *daf-2(e1368)* (47 out of 236), but shows stronger overlap with *daf-2(e1370)* (110 out of 236). Overall, DAF-16A/F has a larger influence on global transcriptional regulation in the context of *daf-2(e1370)* mutation than *daf-2(e1368)* or germline ablation (Figure 3.3D), consistent with the greater longevity of *daf-2(e1370)* mutants.

We then identified 44 transcripts that correlate with longevity in all three contexts. The effects of *mu86* and *mg54* alleles on the expression of these 44 transcripts were strongly correlated ($R^2 = 0.9642$), consistent with specific regulation by DAF-16A/F (Figure 3.3E).

Of these 44, four constitute operons composed of two genes each, and two transcripts corresponded to the same gene. Therefore, we identified a total of 47 genes (Table 3.1).

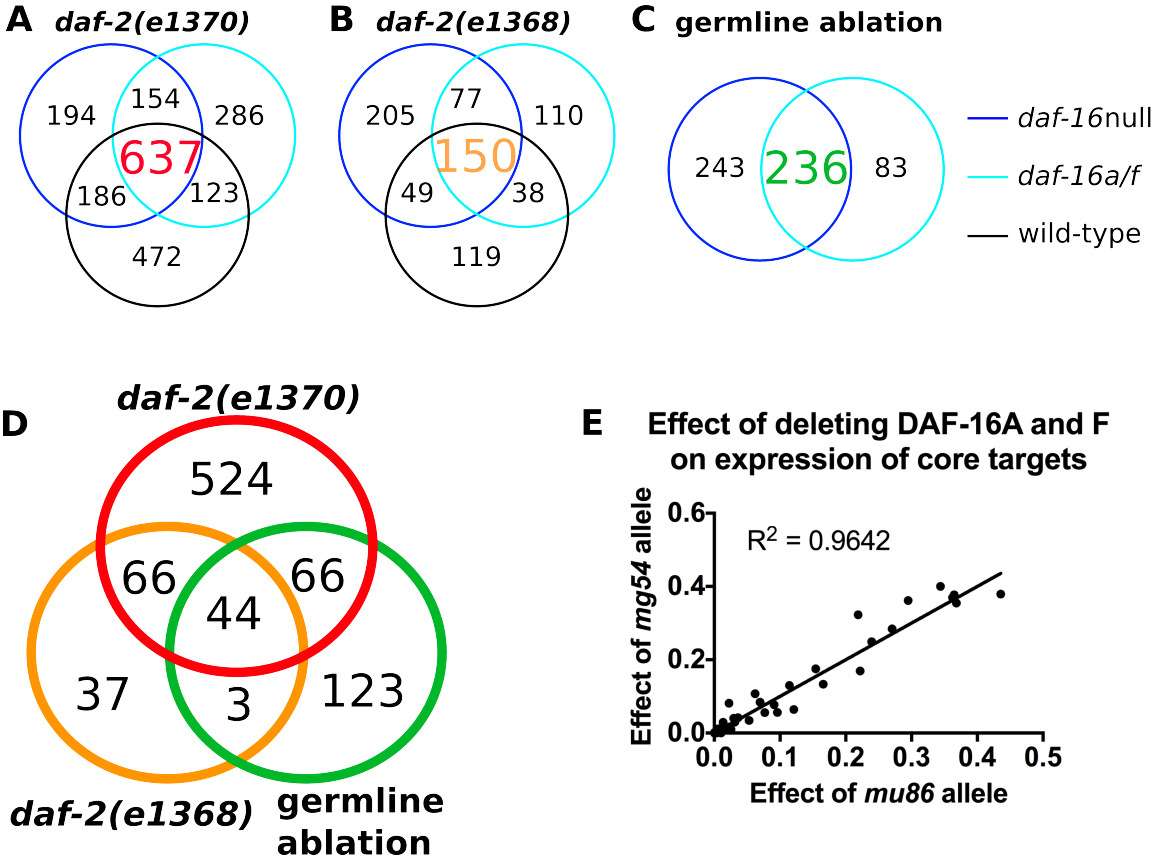
We performed global validation of our data set by quantitative PCR in wild-type and *daf-2(e1370)* animals (Figure 3.4A). 46 of 47 genes showed the expected change in expression. The only exception was *ceh-89* which is part of the *akt-2* transcript and therefore constitutes a bioinformatic artifact. Importantly, the magnitude of change measured by qPCR and by RNA-seq was highly correlated ($R^2 = 0.900$), demonstrating that RNA-seq gives reliable quantitative measurements. One major advantage of RNA-seq over microarray is larger dynamic range and lower background noise, allowing for more precise quantification [27]. More thorough validation of three targets showed the expected changes with both *daf-2* mutations and both *daf-16* mutations (Figure 3.4B-D).

We compared our set of 46 validated targets to DAF-16/FoxO targets previously identified by microarrays (Table 3.1). Our set includes "gold standard" DAF-16/FoxO targets such as *sod-3, mtl-1, dod-3*, and *hsp-12.6* [13,24,35,36]. Only 11 out of 46 genes were found in a previous microarray-based analysis of germline-regulated DAF-16/FoxO target genes [37]. 32 were found in at least one previous microarray study of DAF-2-regulated DAF-16/FoxO target genes, which is much higher than the 6 genes expected by chance [23–25,38]. DAF-16/FoxO was previously shown to directly bind to the promoters of 16 of the genes (by DamID and ChIP-seq) [39,40]. A GO term analysis using DAVID bioinformatics software indicated the only enriched terms were "aging", "multicellular organismal aging", and "determination of adult life span" (false discovery rate less than 0.0001) [41], consistent with the overlap with previous microarray studies of DAF-16 targets.

15 genes were not previously identified by any individual microarray study. Of those, 8 were suggested by a meta-analysis of all existing DAF-2-related microarrays [36], suggesting that individual microarray studies are not sufficiently powered to detect some of the genes we found. Of the remaining six, four were not present on previous microarray chips [36].

Taken together, it is highly likely that most of the 46 genes are *bona fide* DAF-16/FoxO targets, and this gene set represents an opportunity to identify genes with novel roles in life span control. Only 11 were previously tested for roles in life span extension by RNAi [13,24,25,42], but none of those were confirmed by mutation (Table 3.1). 9 of the genes have clear human homologs, and another 20 have domains conserved in humans. In Chapter 5, we will functionally test these genes together with those identified in Chapter 4.

Figure 3.3. Identification of 44 transcripts (47 genes) that correlate with longevity in 8 different long- vs. short-lived comparisons.
(A-C) We applied our genetic filter to identify DAF-16A/F targets in the contexts of (A) *daf-2(e1370)* mutation, (B) *daf-2(e1368)* mutation, and (C) germline ablation. These sets of DAF-16A/F targets were compared in (D) which identified 44 transcripts common to all three sets. (E) shows fold-change due to *daf-16* null (*mu86*) or *daf-16a/f* (*mg54*) in the *daf-2(e1370)* background. The trend is similar in *daf-2(e1368)* (R^2 = 0.9139) and germline ablation (R^2 = 0.8491).



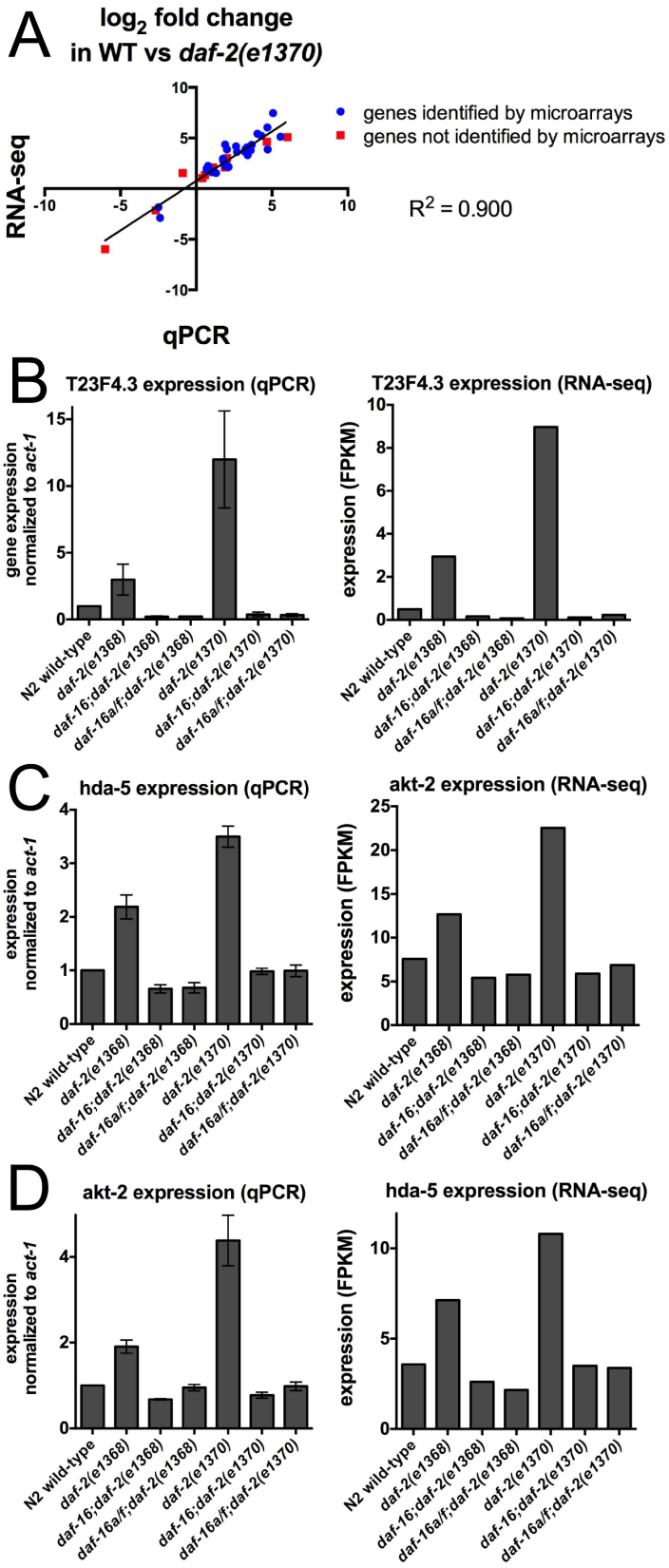


Figure 3.4. Validation of DAF-16/FoxO targets.
(A) Comparison of relative expression measured by qPCR and absolute expression measured by RNA-seq (FPKM = fragments per kilobase of exon per million fragments mapped). (B-D) Detailed comparisons between qPCR and RNA-seq for three different DAF-16/FoxO targets. On the left, average measurements for three independent cohorts are plotted. Error bars correspond to SD, and $p < 0.05$ for all relevant comparisons. On the right, average measurements for five independent cohorts are plotted. FDR < 0.05 for all relevant comparisons.

(caption on next page)

gene	Description	conservation (human)	Fold-change *e1370 vs* WT	previous microarrays			DAF-16 bind	previous LS test
				glp-1	*daf-2*	meta		
F56D6.9	63 aa protein		inf			X	✔	
T12D8.5	135 aa protein		inf		✔	✔	✔	
T02B5.1	esterase/lipase	domain	207		✔	✔		
Y6G8.9	F-box-associated domain	domain	118			X		
dct-8	69 aa protein		90			✔	✔	
Y39G8B.7	ShK domain		82		✔	✔		
R05H10.7			63			✔		
mtl-1	Metallothionein	domain	58	✔	✔	✔		✔*
F15B9.6			53		✔	✔		
F21C10.11	80 aa protein		38			✔		
K03D3.2	144 aa protein		26		✔	✔		
lipl-2	Triacylglycerol lipase	homolog	24	✔		✔		
sdz-24	RPA2 fold	domain	24		✔	✔		
C08E8.4	DUF1768		23		✔	✔	✔	
dod-3	106 aa protein		22	✔	✔	✔	✔	✔*
T23F4.3	Dienelactone hydrolase	homolog	18		✔	✔	✔	
C08F11.3			16		✔	✔		
sod-3	Superoxide dismutase	homolog	15	✔	✔	✔	✔	✔*
Y20C6A.1	F-box	domain	15		✔	✔		
Y6G8.2		domain	15		✔	✔		
gst-29	Glutathione S-transferase	homolog	13		✔	✔	✔	
lys-7	Lysozyme	domain	13		✔	✔		✔*
hen-1	LDL receptor motif	domain	13		✔	✔		
ftn-1	Ferritin heavy chain	homolog	13	✔	✔	✔	✔	✔
sru-40	Serpentine receptor	domain	10		✔	✔		
glb-1	Globin	domain	8	✔	✔	✔	✔	✔
spp-2	Antimicrobial peptide	domain	7	✔		✔		
Y34F4.2	Claudin 3 superfamily	domain	7	✔	✔	✔		
btb-16	BTB domain	domain	7			✔	✔	
M01B2.13	interacting protein-like	domain	6			X		
btb-17	BTB domain	domain	5		✔	✔	✔	
cpg-7	116 aa protein		4			✔	✔	
cutl-24	Zona pellucida domain	domain	4			✔		
Y38C1AA.6			4			✔		
ttr-44	DUF290	domain	4		✔	✔		
icl-1	Isocitrate lyase	homolog	4	✔	✔	✔		✔*
F48D6.4	98 aa protein		4	✔	✔	✔		✔
hsp-12.6	Heat shock protein	homolog	3		✔	✔	✔	✔*
hda-5	Histone deacetylase	homolog	3			✔		
ttr-5	Transthyretin-like domain	domain	3		✔	✔		
akt-2	Akt/Protein Kinase B	homolog	3		✔	✔	✔	
ceh-89	Homeodomain		3					
Y2H9A.4	DUF713		2				✔	
dod-23	168 aa protein		0.30		✔	✔		✔
srh-70	Serpentine receptor	domain	0.25			✔		
F35E12.5	CUB domain	domain	0.18	✔	✔	✔		✔*
K12B6.11	69 aa protein		0.07			X		

Table 3.1. List of genes regulated by DAF-16/FoxO in both contexts of reduced insulin-like signaling and germline ablation.
For the conservation column, "homolog" indicates the gene is unambiguously homologous to a human protein, while "domain" indicates that the gene possesses a domain conserved in human proteins. Fold-change indicates expression in *daf-2(e1370)* divided by N2 wild-type. For comparison to previous microarrays, "*glp-1"* refers to McCormick *et al.* 2012, "*daf-2"* refers to combined results of Murphy *et al.* 2003, McElwee *et al.* 2003, and Troemel *et al*. 2006, and "meta" refers to the meta-analysis performed by Tepper *et al.* 2013. See text for details. "DAF-16 bind" indicates genes bound DAF-16 *in vivo* identified by either ChIP-seq (Contrino *et al.* 2012) or DamID (Schuster *et al.* 2010). See text for details. Previous LS test indicates genes previously tested by RNAi for roles in life span control, and * indicates those that had a statistically significant effect. Note that only *ceh-89* was eliminated by qPCR validation.

Discussion

The mechanism of DAF-16/FoxO-mediated life span extension remains elusive. One strategy to uncover mechanism is to observe multiple long-lived mutants that rely on DAF-16/FoxO activity for longevity and ask what is common between them. In this study, we combined three novel approaches to identify longevity-associated targets of DAF-16/FoxO: (1) direct comparison of DAF-16/FoxO targets in the contexts of reduced IIS and germline ablation, (2) RNA-sequencing technology and (3) a unique genetic filter based on the fact that only the DAF-16A and DAF-16F isoforms promote longevity. Our results both add to and greatly refine the sets of *daf-2/IGFR*- and germline-dependent DAF-16/FoxO target genes have previously been identified [24,36,37]. The end result was a set of 46 genes validated by qPCR that is tractable for rigorous testing.

We have chosen the most stringent criteria to select our 46 genes: genes must show clear evidence of differential expression in all 8 long- *vs.* short-lived comparisons. Therefore, it is possible other genes in our RNA-seq data set also correlate with longevity but are subject to more subtle regulation by DAF-16/FoxO. However, the 46 genes already identified should be sufficient for functional analysis in Chapter 5.

The filtering itself yielded novel insights into DAF-16/FoxO target gene regulation. The *daf-16a/f* mutation reliably produced distinct sets of target genes compared with *daf-16* null mutation, strongly implying that endogenous DAF-16 isoforms diverge in their transcriptional activity. This is consistent with previous measurements of 10 target genes

in transgenic animals over-expressing DAF-16 isoforms individually [29]. Gene regulatory events reversed by *daf-16* mutation but not by *daf-16a/f* mutation are not sufficient for life span extension. We separately found that *daf-16b* mutation does not reduce *daf-2* longevity (data not shown), raising the possibility that these gene regulatory events are also not required.

Confirmation that disparities between *daf-16a/f* and null *daf-16* correspond to DAF-16B targets will require profiling the *daf-16b*-specific mutant. The other possibilities to explain strain-specific differences include: (1) genetic background and (2) biological and technical noise. However, these are not likely to explain why DAF-16-downregulated genes (Class 2) are disproportionately filtered out by *mg54/mu86* comparisons, indicating a biologically significant difference. Thus, it is far more likely that *mg54* filters out DAF-16B-specific targets, which are enriched in Class 2 genes.

We also compared the gene expression profiles of germline-ablated animals and two types of IIS mutants. First, *daf-2(e1370)* exhibited gene expression changes that included nearly all those seen in *daf-2(e1368)*, and many more. This is consistent with *daf-2(e1370)* being a stronger loss-of-function allele that causes more marked longevity and developmental phenotypes. Second, many regulatory events that occur in *daf-2(e1370)*, but not *daf-2(e1368)*, are shared with germline-ablated animals. Thus, the extraordinary longevity of *daf-2(e1370)* may result from longevity assurance mechanisms shared by both *daf-2(e1368)* and germline-ablated animals, as well as additional mechanisms. Finally, the 123 gene regulatory events unique to germline-ablated animals may explain why germline ablation extends the longevity of both *daf-2* mutants [15,19].

Our identification of significant overlap (~110 transcripts) between germline ablation and reduced IIS is important. Thus, nearly half of the DAF-16/FoxO targets controlled by the germline are shared with reduced IIS. This is much higher than the 30 genes found in one rigorous IIS study that are included the set of 230 DAF-16/FoxO targets found by another rigorous germline-ablation study [24,37]. This is surprising because germline ablation and reduced IIS constitute significantly divergent contexts for DAF-16/FoxO action.

Germline ablation only activates intestinal DAF-16/FoxO to promote longevity, while reduced IIS engages system-wide insulin-like ligand networks to activate DAF-16/FoxO in many tissues, especially the intestine, hypodermis, and neurons [43–45]. Furthermore, germline ablation and reduced IIS activate distinct sets of transcription factors that work in concert with DAF-16/FoxO to promote longevity. HSF-1 and SKN-1 are activated by reduced IIS, are required for the longevity of *daf-2/IGFR* mutants, and co-regulate at least some target genes with DAF-16/FoxO [46–48]. In contrast, NHR-80, PHA-4, DAF-12, and TCER-1 transcriptional regulators are critical for longevity in animals lacking a germline [21,49,50]. Finally, the germline constitutes half the mass of adult *C. elegans* [51], and therefore germline ablation itself is likely to dramatically alter the organism-wide transcriptome. Thus, it is intriguing that DAF-16/FoxO controls many of the same genes in these two very different contexts, and these similarities may explain their common life span extension.

To test whether DAF-16/FoxO promotes longevity through overlapping mechanisms in germline-ablated animals and IIS mutants, we will need to determine the contributions of the 46 shared DAF-16/FoxO target genes (Table 3.1) to life span control. In Chapter 5, we will functionally test these genes as well as those found in Chapter 4. It is plausible that these simply correlate with DAF-16/FoxO activation. However, given that almost no DAF-16/FoxO targets have been rigorously tested using genetic mutants, even ruling out individual contributions within these 46 genes would yield novel insight into the mechanism of DAF-16/FoxO-mediated life span extension. More likely, some of these 46 genes are required for longevity across many contexts. Given that at least 29 of these likely possess functions conserved in humans, identification of these genes may shed light on determinants of human longevity.

Materials and Methods

C. elegans strains and maintenance

Strains used in this study are listed in Table 3.2. Animals were maintained at 15°C on nematode growth media (NGM) plates seeded with *Escherichia coli* OP50. Double mutants were constructed using standard genetic techniques. Percival I-36NL incubators (Percival Scientific, Inc., Perry, IA) were employed for maintenance and life span assays.

Strain	Genotype	Outcrossed	Reference
N2 Bristol	wild-type		
DR1572	*daf-2(e1368)*	6X	Kimura *et al.* 1997
CB1370	*daf-2(e1370)*	6X	Kimura *et al.* 1997
CB4037	*glp-1(e2141)*	6X	Priess *et al.* 1987
CF1038	*daf-16(mu86)*	6X	Lin *et al.* 1997
GR1308	*daf-16(mg54)*	6X	Ogg *et al.* 1997

Table 3.2. List of strains used in Chapter 3.

Life span assays

Life span assays were performed as previously described [52], with minor modifications. Wild-type and *daf-2* animals derived from a synchronized 4 hr egglay were grown at 15°C until the early L4 larval stage and then shifted to 20°C. Plates harboring any males were discarded. Animals were grown for an additional 20-24hr to day 1 of adulthood and then placed on life span plates containing 25μg/mL 5-fluoro-2'-deoxyuridine (FUDR; Sigma-Aldrich, St. Louis, MO) to prevent progeny growth. Egglay of *daf-2(e1370)* was performed 6 hours earlier to adjust for slower growth. Preparation of *glp-1* mutant animals was similar except they were raised at the restrictive temperature (25°C) for 48 hrs to ablate the germline. Statistical significance was assessed using the standard chi-square-based log-rank test in GraphPad Prism.

RNA isolation

The remaining animals from populations raised for life span assays were washed twice in M9 buffer. Total RNA was isolated using TRIzol® reagent (Invitrogen) and purified

using an RNeasy kit (QIAGEN Inc., Valencia, CA) according to manufacturers' instructions.

Quantitative real-time reverse-transcriptase PCR (qPCR)

cDNA was synthesized using a SuperScript® III Reverse Transcriptase kit and random hexamers (Invitrogen, Carlsbad, CA). Real-time PCR was then performed in triplicate using *Power* SYBR® Green PCR master mix (Applied Biosystems, Warrington, UK) and a Mastercycler® ep *realplex* thermal cycler (Eppendorf North America, Westbury, NY). 10ng of cDNA was used as a template in 15µl reaction volume. Relative expression levels and technical error were determined by the $\Delta\Delta 2C_t$ method [53]. Gene expression levels were normalized to actin (*act-1*), and the ratio of expression relative to *act-1* was then compared to the same ratio in N2 Bristol wild-type. Statistical analysis was performed in GraphPad Prism (GraphPad Software, La Jolla, CA) using the paired ratio *t*-test.

Whole transcriptome profiling (RNA-seq)

5 biological replicates were collected for every genotype. The Agilent TapeStation was used to assess RNA quality. Samples with RINs (RNA Integrity Numbers) of eight or greater were prepped using the Illumina TruSeq mRNA Sample Prep v2 kit (Catalog #RS-122-2001 and RS-122-2002). mRNA was isolated from 0.1-3 µg of total RNA by polyA$^+$ purification, fragmented, and copied into first strand cDNA using reverse transcriptase and random primers. 3' cDNA ends were then adenylated and adapters ligated. One of the adapters contained a 6-nucleotide barcode to enable multiplexing of samples. Products were purified and enriched by PCR to create the final cDNA library. Libraries were checked for quality and quantity by Agilent TapeStation and qPCR using a library quantification kit for Illumina sequencing platforms (catalog #KK4835, Kapa Biosystems, Wilmington, MA). Clonal clusters were generated using cBot (Illumina, Inc., San Diego, CA). Quadriplexed samples were sequenced using the HiSeq 2000 system

(Illumina) with a 100-cycle paired-end run in high output mode using Version 3 reagents according to manufacturer's protocols.

Individual reads files for each sample were concatenated into a single .fastq file. Raw reads data for each sample were checked using FastQC (Version 0.10.0, Babraham Bioinformatics, Cambridge, United Kingdom; http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) to identify features potentially indicative of quality issues (*e.g.* low quality scores, over-represented sequences, and inappropriate GC content). We used the Tuxedo Suite [54–56] for alignment, differential expression analysis, and post-analysis diagnostics. Briefly, reads were aligned to the reference genome (UCSC ce10; http://genome.ucsc.edu/) using TopHat (version 2.0.9) [54] and Bowtie (version 2.1.0.0) [57]. We used default parameter settings for alignment, with the exception of: "--b2-very-sensitive" and "--no-coverage-search." A second round of quality control was then performed using FastQC to ensure that only high quality data was analyzed further. Cufflinks/CuffDiff (Version 2.1.1) [54] was used for quantification of expression and differential expression analysis, using UCSC ce10.fa as the reference genome and UCSC ce10.gtf as the reference transcriptome (http://genome.ucsc.edu/). For this analysis, we used parameter settings: "--multi-read-correct" to adjust expression calculations for reads that map to more than one locus, as well as "--compatible-hits-norm" and "--upper-quartile –norm" for normalization of expression values. We generated diagnostic plots using the CummeRbund package [55] to confirm that each experiment yielded high quality data.

We used locally developed scripts to format and annotate the differential expression data output from CuffDiff. Genes and isoforms were annotated using NCBI Entrez GeneIDs and text descriptions.

RNA-seq analysis

Gene expression data output from CuffDiff comprised 8 comparisons in 3 groups: (1) *daf-2(e1370)* compared to wild-type, *daf-16(mu86);daf-2(e1370)*, and *daf-16(mg54);daf-*

2(e1370), (2) *daf-2(e1368)* compared to wild-type, *daf-16(mu86);daf-2(e1368)*, and *daf-16(mg54);daf-2(e1368)*, and (3) *glp-1(e2141)* compared to *daf-16(mu86);glp-1* and *daf-16(mg54);glp-1*. We defined DAF-16A/F targets in the *daf-2* context as meeting all of the following criteria: (1) test status = "OK", (2) fold change (FC) $\geq \pm 1.5$ for wild-type *vs. daf-2*, (3) FC ≥ 1.5 in the opposite direction as wild-type for *daf-2 vs. daf-16(mu86);daf-2,* (4) FC ≥ 1.5 in the opposite direction as wild-type for *daf-2 vs. daf-16a/f(mg54);daf-2,* and (5) FDR < 0.05 for all three comparisons. The criteria for *glp-1* animals was similar except there was no wild-type comparison. We noted that many genes previously validated as DAF-16/FoxO targets did not meet our FDR criteria, and that RNA-seq is biased against short, low-expressed genes even if normalized for length [58]. Therefore, we also included genes that did not meet the FDR requirement but did fulfill a more stringent FC > 3 criterion for all comparisons. 4 genes in our final list did not meet the FDR cutoff for any of the 8 comparisons. 19 genes in our final list did not meet the FDR cutoff for one or more of the 8 comparisons. However, all of our genes met the fold change cutoff for all 8 comparisons.

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Chapter 4 Hierarchical Action of DAF-16/FoxO Isoforms in *C. elegans* Life Span Control

Abstract

FoxO transcription factors control development, metabolism, and aging across taxa. The *Caenorhabditis elegans* FoxO transcription factor DAF-16 promotes longevity in the contexts of reduced DAF-2 insulin-like growth factor receptor (IGFR) signaling and germline ablation. How it does so is poorly understood. The *daf-16* genomic locus encodes four distinct isoforms (DAF-16A, D, F, and H) that are capable of extending life span. Using isoform-specific *daf-16/FoxO* mutant alleles, we show that the D, F, and H isoforms of DAF-16/FoxO are collectively not required for longevity in animals either with reduced DAF-2/IGFR signaling or lacking a germline, but function redundantly with DAF-16A to prolong life. In contrast, DAF-16A is necessary for full life span extension in these same contexts. Whole transcriptome profiling in animals with reduced DAF-2/IGFR activity reveals a dominant role for DAF-16A over DAF-16D/F/H in the regulation of target gene expression. Our results unveil a hierarchy of isoform action in DAF-16/FoxO-dependent life span control and gene regulation, establishing DAF-16A as the primary *C. elegans* FoxO isoform that controls aging. As FoxO transcription factors have conserved functions in promoting longevity and may be dysregulated in aging-related diseases, these findings promise to illuminate fundamental principles underlying aging in animals.

Introduction

FoxO transcription factors control development, metabolism, stress responses, and aging in diverse animal species [1-4]. FoxO promotes longevity in invertebrates with reduced insulin-like signaling [5-9]. Phenotypic analysis of knockout mice implicates FoxO transcription factors in the pathogenesis of aging-related diseases such as cancer [10-12], Type 2 diabetes [13-16], osteoporosis [17,18], and atherosclerosis [19,20]. In humans, FoxO1 and FoxO3 polymorphisms are associated with extreme longevity in multiple independent cohorts of centenarians [21-26], suggesting that the role of FoxO in controlling aging may be conserved in mammals. Therefore, understanding the molecular basis for FoxO transcription factor action will likely illuminate fundamental mechanisms that govern aging in animals.

The well-established role of FoxO transcription factors as targets of insulin-like signaling first came to light from studies in *C. elegans,* where the insulin/insulin-like growth factor receptor (IGFR) ortholog DAF-2 promotes reproductive development and limits adult life span by inhibiting the FoxO transcription factor DAF-16 via a conserved phosphoinositide 3-kinase (PI3K)/Akt pathway-dependent mechanism. Engagement of DAF-2/IGFR by agonist insulin-like ligands activates the PI3K/Akt pathway, resulting in Akt-dependent phosphorylation of three DAF-16/FoxO amino acid residues that lie within conserved RxRxxS/T motifs. Phosphorylated DAF-16/FoxO is subsequently exported from the nucleus and sequestered in the cytoplasm. When DAF-2/IGFR pathway activity is reduced, unphosphorylated DAF-16/FoxO translocates to the nucleus, where it regulates the expression of numerous genes, including those that control metabolism, immunity, and detoxification [27]. The inhibition of FoxO by insulin-like signaling is evolutionarily conserved, as reduction of FoxO activity ameliorates biological phenotypes associated with reduced insulin-like signaling in flies [8,9,28] and mice [13-16].

DAF-16/FoxO also promotes life span extension in animals lacking a germline [29]. Although DAF-2/IGFR and the germline both control DAF-16/FoxO activity by

regulating its subcellular localization [30-32], they may do so through distinct mechanisms, as the molecular requirements for DAF-16/FoxO regulation by DAF-2/IGFR signaling and the germline differ [33,34].

The *daf-16* genomic locus encodes three groups of transcripts (a, b, and d/f/h) that are transcribed from distinct promoters [6,7,35]. *daf-16d, f,* and *h* are transcribed from the same promoter but have distinct 5' ends and translational start sites [Supplemental Figure 4.1,Supplemental Figure 4.2]; WormBase (www.wormbase.org)] [27,35]. For clarity, we refer to the *d/f/h* group of transcripts and polypeptides collectively as *daf-16f* and DAF-16F, respectively.

In animals with diminished DAF-2/IGFR signaling, mutations that reduce *daf-16a* and *f* but not *daf-16b* activity shorten life span to the same extent as *daf-16* null mutations [31]. Furthermore, overexpression of DAF-16B under the control of its endogenous promoter does not extend the life span of *daf-16/FoxO* null mutants [31,35]. These data implicate DAF-16A and DAF-16F as the critical targets of DAF-2/IGFR in life span control.

DAF-16A and DAF-16F share their C-terminal 428 amino acids, including the Forkhead DNA binding domain and two of the three conserved Akt-dependent phosphorylation motifs (Figure 4.1A) [27,35]. However, the A and F isoforms diverge in their N-termini (Figure 4.1A). Whereas DAF-16A contains a conserved RxRxxS/T motif likely to be recognized by Akt and/or Akt-related kinases [36], the N-terminus of DAF-16F lacks this motif but harbors a QxRxxS/T motif that is phosphorylated *in vivo* (Figure 4.1B) [27,35,37]. Since phosphorylation of the N-terminal RxRxxS/T site in murine FoxO1 is important in controlling FoxO subcellular localization [38], this difference suggests that nucleocytoplasmic shuttling of DAF-16A and DAF-16F may be controlled by distinct upstream inputs.

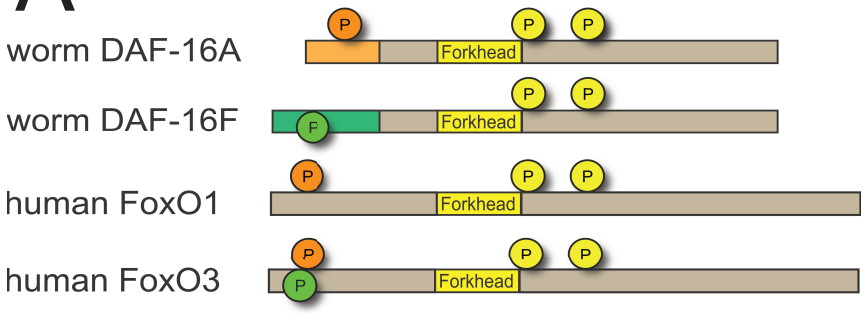
Comparative sequence analysis indicates that DAF-16F is conserved in the related nematode *C. briggsae* [39]. Intriguingly, *Drosophila* and mammalian FoxO transcription factors possess both RxRxxS/T and QxRxxS/T motifs in their N-termini (Figure 4.1B),

and these sites are phosphorylated in intact cells [40,41], suggesting that conserved upstream inputs that regulate distinct FoxO isoforms in *Caenorhabditis* species may converge on a single FoxO isoform in flies and mammals.

Transgene-based experiments demonstrate that, whereas both DAF-16A and DAF-16F are capable of extending life span in animals harboring a *daf-16/FoxO* null mutation when overexpressed under the control of their native promoters, overexpression of either isoform using the *daf-16f* promoter increases life span to a greater extent than overexpression of the same isoforms using the *daf-16a* promoter [35]. These findings have led to the conclusion that DAF-16F is the major FoxO isoform that controls *C. elegans* life span [35,42]. However, the lack of mutant alleles that specifically inactivate either *daf-16a* or *daf-16f* has precluded an analysis of the functions of each isoform in the presence of physiologic levels of expression of the other isoform. In this study, we use isoform-specific *daf-16/FoxO* mutant alleles to establish the roles of DAF-16A and DAF-16F in the control of *C. elegans* development, longevity, and gene expression.

Figure 4.1. *daf-16/FoxO* isoforms and isoform-specific mutations (next page).
(A) Protein schematic comparing *C. elegans* DAF-16A and DAF-16F isoforms with human FoxO1 and FoxO3 proteins. The identical C-termini of DAF-16A and DAF-16F share two highly conserved RxRxxS/T Akt-family consensus phosphorylation motifs (yellow) with human FoxO. The N-terminus of DAF-16A includes an RxRxxS/T phosphorylation motif (orange) conserved in human FoxO1 and FoxO3, while the unique N-terminus of DAF-16F harbors a QxRxxS motif (green) conserved in FoxO3. (B) N-terminal phosphorylation motifs in DAF-16A and DAF-16F are conserved in *Drosophila* dFoxO and human FoxO1, FoxO3, and FoxO4. Asterisks indicate conserved phosphoacceptor sites. Residues known to be phosphorylated *in vivo* (DAF-16F) or in intact cells (dFoxO, FoxO3, and FoxO4) are emboldened and underlined. See text for details. (C) *C. elegans daf-16/FoxO* genomic structure with isoform-specific mutations. Colors indicate unique N-terminal exons and Forkhead domains that correspond to DAF-16/FoxO protein domains in Figure 1A. (D) Summary of isoform-specific mutant alleles. See text for details.

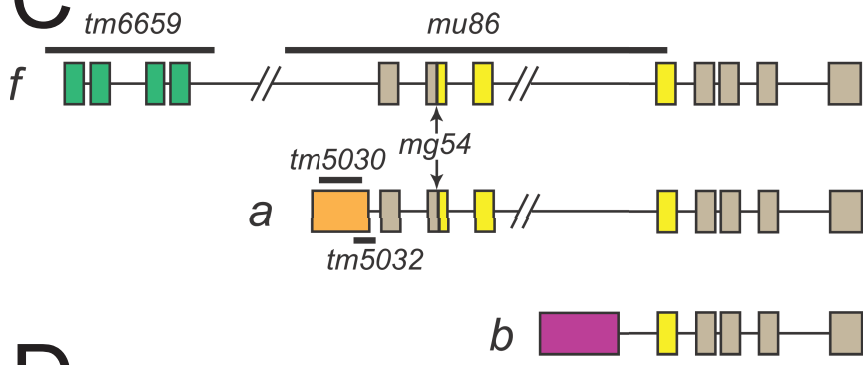
A



B

```
                                  *  *
worm DAF-16A IPRDRCNTWP
worm DAF-16F QERSASFGGV
   fly dFoxO QTRARSNTWP
 human FoxO1 LPRPRSCTWP
 human FoxO3 QSRPRSCTWP
 human FoxO4 QSRPRSCTWP
```

C



D

Allele	Referred to as	Isoform(s) affected	Predicted effect on protein
mu86	*daf-16(null)*	a, b, f	elimination of DAF-16F Forkhead domain, elimination of all DAF-16A and DAF-16B
mg54	*daf-16a/f*	a, f	Y 147 ➔ STOP
tm5030	*daf-16a #1*	a	frameshift after 7 aa ➔STOP after 91 aa
tm5032	*daf-16a #2*	a	translation into intron after 58 aa ➔STOP after 6 aa
tm6659	*daf-16f*	f	no protein due to elimination of transcriptional and translational start sites

Results

Molecular characterization of isoform-specific *daf-16/FoxO* mutants

To isolate *daf-16a*- and *daf-16f*-specific mutant alleles, we screened for mutants with deletions in the unique N-terminal exons of each isoform [43]. Two independent alleles, *tm5030* and *tm5032*, are deletions in the *daf-16a*-specific exon (Figure 4.1C-D). 5' rapid amplification of cDNA ends (RACE) analysis shows that these mutations result in frameshifts and predicted early translation termination (Figure 4.1D and Supplemental Figure 4.3-5). As expected, both *tm5030* and *tm5032* significantly reduced *daf-16a* transcript levels as measured by quantitative real-time reverse transcriptase PCR (qPCR; Supplemental Figure 4.6A-B; Supplemental Table 4.1), consistent with nonsense-mediated decay secondary to premature translation termination. Neither mutation influenced the integrity or quantity of *daf-16b* or *daf-16f* transcripts (Supplemental Figure 4.6C-D; Supplemental Table 4.1).

tm6659 is a deletion that spans all four *daf-16f*-specific exons, including all three putative transcriptional and translational start sites (Figure 4.1C-D and Supplemental Table 4.1) [27,35]. *daf-16f* transcripts were undetectable in animals harboring *tm6659* by 5' RACE and qPCR (Supplemental Figure 4.3,6D; Supplemental Table 4.1), and neither *daf-16a* nor *daf-16b* transcripts were affected (Supplemental Figure 4.6A-C; Supplemental Table 4.1). Total *daf-16* levels are sharply reduced in *tm6659* mutants (Supplemental Figure 4.6E-F; Supplemental Table 4.1). Examination of *tm6659* RNA-sequencing data shows no reads originating from the *daf-16f*-specific exons. Importantly, our data show no compensatory increase in *daf-16f* transcripts in either *daf-16a(tm5030)* or *daf-16a(tm5032)* (Supplemental Figure 4.6D), and no increase in *daf-16a* transcripts in *daf-16f(tm6659)* (Supplemental Figure 4.6A-B).

Taken together, these data strongly suggest that *tm5030, tm5032,* and *tm6659* are *bona fide* isoform-specific loss-of-function alleles. These isoform-specific alleles are henceforth referred to as *daf-16a #1 (tm5030), daf-16a #2 (tm5032),* and *daf-16f (tm6659)*.

DAF-16A promotes dauer arrest in animals with reduced DAF-2/IGFR signaling

In response to adverse environmental conditions, *C. elegans* larvae undergo developmental arrest in an alternative larval stage known as dauer [44]. *daf-2/IGFR* and *daf-16/FoxO* mutants were first isolated in genetic screens for animals with dauer-constitutive (Daf-c) and dauer-defective (Daf-d) phenotypes, respectively [45]. *daf-2/IGFR* mutants constitutively arrest as dauers at 25°C in a *daf-16/FoxO*-dependent manner [45-47].

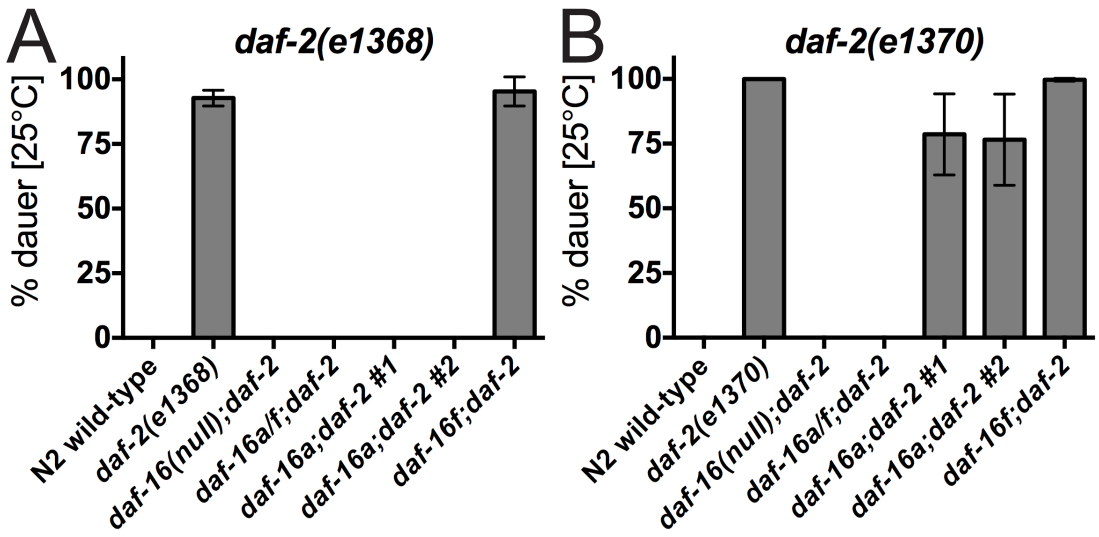
To examine the roles of distinct DAF-16/FoxO isoforms in dauer regulation, we determined the influence of isoform-specific *daf-16/FoxO* mutations on dauer-constitutive phenotypes in two *daf-2/IGFR* mutant backgrounds: *e1368,* a missense mutation in the DAF-2/IGFR extracellular ligand-binding domain, and *e1370,* a missense mutation in the cytoplasmic tyrosine kinase domain [48]. *e1370* may be a stronger loss-of-function allele than *e1368*, as it causes a more penetrant dauer-constitutive phenotype and extends life span to a greater extent than *e1368* (Figure 4.2-3) [49].

As previously shown, the dauer-constitutive phenotype caused by both *daf-2/IGFR* mutations is fully suppressed by the null *daf-16/FoxO* allele *mu86 [6]* as well as by *mg54* (Figure 4.2; Supplemental Table 4.2)*,* a nonsense mutation that affects *daf-16a* and *daf-16f* but not *daf-16b* (Figure 4.1C-D) [7,31]. *daf-16(mu86)* and *daf-16(mg54)* are henceforth referred to as "*daf-16* null" and "*daf-16a/f* mutation" for purposes of clarity. These results indicate that DAF-16B does not suffice to promote dauer arrest in animals with reduced DAF-2/IGFR signaling, implicating DAF-16A and/or DAF-16F in dauer regulation.

Both *daf-16a* mutations completely suppress the dauer-constitutive phenotype of *daf-2(e1368)* mutants, whereas *daf-16f* mutation does not influence *daf-2(e1368)* dauer arrest (Figure 4.2A; Supplemental Table 4.2; Supplemental Table 4.3). Thus, in this context, DAF-16A is the critical isoform that regulates dauer arrest. In *daf-2(e1370)* mutants, *daf-16a #1* and #2 mutations suppress dauer arrest by 22% ($p = 0.0204$) and 24% ($p = 0.0408$) respectively, whereas *daf-16f* mutation has no effect on dauer arrest (Figure

4.2B; Supplemental Table 4.2). Since *daf-16a/f* mutation fully suppresses *daf-2(e1370)* dauer arrest (Figure 4.2B; Supplemental Table 4.2), DAF-16A and DAF-16F act redundantly to promote dauer arrest in *daf-2(e1370)* mutant animals.

Figure 4.2. *daf-16a*-specific mutations suppress dauer arrest in *daf-2/IGFR* mutants. (A) The dauer-constitutive phenotype of *daf-2(e1368)* animals is fully suppressed by *daf-16a/f* mutation and both *daf-16a* mutations but is unaffected by *daf-16f* mutation. (B) The dauer-constitutive phenotype of *daf-2(e1370)* animals is fully suppressed by *daf-16a/f* mutation, partially suppressed by both *daf-16a* mutations, and unaffected by *daf-16f* mutation. Mean and standard deviation for at least three biological replicates are presented. Statistics and raw data are presented in Supplemental Table 4.2, Supplemental Table 4.3, and Supplemental Table 4.4.



DAF-16A promotes longevity in animals with reduced DAF-2/IGFR signaling

DAF-16/FoxO is required for life span extension both in animals with reduced DAF-2/IGFR activity [5] and in animals lacking a germline [29]. Experiments involving RNAi-based knockdown and transgenic overexpression of DAF-16/FoxO isoforms suggest that both DAF-16A and DAF-16F promote longevity in *daf-2/IGFR* mutants [35]. We determined the effect of *daf-16a* and *daf-16f* mutations on life span in *daf-2(e1368)* and *daf-2(e1370)* mutants. As previously shown, life span extension induced by *daf-2/IGFR* mutation was fully suppressed by *daf-16* null mutation as well as by *daf-16a/f* mutation (Figure 4.3A-D; Supplemental Table 4.5-6) [31,32].

In *daf-2(e1368)* mutants, *daf-16a #1* and *#2* mutations reduced mean life span by 19.3% and 17.3% respectively (p<0.0001), whereas *daf-16a/f* mutation decreased mean life span by 39.5% (Figure 4.3A; Supplemental Table 4.5-6). Thus, on average, *daf-16a* mutations shortened *daf-2(e1368)* life span ~46% as much as *daf-16a/f* mutation did (18.3/39.5). Similarly, in *daf-2(e1370)* mutants, *daf-16a #1* and #2 mutations shortened mean life span by 26.3% and 26.6% respectively (p<0.0001), whereas *daf-16a/f* mutation reduced mean life span by 65.6% (Figure 4.3C; Supplemental Table 4.5-6). Therefore, *daf-16a* mutation was on average 40% as potent as *daf-16a/f* mutation in decreasing *daf-2(e1370)* life span (26.45/65.6). These results indicate that DAF-16A is partially required for the longevity of *daf-2/IGFR* mutants. In contrast, *daf-16f* mutation did not reproducibly influence life span in either *daf-2/IGFR* mutant background, shortening *daf-2(e1368)* mean life span by 5% and 9% in two of three trials (p = 0.0356, 0.0013) while having no significant effect on *daf-2(e1370)* life span in three trials (Figure 4.3B,D; Supplemental Table 4.5-6). This finding was unexpected in light of previous studies implicating DAF-16F in life span control by the DAF-2/IGFR pathway [35,42]. Taken with our finding that the *tm6659* mutation likely fully eliminates *daf-16f* activity (Figure 4.1, Supplemental Figure 4.1, Supplemental Figure 4.6D), our data are consistent with a hierarchical model of DAF-16/FoxO isoform function in promoting longevity in the context of reduced DAF-2/IGFR signaling. DAF-16A is necessary for full life span extension, as *daf-16a*-specific mutation reduces life span extension by ~40-50%. However, DAF-16F is

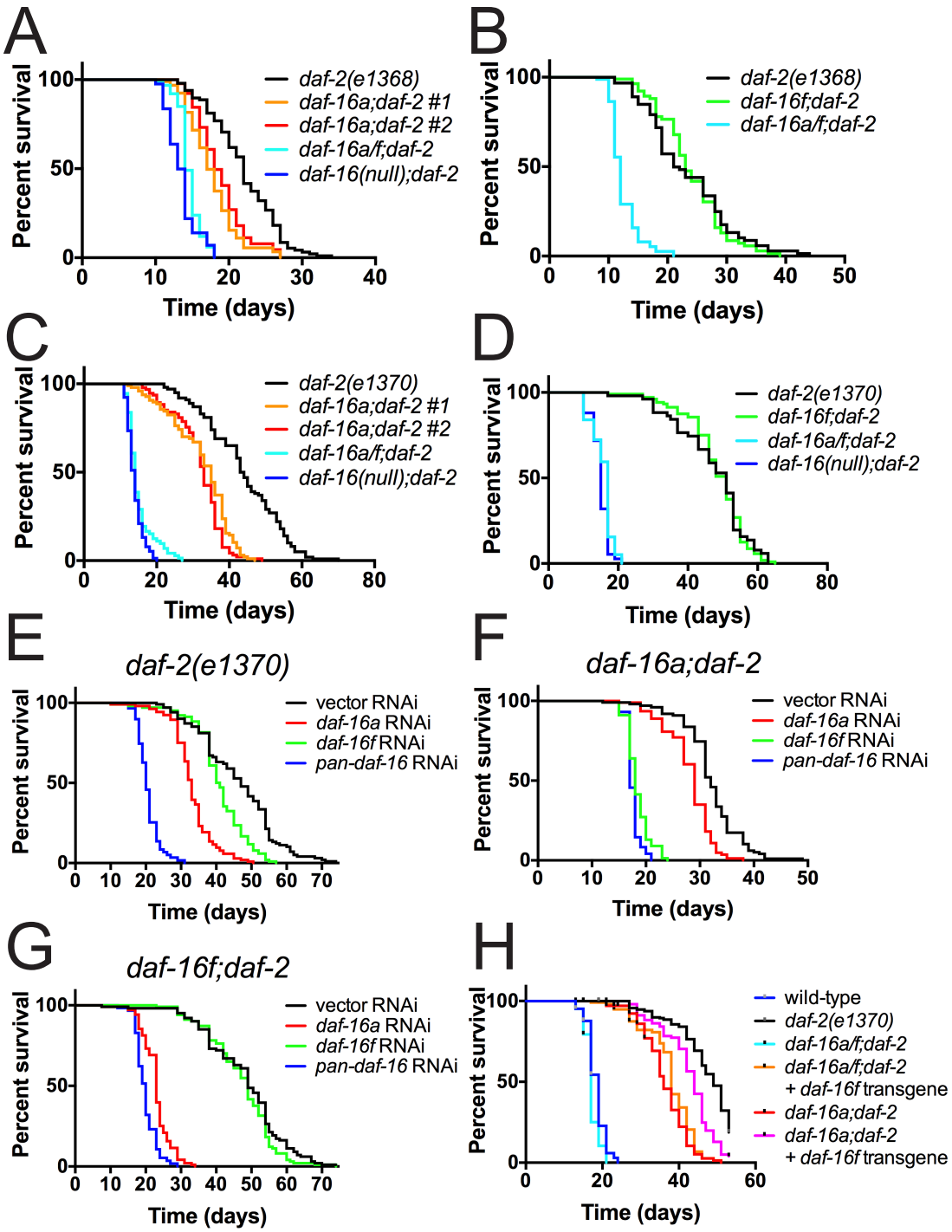
dispensable for longevity, as DAF-16A is sufficient to fully extend life span even when *daf-16f* is mutated.

Given that *daf-16a/f* mutation shortens life span in animals with reduced DAF-2/IGFR activity to a much greater extent than *daf-16a* mutation alone, we tested the possibility that DAF-16F is required for life span extension in the absence of DAF-16A. Thus, we performed isoform-specific *daf-16/FoxO* RNAi [35]. As previously shown, inactivation of either *daf-16a* or *daf-16f* alone by RNAi had a modest effect on the longevity of *daf-2/IGFR* mutant animals compared to RNAi of all *daf-16/FoxO* isoforms ("pan-*daf-16* RNAi") [35]. *daf-2(e1370)* mean life span is reduced 25% by *daf-16a* RNAi and 16.7% by *daf-16f* RNAi, corresponding to 45% and 30% of the effect of *pan-daf-16* RNAi (55.0% reduction of mean life span; Figure 4.3E; Supplemental Table 4.7). However, *daf-16f* RNAi shortened the mean life span of *daf-16a;daf-2* double mutant animals by 46%, which is 93% of the effect of *pan-daf-16* RNAi (49.5%; Figure 4.3F; Supplemental Table 4.7). Therefore, although DAF-16F is dispensable for longevity in the presence of DAF-16A, it is required for life span extension in animals lacking DAF-16A. The life-span-shortening effect of *daf-16a* RNAi in *daf-16a;daf-2* double mutants (10.8% reduction in mean life span) may be a consequence of off-target RNAi effects [50].

We also wished to determine the extent to which longevity in *daf-16f;daf-2* double mutants requires DAF-16A. To address this question, we performed isoform-specific *daf-16/FoxO* RNAi on *daf-16f;daf-2* double mutants. *daf-16a* RNAi shortened the mean life span of *daf-16f;daf-2* double mutant animals by 53.8%, constituting 90% of the effect of *pan-daf-16* RNAi (59.9%; Figure 4.3G; Supplemental Table 4.7). In contrast, *daf-16f* RNAi increased *daf-16f;daf-2* mean life span by 1.1%.

Therefore, when DAF-16F is absent, DAF-16A is likely the sole FoxO isoform that promotes longevity. DAF-16A is the primary isoform that controls *C. elegans* aging in the context of reduced DAF-2/IGFR signaling. DAF-16F is not required for longevity when DAF-16A is present, but it promotes long life in the absence of DAF-16A.

Figure 4.3. DAF-16A, but not DAF-16F, is sufficient for full life span extension in *daf-2/IGFR* mutants.
(A-D) Effects of *daf-16a* (A, C) and *daf-16f* (B, D) mutations on life spans of *daf-2(e1368)* (A-B) and *daf-2(e1370)* (C-D). (E-G) *daf-16a* is required for *daf-16f;daf-2* longevity and vice versa. Survival curves are presented for (E) *daf-2(e1370)*, (F) *daf-16a;daf-2*, (G) *daf-16f;daf-2* mutant animals upon exposure to isoform-specific *daf-16* RNAi. (H) Single-copy *daf-16f* rescue in *daf-16a/f;daf-2* recapitulates *daf-16a;daf-2* longevity, and doubling the gene dosage of *daf-16f* extends life span. See text for details. Statistics and raw data are presented in Supplemental Table 4.5-8.



Rescue using *daf-16a* and *daf-16f* single-copy transgenes

Our analysis demonstrates that DAF-16A promotes longevity in *daf-2/IGFR* mutants more strongly than DAF-16F, but previous studies suggested that DAF-16F plays a stronger role [35,42]. These studies employed transgenic strains harboring multiple cDNA copies of either *daf-16a* or *daf-16f*. Thus, we generated single-copy transgenic strains with the aim of achieving endogenous levels of expression of *daf-16a* and *daf-16f*. A *daf-16a/f;daf-2* mutant with a *daf-16f* single-copy transgene exhibited the same life span as the *daf-16a;daf-2* mutant (Figure 4.3H), providing further evidence that *daf-16a* is required for full life span extension of *daf-2/IGFR* mutants. Furthermore, *daf-16f* rescue in a *daf-16a;daf-2* mutant effectively doubles the gene dosage of *daf-16f* in the absence of *daf-16a*, and this mutant with four *daf-16f* copies lived longer than a strain with two *daf-16f* copies (Figure 4.3H). This strongly suggests that multi-copy expression of *daf-16f* in previous studies over-stated the role of *daf-16f*. However, our *daf-16a* single-copy transgene only partially rescued the dauer and life span phenotypes of *daf-16a* mutation (Supplemental Discussion). Taken together, these results likely reconcile the previous study by Kwon *et al.* and our results- see Supplemental Discussion for further details.

DAF-16A promotes stress resistance

Given the lack of effect of *daf-16f* mutation on *daf-2/IGFR* mutant dauer arrest (Figure 4.2) and longevity (Figure 4.3) when *daf-16a* is intact, we attempted to identify a *daf-16f* phenotype. DAF-16/FoxO promotes resistance to a wide variety of stresses [2]. We found that *daf-16a* mutation partially reduces the resistance of *daf-2(e1370)* mutants to ultraviolet (UV), oxidative, and heat stress (Supplemental Figure 4.7, Supplemental Table 4.10), similar to the effect of *daf-16a* mutation on longevity. In contrast, *daf-16f* mutation does not have any significant effect on resistance to these same stresses (Supplemental Figure 4.7, Supplemental Table 4.10).

DAF-16A promotes longevity in animals lacking a germline

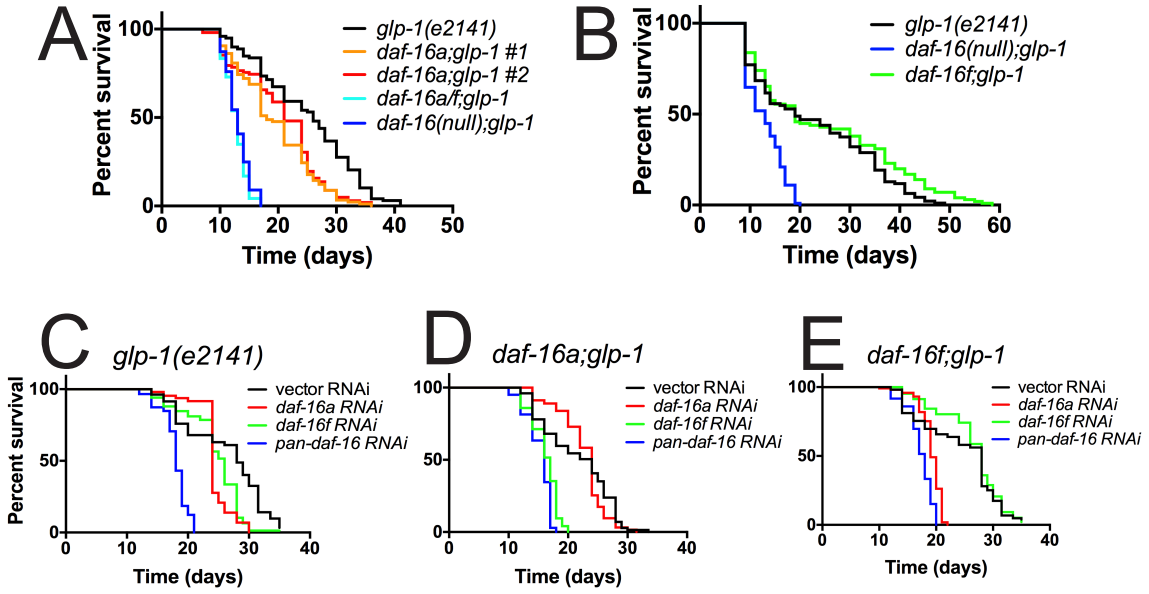
Although DAF-16/FoxO is also required for longevity in animals lacking a germline [29], life span extension caused by germline ablation requires molecules such as KRI-1 and TCER-1 that are not necessary for longevity in *daf-2/IGFR* mutants [33,34]. These observations suggest that DAF-2/IGFR and the germline could control life span by coupling to distinct DAF-16/FoxO isoforms. To test this hypothesis, we determined the effect of isoform-specific *daf-16/FoxO* mutations on life span in animals harboring a temperature-sensitive *glp-1* mutation that develop without a germline when grown at 25°C [51]. As we observed in animals with reduced DAF-2/IGFR activity, the life spans of germline-ablated animals were modestly reduced by *daf-16a* mutation; *daf-16a #1* and *#2* mutations shortened mean life spans of germline-ablated animals by 17.5% and 18.2% respectively ($p<0.0001$), compared to a 45.2% reduction in mean life span due to *daf-16a/f* mutation (Figure 4.4A; Supplemental Table 4.8). Therefore, the effect of *daf-16a* mutation on mean life span is 39% of the effect of combined *daf-16a/f* mutation on mean life span of animals lacking a germline. *daf-16f* mutation did not influence the life span of germline-ablated animals in a consistent manner, increasing mean life span by 11% in one of three trials ($p = 0.0454$; Figure 4.4B; Supplemental Table 4.8).

We also tested whether DAF-16F is required for life span extension of germline-ablated animals in the absence of DAF-16A and vice versa, similar to requirements in the context of *daf-2/IGFR* mutation. In germline-ablated animals where all *daf-16* isoforms are intact, mean life span is reduced 13.8% by *daf-16a* RNAi and 4.8% by *daf-16f* RNAi, corresponding to 40% and 14% of the effect of *pan-daf-16* RNAi (34.7% reduction of mean life span; Figure 4.4C; Supplemental Table 4.9). However, *daf-16f* RNAi shortened the mean life span of *daf-16a;daf-2* double mutant animals by 21.8%, which is 84% of the effect of *pan-daf-16* RNAi (26.0%; Figure 4.4D; Supplemental Table 4.9). *daf-16a* RNAi shortened the mean life span of *daf-16f;daf-2* double mutant animals by 25.3%, constituting 83% of the effect of *pan-daf-16* RNAi (30.6%, Figure 4.4E; Supplemental Table 4.9). Intriguingly, *daf-16a* and *daf-16f* RNAi appears to modify life span variation in germline-ablated animals, where a subset of the population experiences early death

[29]. This may reflect dosage-dependent effects of *daf-16/FoxO*, or it may be the result of off-target effects of RNAi.

Therefore, DAF-16A is also the main FoxO isoform that promotes longevity in animals lacking a germline, and DAF-16F is not required for life span extension in this context. Furthermore, the distinct molecular requirements for life span extension in *daf-2/IGFR* mutants and germline-ablated animals cannot be explained by the coupling of these upstream pathways to disparate DAF-16/FoxO isoform outputs.

Figure 4.4. DAF-16A, but not DAF-16F, is sufficient for full life span extension in animals lacking a germline.
(A-B) Effects of *daf-16a* (A) and *daf-16f* (B) mutations on life spans of *glp-1(e2141)* animals raised at the restrictive temperature to ablate the germline (C-E) *daf-16a* is required for *daf-16f;glp-1* longevity and vice versa. Survival curves are presented for (E) *glp-1(e2141)*, (F) *daf-16a;glp-1*, (G) *daf-16f;glp-1* mutant animals upon exposure to isoform-specific *daf-16* RNAi. See text for details. Statistics and raw data are presented in Supplemental Table 4.8 and Supplemental Table 4.9.



DAF-16/FoxO target gene regulation by DAF-16A and DAF-16F

In order to illuminate the mechanistic basis for DAF-16/FoxO isoform-specific functions in life span control, we performed whole transcriptome profiling of young adult *daf-2(e1370)* mutant animals in the context of wild-type *daf-16/FoxO* and isoform-specific *daf-16/FoxO* mutant alleles. A subset of animals from each experimental replicate was subjected to life span assays to confirm that all mutants from which RNA was isolated had the expected life span phenotypes. Identification of genes that were differentially expressed in wild-type and *daf-2(e1370)* and differentially expressed in the opposite direction in both *daf-16(null);daf-2* and *daf-16a/f;daf-2* double mutants compared to *daf-2* mutants defined a set of 399 genes that are targets of DAF-16A and/or DAF-16F (Figure 4.5; henceforth referred to as "DAF-16A/F target genes"). We validated our profiling results by measuring transcript levels corresponding to twelve genes that emerged from this analysis using qPCR. DAF-16/FoxO-dependent regulation was confirmed for all twelve of these genes (Figure 4.6; Supplemental Figure 4.8; Supplemental Table 4.11).

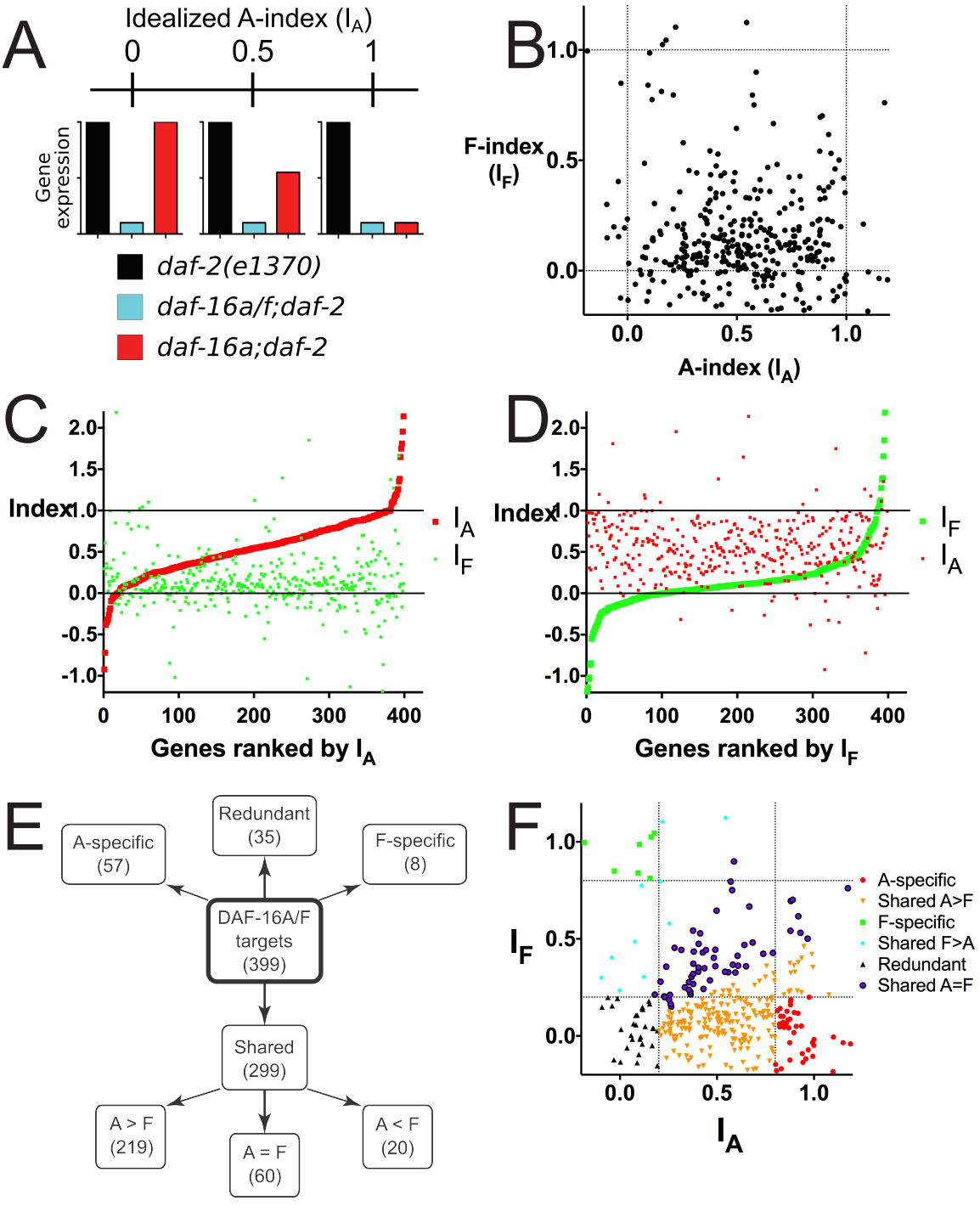
To gain insight into the relative magnitude of DAF-16A- and DAF-16F-specific contributions to the regulation of individual DAF-16A/F target genes, we compared the effect of either *daf-16a* or *daf-16f* mutation on DAF-2/IGFR-dependent gene regulation to the effect of *daf-16a/f* mutation on DAF-2/IGFR-dependent gene regulation, thus calculating an "A-index" (I_A) and "F-index" (I_F) for each DAF-16A/F target gene (see Materials and Methods). Hypothetically, an idealized DAF-16A-specific target gene would have $I_A = 1.0$ and $I_F = 0$, whereas a DAF-16F-specific target gene would have $I_A = 0$ and $I_F = 1.0$ (Figure 4.5A). I_A and I_F calculated using fragments per kilobase per million reads (FPKM) values from whole transcriptome profiling correlated well with I_A and I_F derived from qPCR data for twelve genes tested (Supplemental Table 4.12).

We first generated a scatter plot of I_A and I_F for the entire set of 399 DAF-16A/F target genes (Figure 4.5B). This depiction indicates that $I_A > I_F$ for most genes, suggesting that DAF-16A plays a larger role in gene regulation than DAF-16F.

To further explore this question, we plotted I_A and I_F of the entire set of DAF-16A/F target genes from lowest to highest I_A (Figure 4.5C) and I_F (Figure 4.5D). This allowed us to visualize the magnitude of isoform-specific regulation for both DAF-16A and DAF-16F across the entire set of target genes while potentially revealing relationships between the isoforms in target gene regulation. These illustrations confirm that for most target genes, DAF-16A has a greater impact on expression than DAF-16F. Furthermore, they reveal no obvious global relationship between the degree of regulation of any single gene by one isoform and the impact of the other isoform on its expression.

Figure 4.5. DAF-16A and DAF-16F target genes identified by whole transcriptome profiling (next page).

(A) Depiction of the A-index (I_A) for three hypothetical target genes with I_A = 0, 0.5, and 1.0. Idealized expression profiles in *daf-2(e1370), daf-16a/f;daf-2,* and *daf-16a;daf-2* are shown for all three genes. (B) Scatterplot comparing I_A and I_F for DAF-16A/F target genes. Dashed lines indicate I_A and I_F = 0 or 1. Only genes with indices from -0.2 to 1.2 are shown; a scatter plot with a wider range of indices is shown in Supplemental Figure 4.9. (C-D) Plots of I_A and I_F for all DAF-16A/F target genes from lowest to highest I_A (C) or I_F (D). Solid lines correspond to indices of 0 and 1. Three genes with indices greater than 2.2 or less than -1.2 were omitted for presentation purposes. (E) Tree diagram summarizing the categorization system for DAF-16A and DAF-16F target genes. See text and Materials and Methods for details. (F) Scatterplot from (B), with individual genes color-coded according to category. Dashed lines indicate I_A or I_F values of 0.2 or 0.8, corresponding to the cutoffs used to define redundantly regulated, A-specific, and F-specific targets.



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Categorization of DAF-16A/F target genes

The life span phenotypes of *daf-16a/f, daf-16a,* and *daf-16f* mutants (Figure 4.3 and Figure 4.4) suggested that sorting of DAF-16A/F target genes into categories based on their regulation by DAF-16A and/or DAF-16F might shed light on which genes are likely to contribute significantly to longevity. Therefore, we placed each DAF-16A/F target gene into one of four categories based on the impact of each isoform on expression: DAF-16A-specific ($I_A > 0.8$ and $I_F < 0.2$), DAF-16F-specific ($I_F > 0.8$ and $I_A < 0.2$), redundant (I_A and I_F both < 0.2), and shared (all others). We further subdivided genes in the "shared" category into three subgroups: genes for which DAF-16A plays a greater role than DAF-16F in regulation (shared A > F; $I_A/I_F > 2.0$), genes for which DAF-16F has a greater impact on regulation than DAF-16A (shared F > A; $I_F/I_A > 2.0$), and genes that are regulated by both isoforms (shared A = F; $0.5 \leq I_A/I_F \leq 2.0$; Figure 4.5E-F).

57 genes are DAF-16A-specific targets (Figure 4.5E-F). This group includes *far-3* (Figure 4.6A; Supplemental Table 4.11), which encodes a fatty acid/retinol binding protein [52], as well as the *lipl-1* gene encoding a lysosomal acid lipase, which is transcriptionally upregulated and promotes the mobilization of lipid stores in response to starvation [53]. Eight genes are DAF-16F-specific targets (Figure 4.5E-F), including *lea-1* (Figure 4.6B; Supplemental Table 4.11), which encodes a homolog of human perilipin-4 that promotes resistance to dehydration stress [54].

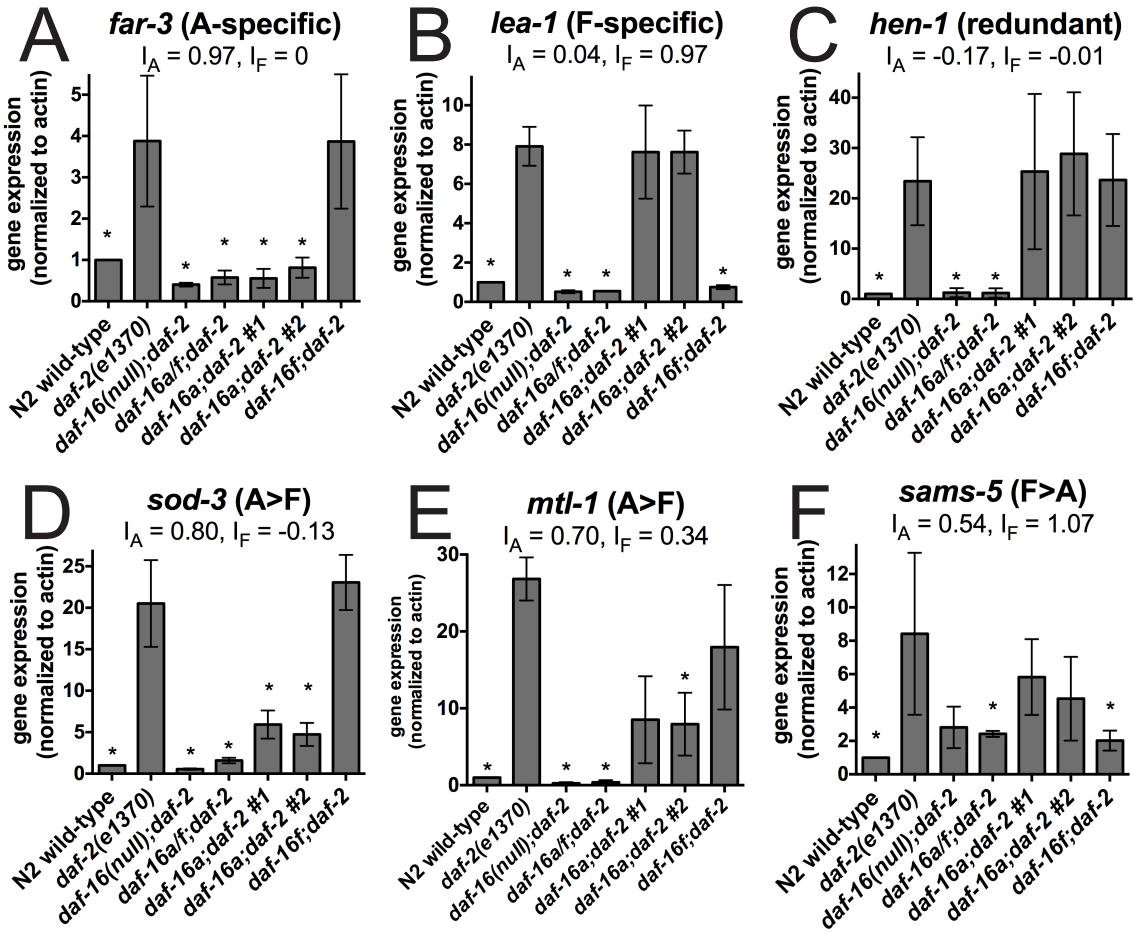
Most DAF-16A/F target genes are regulated by both DAF-16A and DAF-16F (Figure 4.5E-F). 35 genes are redundantly regulated by DAF-16A and DAF-16F (Figure 4.5E-F), including *hen-1* (Figure 4.6C; Supplemental Table 4.11), a secreted protein required for sensory integration and learning [55], and the established DAF-16/FoxO target genes *lys-7* and *dod-17* (Supplemental Figure 4.8) [56].

The remaining 299 DAF-16A/F target genes are categorized as those with "shared" regulation by DAF-16A and DAF-16F (Figure 4.5E-F). 73 percent of these (219/299) are primarily regulated by DAF-16A (Figure 4.5E-F). This subgroup comprises the largest subset of target genes and includes the established DAF-16/FoxO targets *sod-3* (Figure

4.6D, Supplemental Table 4.11), *mtl-1* (Figure 4.6E), *fat-7* (Supplemental Figure 4.8), *hsp-12.6, dod-3, dod-23,* and *dod-24* [56] and the lipase-like gene *lipl-2* (Supplemental Figure 4.8). It also includes *aakg-4,* which encodes an atypical AMP kinase gamma subunit that participates in a positive feedback loop to promote DAF-16/FoxO activity [57]. Among the 20 "shared" target genes that are primarily regulated by DAF-16F are the S-adenosyl methionine synthase gene *sams-5* (Figure 4.6F) and six collagen genes (Figure 4.5E-F). Finally, 60 target genes are regulated to a comparable extent by DAF-16A and DAF-16F (Figure 4.5E-F). Within this subgroup are *lipl-3* and *lipl-4,* which encode lipases that, along with the DAF-16A-specific target *lipl-1,* are transcriptionally induced in response to fasting [53].

Collectively, these classifications indicate that DAF-16A plays an important role in regulating 93% of DAF-16A/F target genes ("DAF-16A-specific," "redundant," "shared A > F," and "shared A = F" categories; 371/399 genes), whereas DAF-16F strongly influences the expression of 30% of target genes ("DAF-16F-specific," "redundant," "shared F > A," and "shared A = F" categories; 123/399 genes).

Figure 4.6. qPCR validation of target gene regulation by DAF-16A and DAF-16F.
(A-F) Expression of six DAF-16A/F target genes quantified by qPCR using RNA isolated from Day 1 young adult animals. Values represent the mean from three biological replicates. Error bars represent standard deviation. Asterisks indicate statistically significant changes ($p < 0.05$ by paired ratio *t*-test). I_A and I_F were calculated using mean expression values measured by qPCR. Statistics and data are summarized in Supplemental Table 4.11.



Discussion

We have used isoform-specific *daf-16/FoxO* mutant alleles to elucidate the biological functions of specific DAF-16/FoxO isoforms in development, life span control, and gene regulation. Our results indicate that DAF-16A is the major FoxO isoform that controls dauer arrest (Figure 4.2) and longevity (Figure 4.3) in the context of reduced DAF-2/IGFR signaling. Furthermore, we show for the first time that DAF-16A is also the primary FoxO isoform that promotes longevity in animals lacking a germline (Figure 4.4). Importantly, a deletion mutant that lacks all exons specific to *daf-16f* does not significantly influence dauer arrest (Figure 4.2) or longevity in either *daf-2/IGFR* mutants or in germline-ablated animals (Figure 4.3 and Figure 4.4). These results indicate that in the presence of physiologic levels of DAF-16A, DAF-16F is dispensable for dauer regulation and life span control. In the absence of DAF-16A, DAF-16F promotes dauer arrest and longevity, as demonstrated by the incomplete suppression of dauer-constitutive (Figure 4.2B) and life span extension phenotypes by *daf-16a* mutation (Figure 4.3A,C and Figure 4.4A) and the influence of *daf-16f* RNAi on life span in *daf-2/IGFR* mutants and germline-ablated animals that lack *daf-16a* (Figure 4.3F and Figure 4.4D).

Our data are in agreement with a previous study implicating both DAF-16A and DAF-16F in dauer regulation and stress resistance in the *daf-2(e1370)* mutant background [35]. However, our results contradict the contention that DAF-16F plays a more prominent role in life span control than DAF-16A [35,42]. This discrepancy is likely a consequence of distinct experimental strategies used to assess the function of specific DAF-16/FoxO isoforms in life span control. We used isoform-specific deletion mutants in which other *daf-16/FoxO* isoforms remained under the control of endogenous regulatory elements and continued to be expressed at physiological levels (Supplemental Figure 4.6). In contrast, Kwon *et al.* based their analysis on strains harboring a *daf-16/FoxO* null mutation in which cDNAs encoding individual *daf-16/FoxO* isoforms were overexpressed transgenically [35]. Results utilizing single-copy transgenes to rescue the isoform-specific mutants likely reconcile these seemingly conflicting results- see Supplemental Discussion for further details.

The expression profiling experiments presented here are the first to define the relative contributions of specific DAF-16/FoxO isoforms to the regulation of DAF-16/FoxO target genes. They reveal a dominant role for DAF-16A relative to DAF-16F in regulating gene expression in young adult animals (Figure 4.5) that is commensurate with the relative influence of DAF-16A and DAF-16F on adult life span (Figure 4.3). These results further support the conclusion that DAF-16A is the major FoxO isoform that promotes longevity in *C. elegans*.

Although the prolongevity function of DAF-16/FoxO is well established, the question of which DAF-16/FoxO target genes are important in life span control remains unanswered. Multiple studies have identified thousands of DAF-16/FoxO target genes that are regulated by DAF-2/IGFR signaling and the germline [56,58-66]. In light of these data, a commonly accepted model is that DAF-16/FoxO promotes longevity by controlling the expression of several hundreds of genes, the products of which collectively contribute to life span extension. Although this model may be intuitively appealing, it remains untested; the possibility exists that a relatively small subset of DAF-16/FoxO target genes plays a disproportionately prominent role in influencing longevity.

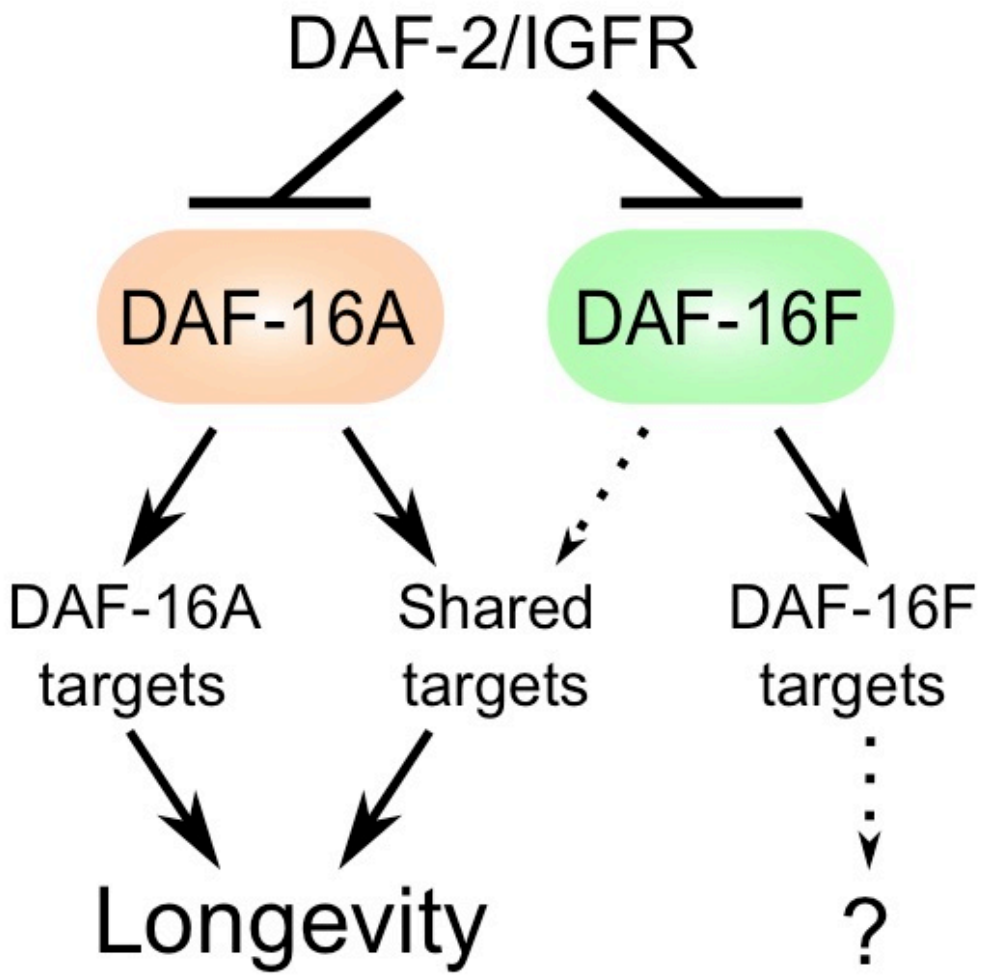
Few DAF-16/FoxO target genes have been rigorously analyzed for roles in life span control. This is likely a consequence of the large number of genes that are regulated by DAF-16/FoxO [64]. Our phenotypic analysis and transcriptional profiling data define subsets of DAF-16/FoxO targets that may be more or less likely to include genes that contribute significantly to longevity. Since *daf-16f* mutation does not influence life span in the presence of DAF-16A (Figure 4.3B,D and Figure 4.4B), genes that are primarily regulated by DAF-16F (*i.e.,* genes in the "DAF-16F-specific" and "shared F > A" categories; Figure 4.5E-F) are unlikely to play prominent roles in promoting longevity. Conversely, since *daf-16a* mutation significantly shortens life span and *daf-16a/f* mutation suppresses longevity to the same extent as *daf-16/FoxO* null mutation (Figure 4.3A,C and Figure 4.4A), genes that are primarily regulated by DAF-16A (*i.e.,* "DAF-16A-specific" and "shared A > F" genes; Figure 4.5E-F) or that are redundantly regulated by both DAF-16A and DAF-16F ("redundant" genes) may be more likely to function as

DAF-16/FoxO effectors in promoting longevity. Consistent with this notion, *aakg-4,* which emerged from our analysis as a "shared A > F" gene, was recently shown to be important for life span extension in *daf-2/IGFR* mutants [57]. Thus, our analysis provides a useful framework for prioritizing the evaluation of specific DAF-16/FoxO target genes for potential roles in life span control.

Based on our results, we propose a hierarchical model of DAF-16/FoxO isoform function in life span control (Figure 4.7). DAF-2/IGFR inhibits both DAF-16A and DAF-16F [35]. In the context of reduced DAF-2/IGFR signaling, both DAF-16A and DAF-16F contribute to longevity and the regulation of DAF-16/FoxO target genes. In the absence of DAF-16A, the altered expression of genes regulated mainly by DAF-16A shortens life span. When DAF-16F is inactive, genes regulated primarily by DAF-16F are misregulated, but this does not influence life span. When neither DAF-16A nor DAF-16F is present, DAF-2/IGFR mutation does not promote longevity due to the misregulation of both DAF-16A-specific target genes as well as genes that are regulated by both DAF-16A and DAF-16F.

The vital role of IGFR signaling and FoxO transcription factors in life span control was discovered in *C. elegans* decades ago [5,67-69]. IGFR signaling is now known to influence aging in mammals [70,71] and possibly humans [72,73], and the prolongevity function of FoxO that is conserved in invertebrates [5,8,9,74,75] is likely relevant to mammalian aging and aging-related diseases. In establishing the primacy of DAF-16A in *C. elegans* life span control and gene regulation, we provide a foundation for understanding phyletically general functions of FoxO transcription factors in controlling aging.

Figure 4.7. Model of life span control and gene regulation by DAF-16A and DAF-16F. See text for details.



Materials and Methods

C. elegans strains and maintenance

Strains used in this study are listed in Supplemental Table 4.13. Animals were maintained at 15°C on nematode growth media (NGM) plates seeded with *Escherichia coli* OP50. Double mutants were constructed using standard genetic techniques. Genotypes were confirmed by PCR amplification to detect restriction fragment length or PCR polymorphisms. Percival I-36NL incubators (Percival Scientific, Inc., Perry, IA) were used for maintenance, dauer arrest assays, and life span assays.

RNA isolation

Animals were washed twice in M9 buffer. Total RNA was isolated using TRIzol® reagent (Invitrogen) and purified using an RNeasy kit (QIAGEN Inc., Valencia, CA) according to manufacturers' instructions.

Quantitative real-time reverse-transcriptase PCR (qPCR)

cDNA was synthesized using a SuperScript® III Reverse Transcriptase kit and random hexamers (Invitrogen, Carlsbad, CA). Real-time PCR was then performed in triplicate using *Power* SYBR® Green PCR master mix (Applied Biosystems, Warrington, UK) and a Mastercycler® ep *realplex* thermal cycler (Eppendorf North America, Westbury, NY). 10ng of cDNA was used as a template in 15µl reaction volume. Primers were selected initially using GETPrime [76] and Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and subsequently validated by melt curve analysis and agarose gel electrophoresis. Primer sequences are listed in Supplemental Table 4.14. Relative expression levels and technical error were determined by the $\Delta\Delta 2C_t$ method [77]. Gene expression levels were normalized to actin (*act-1*), and the ratio of expression relative to *act-1* was then compared to the same ratio in N2 Bristol wild-type. Statistical analysis was performed in GraphPad Prism (GraphPad Software, La Jolla, CA) using the paired ratio *t*-test.

Rapid amplification of cDNA ends (RACE)

Total RNA was isolated from young adult animals. *daf-16a* and *daf-16f* cDNA was prepared using a 5' RACE System Version 2.0 (Invitrogen). First-strand cDNA was synthesized using a *daf-16a/f* gene-specific primer and SuperScript™ II. The original mRNA template was degraded by RNase H and RNase T1. After purification, a homopolymeric tail was added using terminal deoxynucleotidyl transferase to the 3' end of cDNA. Standard PCR was performed using *Taq* DNA polymerase, a nested *daf-16a* or *daf-16f*-specific primer, and the abridged anchor primer complementary to the homopolymeric tail. After visualization of products on a standard agarose gel, the reaction mix was cloned into pCR™4-TOPO® vector using a TOPO TA Cloning® kit (Invitrogen) and transformed into chemically competent *E. coli* DH5α. Clones were selected on LB plates containing 50μg/mL ampicillin, and plasmids were analyzed by Sanger sequencing. Numbers of clones analyzed for each strain are indicated in Supplemental Table 4.15.

Dauer arrest assays

Dauer assays were performed at 25°C as previously described [78]. Briefly, animals were synchronized in a 4 hr egglay at 15°C and grown at 25°C on NGM plates. Animals were scored when wild-type animals were gravid adults and *daf-2* animals had arrested as dauers (~60-72 hours after egglay). Statistical significance was assessed using a two-tailed, unpaired *t*-test with Welch's correction.

Life span assays

Life span assays were performed as previously described [79], with minor modifications. Animals derived from a synchronized 4 hr egglay were grown at 15°C until the L4 larval stage and then shifted to 20°C. Plates harboring any males were discarded. Animals were grown for an additional 20-24 hr to day 1 of adulthood and then placed on life span plates containing 25μg/mL 5-fluoro-2'-deoxyuridine (FUDR; Sigma-Aldrich, St. Louis, MO) to prevent progeny growth. *glp-1* mutant animals were raised at the restrictive temperature to ablate the germline. Statistical significance was assessed using the standard chi-square-based log-rank test in GraphPad Prism.

RNAi

RNAi clones were constructed to coincide with previously published isoform-specific and pan-*daf-16* RNAi clones [35]. The *daf-16a/f* clone was obtained from Addgene. Feeding RNAi was performed using standard procedures [80]. All RNAi NGM plates contained 5mM IPTG and 25μg/mL carbenicillin. NGM plates were seeded with an overnight culture of *E. coli* HT115 with either control L4440 vector or RNAi plasmid. For RNAi life span assays, HT115 was concentrated 5X. Plasmids from *E. coli* clones were sequenced for every experimental replicate to confirm their identity.

Stress assays

For all stress assays, animals derived from a synchronized 4 hr egglay were grown at 15°C until the late L3 larval stage and then shifted to 20°C and grown for an additional 12 hours until the L4 larval stage. Plates harboring any males were discarded.

For oxidative stress, animals were transferred to plates containing 7.5mM *tert*-Butyl hydroperoxide (*t*-BOOH) (Sigma-Aldrich) and scored ~3 times per day for survival. For ultraviolet (UV) stress, animals were transferred to plates lacking bacteria and irradiated with 1200 J/m^2 UV-C using a Stratalinker 2400 UV crosslinker (Stratagene, La Jolla, CA). UV-treated animals were then transferred to seeded plates containing 25 μg/mL

FUDR and scored daily for survival. For thermotolerance, animals were transferred to seeded plates containing 25 μg/mL FUDR, grown for an additional 12 hours at 20°C, shifted to 33°C, and scored ~4 times per day for survival.

For all assays, animals that desiccated on the side of plates were censored. Statistical significance was assessed in GraphPad Prism using the standard chi-square-based log-rank test.

Whole transcriptome profiling (RNA-Seq)

Animals were grown as described for life span assays. After picking a subset of the population for life span assays, the remaining animals were harvested for isolation of total RNA. The Agilent TapeStation was used to assess RNA quality. Samples with RINs (RNA Integrity Numbers) of eight or greater were prepped using the Illumina TruSeq mRNA Sample Prep v2 kit (Catalog #RS-122-2001 and RS-122-2002). mRNA was isolated from 0.1-3 μg of total RNA by polyA$^+$ purification, fragmented, and copied into first strand cDNA using reverse transcriptase and random primers. 3' cDNA ends were then adenylated and adapters ligated. One of the adapters contained a 6-nucleotide barcode to enable multiplexing of samples. Products were purified and enriched by PCR to create the final cDNA library. Libraries were checked for quality and quantity by Agilent TapeStation and qPCR using a library quantification kit for Illumina sequencing platforms (catalog #KK4835, Kapa Biosystems, Wilmington, MA). Clonal clusters were generated using cBot (Illumina, Inc., San Diego, CA). Quadriplexed samples were sequenced using the HiSeq 2000 system (Illumina) with a 100-cycle paired-end run in high output mode using Version 3 reagents according to manufacturer's protocols.

Individual reads files for each sample were concatenated into a single .fastq file. Raw reads data for each sample were checked using FastQC (Version 0.10.0, Babraham Bioinformatics, Cambridge, United Kingdom; http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/) to identify features potentially indicative of quality issues (*e.g.* low quality scores, over-represented sequences, and

inappropriate GC content). We used the Tuxedo Suite [81-83] for alignment, differential expression analysis, and post-analysis diagnostics. Briefly, reads were aligned to the reference genome (UCSC ce10; http://genome.ucsc.edu/) using TopHat (version 2.0.9) [83] and Bowtie (version 2.1.0.0) [84]. We used default parameter settings for alignment, with the exception of: "--b2-very-sensitive" and "--no-coverage-search." A second round of quality control was then performed using FastQC to ensure that only high quality data was analyzed further. Cufflinks/CuffDiff (Version 2.1.1) [82] was used for quantification of expression and differential expression analysis, using UCSC ce10.fa as the reference genome and UCSC ce10.gtf as the reference transcriptome (http://genome.ucsc.edu/). For this analysis, we used parameter settings: "--multi-read-correct" to adjust expression calculations for reads that map to more than one locus, as well as "--compatible-hits-norm" and "--upper-quartile –norm" for normalization of expression values. We generated diagnostic plots using the CummeRbund package [85] to confirm that each experiment yielded high quality data.

We used locally developed scripts to format and annotate the differential expression data output from CuffDiff. Genes and isoforms were annotated using NCBI Entrez GeneIDs and text descriptions. Differentially expressed genes were further annotated with Gene Ontology (GO) terms (http://www.geneontology.org/) using NCBI annotation.

RNA-Seq analysis

The annotated gene expression data output from CuffDiff was read into R (http://www.r-project.org/) for six comparisons: *daf-2(e1370)* compared to wild-type, *daf-16(mu86);daf-2, daf-16a/f(mg54);daf-2, daf-16a(tm5030);daf-2, daf-16a(tm5032);daf-2*, and *daf-16f(tm6659);daf-2*. We defined 399 DAF-16A/F targets as meeting all of the following criteria: (1) test status = "OK" for all six comparisons, (2) fold change (FC) ≥ ±1.5 for wild-type *vs. daf-2*, (3) FC ≥ 2 in the opposite direction as wild-type for *daf-2 vs. daf-16(mu86);daf-2,* (4) FC ≥ 2 in the opposite direction as wild-type for *daf-2 vs. daf-16a/f(mg54);daf-2,* and (5) FDR < 0.05 for at least one of the three comparisons of *daf-2 vs.* wild-type, *daf-16(mu86);daf-2*, or *daf-16(mg54);daf-2*. We required FDR < 0.05 for

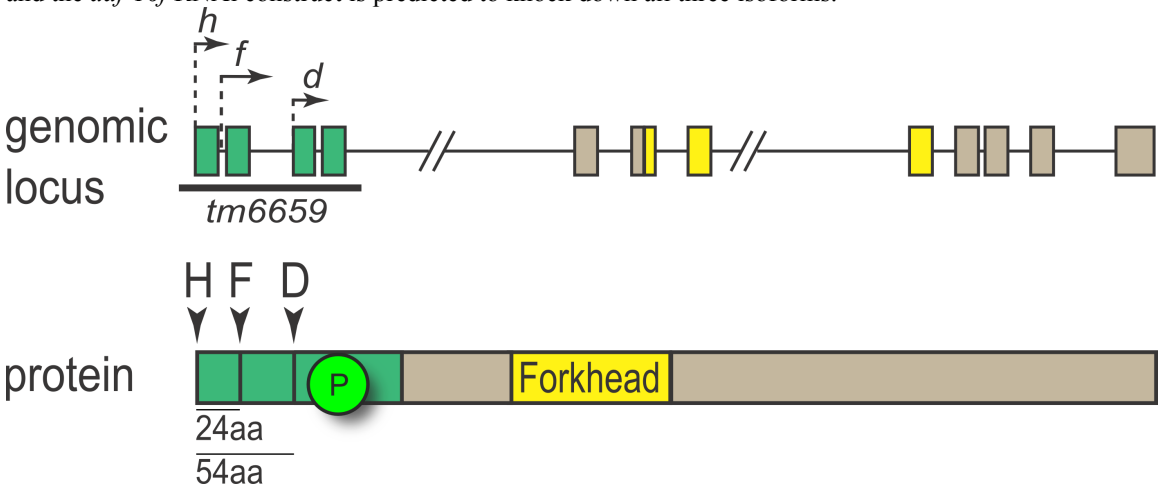
only one of the three comparisons for three reasons: (1) nearly all genes showed concordance in fold-change for all three comparisons even if they did not satisfy FDR < 0.05 for all three; (2) some known DAF-16/FoxO targets only satisfied FDR < 0.05 in one or two comparisons; and (3) the choice of requiring FDR < 0.05 for one, two, or three comparisons did not significantly affect the categorization of DAF-16A/F target genes (Supplemental Table 4.16; see below). FC ≥ 2 instead of 1.5 was chosen for the *daf-2 vs. daf-16;daf-2* comparisons to improve quantification of the effects of *daf-16a* and *daf-16f* mutations on gene expression.

To define classes of DAF-16A/F targets, A- and F-indices (I_A and I_F) were calculated for 399 DAF-16A/F targets. Given high correlation in gene expression between *daf-16a(tm5030);daf-2* and *daf-16a(tm5032);daf-2* (Supplemental Figure 4.10), a combined *daf-16a;daf-2* gene expression profile was generated by calculating the mean FPKM for each gene. I_A for each gene was defined as the absolute FPKM difference between *daf-2(e1370)* and *daf-16a;daf-2*, divided by the absolute FPKM difference between *daf-2(e1370)* and *daf-16a/f(mg54);daf-2*. Likewise, I_F for each gene was defined as the FPKM difference between *daf-2(e1370)* and *daf-16f(tm6659);daf-2*, divided by the FPKM difference between *daf-2(e1370)* and *daf-16a/f(mg54);daf-2*.

Classes of DAF-16A/F targets were defined by the following criteria: DAF-16A-specific, $I_A > 0.8$ and $I_F < 0.2$; DAF-16F-specific, $I_F > 0.8$ and $I_A < 0.2$; and redundantly regulated, $I_A < 0.2$ and $I_F < 0.2$. Genes not binned into these three categories were partitioned into one of the following three groups: shared A-dominant (shared A>F), $I_A/I_F > 2$; shared F-dominant (shared F>A), $I_F/I_A > 2$; or equally shared (shared A=F), $0.5 \leq I_A/I_F \leq 2$.

Supplemental Information

Supplemental Figure 4.1. Distinction between *daf-16d*, *daf-16f*, and *daf-16h*, collectively referred to as *daf-16f* in this study.
daf-16d/f/h transcripts (arrows) differ slightly in their 5' ends. DAF-16D/F/H proteins are translated from distinct translational start sites (arrowheads). Importantly, *daf-16(tm6659)* eliminates all three isoforms, and the *daf-16f* RNAi construct is predicted to knock down all three isoforms.



Supplemental Figure 4.2. *daf-16f* N-terminal cDNA sequence

Legend

Green: Unique *daf-16d* N-terminal exon. Start codon is underlined.
Blue: 7 nucleotides included in two RACE clones due to alternative trans-splicing
Bold: *C. elegans* SL1 trans-spliced leader
Italics: 5'UTR
| exon-exon junction
No *daf-16h* transcripts were detected by 5' RACE.

A. *daf-16d* cDNA sequence – first 3 exons, including 5'UTR

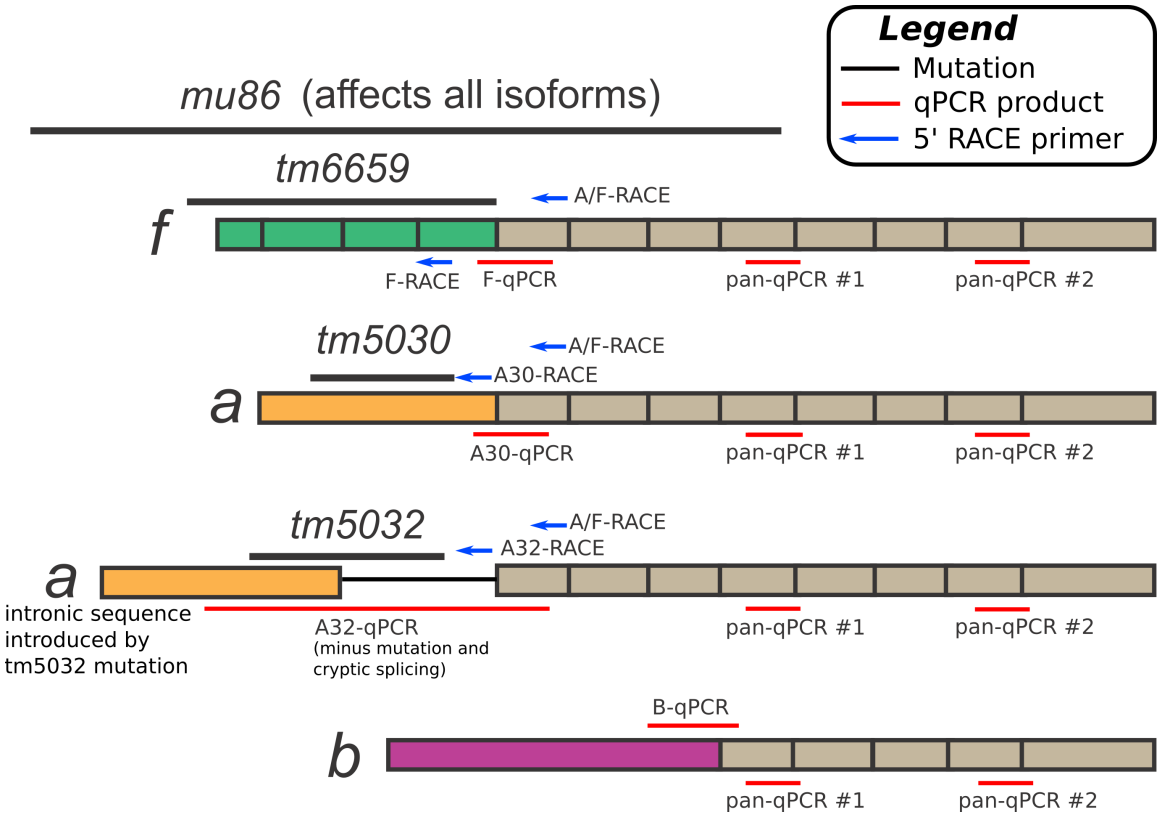
GGTTTAATTACCCAAGTTTGAG*TTTTCAG**CTCGATTCGCCGCTACCATCTGACATCACA*
*CTGCACAATCTCGAACCGGCAAGGCCTGATTCCGGA*ATGAGTTTTTCCACTGATTTTGA
C|GATGATTTCTTCAATCTCGACCTCCATCAACAAGAGCGTTCGGCTTCTTTTGGCGGA
GTAACCCAGTATTCTCAACAATTTCTTCGCGAAGAATGCTCGTTCTCTCCGTATTTCCA
CACATCTTTAGAGACTGTTGACAGCGGAAGAACTAG|CCTATACGGGAGCAATGAGCAA
TGTGGACAGCTCGGCGGAGCATCTTCAAACGGGTCGACAGCAATGCTTCATACTCCAGA
TGGAAGCAATTCTCATCAGACATCGTTTCCTTCGGA

B. *daf-16f* cDNA sequence – first 4 exons, including 5'UTR

GGTTTAATTACCCAAGTTTGAG*CAAAAGTCTTCATTACATTGCTCGAAGTGCCGAAATT*
*TTCTGCAAAAATTCTCACAGGAC*ATGCAAGCGTGGAACTGTCGTGAG|CTCGATTCGCC
GCTACCATCTGACATCACACTGCACAATCTCGAACCGGCAAGGCCTGATTCCGGAATGA
GTTTTTCCACTGATTTTGAC|GATGATTTCTTCAATCTCGACCTCCATCAACAAGAGCG
TTCGGCTTCTTTTGGCGGAGTAACCCAGTATTCTCAACAATTTCTTCGCGAAGAATGCT
CGTTCTCTCCGTATTTCCACACATCTTTAGAGACTGTTGACAGCGGAAGAACTAG|CCT
ATACGGGAGCAATGAGCAATGTGGACAGCTCGGCGGAGCATCTTCAAACGGGTCGACAG
CAATGCTTCATACTCCAGATGGAAGCAATTCTCATCAGACATCGTTTCCTTCGGA

Supplemental Figure 4.3. Overview of strategy for characterizing *daf-16/FoxO* transcripts in isoform-specific mutants.
Spliced transcripts are shown, and exons are separated by black lines. Note that *daf-16a(tm5032)* disrupts a 5' splice site and therefore intronic sequence is included.

For 5' RACE, a semi-synchronized population of young adults was harvested for RNA. RNA was purified and analyzed as described in Methods. The A/F-RACE primer was used for first-strand cDNA synthesis. PCR amplification was performed using either the A30-RACE primer, A32-RACE primer, or the F-RACE primer, in combination with the Abridged Anchor Primer from the Invitrogen RACE kit. For *daf-16(tm5032)*, a different primer A32-RACE was used.



Supplemental Figure 4.4. Effects of *daf-16a* mutations on daf-*16a* N-terminal cDNA sequence

Legend

Orange: Unique *daf-16a* N-terminal exon. Start codon is underlined.
Red: early stop codon in *daf-16a(tm5030)* and *daf-16a(tm5032)* transcripts
Blue: 6 nucleotides included in R13H8.1c but not R13H8.1b by alternative splicing that do not affect the reading frame. R13H8.1c and R13H8.1b are both *daf-16a* transcripts.
Bold: *C. elegans* SL1 trans-spliced leader
Italics: 5'UTR
| exon-exon junction

A. *daf-16a(tm5030)* cDNA sequence – first 3 exons, including 5'UTR

```
wild-type GGTTTAATTACCCAAGTTTGAGAGAACTCACTGATCTTTCAAGCCGAAGCAATCAAGACC
   tm5030 GGTTTAATTACCCAAGTTTGAGAGAACTCACTGATCTTTCAAGCCGAAGCAATCAAGACC

wild-type TCAAAGCCAATCAACTCTACTCACTTTTCTTCAGAACCTTAACTTTTTGTGTCACTTTCC
   tm5030 TCAAAGCCAATCAACTCTACTCACTTTTCTTCAGAACCTTAACTTTTTGTGTCACTTTCC

wild-type CCAAAAACCGTTCAAGCTGCTGCCTTCACTCTCATCCCCTCCTCTTACTCCTTCTTTCTC
   tm5030 CCAAAAACCGTTCAAGCTGCTGCCTTCACTCTCATCCCCTCCTCTTACTCCTTCTTTCTC

wild-type GTCCGCTACTACTGTATCTTCTGGACATCTACCTGTATACACACCAGTGGCCAGTCATCT
   tm5030 GTCCGCTACTACTGTATCTTCTGGACATCTACCTGTATACACACCAGTGGCCAGTCATCT

wild-type GCCATTACAATTTCATCAATTGACACTTCTTCAACAACAACCGCCGTCCTCATTCACTCC
   tm5030 GCCATTACAATTTCATCAATTGACACTTCTTCAACAACAACCGCCGTCCTCATTCACTCC

wild-type CGATTCTTCCTCATCCTCAACATCGTCGTCTTTGGCTGAAATTCCCGAAGACGTTATGAT
   tm5030 CGATTCTTCCTCATCCTCAACATCGTCGTCTTTGGCTGAAATTCCCGAAGACGTTATGAT

wild-type GGAGATGCTGGTAGATCAGGGAACTGATGCATCGTCATCCGCCTCCACGTCCACCTCATC
   tm5030 GGAGATGCTGGTAGAT----------------------------------------------

wild-type TGTTTCGAGATTCGGAGCGGACACGTTCATGAATACACCGGATGATGTGATGATGAATGA
   tm5030 ------------------------------------------------------------

wild-type TGATATGGAACCGATTCCTCGTGATCGGTGCAATACGTGGCCAATGCGTAGGCCGCAACT
   tm5030 ------------------------------------------------------------

wild-type CGAACCACCACTCAACTCGAGTCCCATTATTCATGAACAAATTCCTGAAGAAGATGCTGA
   tm5030 ----------------------------TTCATGAACAAATTCCTGAAGAAGATGCTGA

wild-type |CCTATACGGGAGCAATGAGCAATGTGGACAGCTCGGCGGAGCATCTTCAAACGGGTCGA
   tm5030 |CCTATACGGGAGCAATGAGCAATGTGGACAGCTCGGCGGAGCATCTTCAAACGGGTCGA

wild-type CAGCAATGCTTCATACTCCAGATGGAAGCAATTCTCATCAGACATCGTTTCCTTCGGA|
   tm5030 CAGCAATGCTTCATACTCCAGATGGAAGCAATTCTCATCAGACATCGTTTCCTTCGGA|

wild-type TTTACGAATGTCCGAATCGCCAGACGATACCGTATCGGGAAAAAAGACAACGACCAGACG
   tm5030 TTTACGAATGTCCGAATCGCCAGACGATACCGTATCGGGAAAAAAGACAACGACCAGACG

wild-type GAACGCTTGGGGAAATATGTCATATGCTGAACTTATCACTACAGCCATTATGGCTAGTCC
   tm5030 GAACGCTTGGGGAAATATGTCATATGCTGAACTTATCACTACAGCCATTATGGCTAGTCC

wild-type AGAGAAACGGTTAACTCTTGCACAAG|...
   tm5030 AGAGAAACGGTTAACTCTTGCACAAG|...
                    STOP
```

(continued)

Additional legend- specific to *daf-16a(tm5032)*
lower-case: intronic sequence introduced by *tm5032* mutation
tm5032 #1 *vs.* #2: Two products detected by cDNA sequencing. #1 is the major product, formed by cryptic splice site activation. * denotes cryptic splice site.

B. *daf-16a(tm5032)* cDNA sequence – first exon and intron

```
wild-type GGTTTAATTACCCAAGTTTGAGAGAACTCACTGATCTTTCAAGCCGAAGCAATCAAGACC
tm5032 #1 GGTTTAATTACCCAAGTTTGAGAGAACTCACTGATCTTTCAAGCCGAAGCAATCAAGACC
tm5032 #2 GGTTTAATTACCCAAGTTTGAGAGAACTCACTGATCTTTCAAGCCGAAGCAATCAAGACC

wild-type TCAAAGCCAATCAACTCTACTCACTTTTCTTCAGAACCTTAACTTTTTGTGTCACTTTCC
tm5032 #1 TCAAAGCCAATCAACTCTACTCACTTTTCTTCAGAACCTTAACTTTTTGTGTCACTTTCC
tm5032 #2 TCAAAGCCAATCAACTCTACTCACTTTTCTTCAGAACCTTAACTTTTTGTGTCACTTTCC

wild-type CCAAAAACCGTTCAAGCTGCTGCCTTCACTCTCATCCCCTCCTCTTACTCCTTCTTTCTC
tm5032 #1 CCAAAAACCGTTCAAGCTGCTGCCTTCACTCTCATCCCCTCCTCTTACTCCTTCTTTCTC
tm5032 #2 CCAAAAACCGTTCAAGCTGCTGCCTTCACTCTCATCCCCTCCTCTTACTCCTTCTTTCTC

wild-type GTCCGCTACTACTGTATCTTCTGGACATCTACCTGTATACACACCAGTGGCCAGTCATCT
tm5032 #1 GTCCGCTACTACTGTATCTTCTGGACATCTACCTGTATACACACCAGTGGCCAGTCATCT
tm5032 #2 GTCCGCTACTACTGTATCTTCTGGACATCTACCTGTATACACACCAGTGGCCAGTCATCT

wild-type GCCATTACAATTTCATCAATTGACACTTCTTCAACAACAACCGCCGTCCTCATTCACTCC
tm5032 #1 GCCATTACAATTTCATCAATTGACACTTCTTCAACAACAACCGCCGTCCTCATTCACTCC
tm5032 #2 GCCATTACAATTTCATCAATTGACACTTCTTCAACAACAACCGCCGTCCTCATTCACTCC

wild-type CGATTCTTCCTCATCCTCAACATCGTCGTCTTTGGCTGAAATTCCCGAAGACGTTATGAT
tm5032 #1 CGATTCTTCCTCATCCTCAACATCGTCGTCTTTGGCTGAAATTCCCGAAGACGTTATGAT
tm5032 #2 CGATTCTTCCTCATCCTCAACATCGTCGTCTTTGGCTGAAATTCCCGAAGACGTTATGAT

wild-type GGAGATGCTGGTAGATCAGGGAACTGATGCATCGTCATCCGCCTCCACGTCCACCTCATC
tm5032 #1 GGAGATGCTGGTAGATCAGGGAACTGATGCATCGTCATCCGCCTCCACGTCCACCTCATC
tm5032 #2 GGAGATGCTGGTAGATCAGGGAACTGATGCATCGTCATCCGCCTCCACGTCCACCTCATC

wild-type TGTTTCGAGATTCGGAGCGGACACGTTCATGAATACACCGGATGATGTGATGATGAATGA
tm5032 #1 TGTTTCGAGATTCGGAGCGGACACGTTCATGAATACACCGGATGATGTGATGATGAATGA
tm5032 #2 TGTTTCGAGATTCGGAGCGGACACGTTCATGAATACACCGGATGATGTGATGATGAATGA

wild-type TGATATGGAACCGATTCCTCGTGATCGGTGCAATACGTGGCCAATGCGTAGGCCGCAACT
tm5032 #1 TGATATGGAACCGATTCCTCGTGATCGGTGCAATACGTGGCC------------------
tm5032 #2 TGATATGGAACCGATTCCTCGTGATCGGTGCAATACGTGGCC------------------

wild-type CGAACCACCACTCAACTCGAGTCCCATTATTCATGAACAAATTCCTGAAGAAGATGCTGA
tm5032 #1 ------------------------------------------------------------
tm5032 #2 ------------------------------------------------------------

wild-type ------------------------------------------------------------
tm5032 #1 ttttgtatttttggagcataa*------------------------------------
tm5032 #2 ttttgtatttttggagcataa*gtaatacgactgatatgaacctgaaaaaccaccaatta
                          STOP

wild-type ----------------------------------------|CCTATACGGGAGCA...
tm5032 #1 ----------------------------------------CCTATACGGGAGCA...
tm5032 #2 tatctaattttcccgaacattgtctaatatttctattttcag-CCTATACGGGAGCA...
```

Supplemental Figure 4.5. Predicted DAF-16A proteins encoded by *daf-16a* mutants

For wild-type DAF-16A, 162 out of 510 amino acids are shown. Predicted mutant DAF-16A sequences are aligned to wild-type. Note that R13H8.1b and R13H8.1c are two nearly identical transcripts that both encode DAF-16A.

Legend

| = identity with wild-type sequence
Underline: start of forkhead domain
Bold: RxRxxT AKT family phosphorylation motif
↓ = phosphothreonine
* = early stop
Blue: amino acids included in the protein products of R13H8.1c but not R13H8.1b due to inclusion of 6 nucleotides that do not affect the reading frame (see figure S4). In wild-type, the three indicated amino acids are present in R13H8.1c, but are replaced by a single glutamic acid residue in R13H8.1b. In *daf-16a(tm5030)*, the three indicated amino acids are present in R13H8.1c, but are replaced by a single lysine residue in R13H8.1b.

A. Predicted DAF-16A protein encoded by *daf-16a(tm5030)*

```
                                                                    ↓
wild-type MMEMLVDQGTDASSSASTSTSSVSRFGADTFMNTPDDVMMNDDMEPIPRDRCNTW
 identity ||||||
   tm5030 MMEMLVDFMNKFLKKMLTYTGAMSNVDSSAEHLQTGRQQCFILQMEAILIRHRFL

wild-type PMRRPQLEPPLNSSPIIHEQIPEEDADLYGSNEQCGQLGGASSNGSTAMLHTPDG
 identity
   tm5030 RIYECPNRQTIPYREKRQRPDGTLGEICHMLNLSLQPLWLVQRNG*---------

wild-type SNSHQTSFPSDFRMSESPDDTVSGKKTTTRRNAWGNMSYAELITTAIMASPE...
 identity
   tm5030 ------------------------------------------------------
```

B. Predicted DAF-16A protein encoded by *daf-16a(tm5032)*

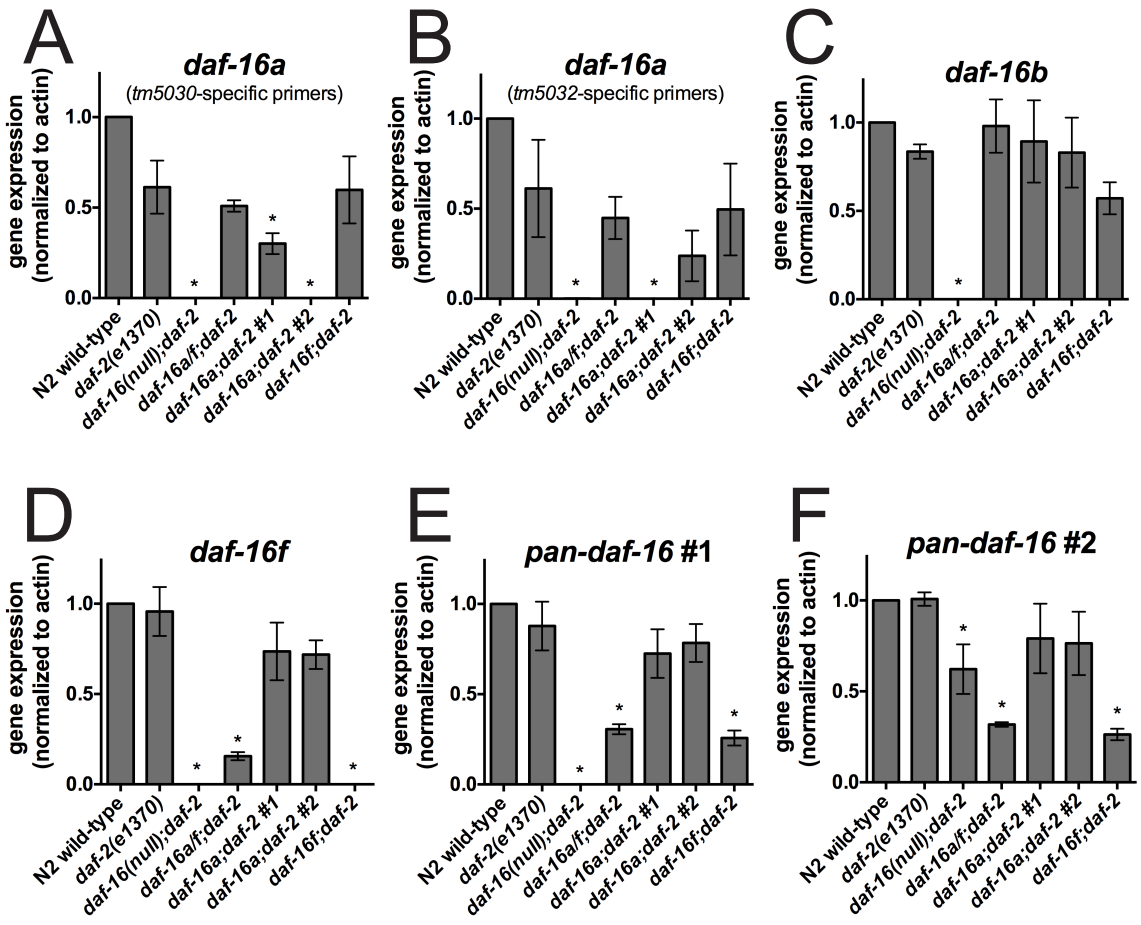
```
                                                                    ↓
wild-type MMEMLVDQGTDASSSASTSTSSVSRFGADTFMNTPDDVMMNDDMEPIPRDRCNTW
 identity ||||||||||||||||||||||||||||||||||||||||||||||||||||||||
   tm5032 MMEMLVDQGTDASSSASTSTSSVSRFGADTFMNTPDDVMMNDDMEPIPRDRCNTW

wild-type PMRRPQLEPPLNSSPIIHEQIPEEDADLYGSNEQCGQLGGASSNGSTAMLHTPDG
 identity |||
   tm5032 PMRFCILEHK*----------------------------------------------

wild-type SNSHQTSFPSDFRMSESPDDTVSGKKTTTRRNAWGNMSYAELITTAIMASPE...
 identity
   tm5032 ------------------------------------------------------
```

Supplemental Figure 4.6. qPCR measurements of *daf-16/FoxO* transcripts in isoform-specific mutants.

Mean values are presented from three biological replicates, error bars represent standard deviation and asterisks indicate samples that display statistically significant changes ($p < 0.05$ by paired ratio *t*-test). Data and statistics are summarized in Table S1. Note that *pan-daf-16* #1 primers anneal within the deletion of the *daf-16(mu86)* null allele, and *pan-daf-16* #2 primers anneal outside of the deletion. Likewise, *tm5030*-specific primers anneal within the *tm5032* deletion and vice versa.



Supplemental Table 4.1. *daf-16/FoxO* isoform-specific qPCR data and statistics.

	Independent cohorts						Statistical Analysis			
	1		2		3		Summary		P value (paired ratio t-test)	Fold change
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
***daf-16a(tm5030*-specific)**										
daf-2(e1370)	0.44	0.26	0.71	0.21	0.68	0.26	0.61	0.15	control	control
daf-16(mu86);daf-2	0.00	0.03	0.00	0.14	0.00	0.14	0.00	0.00	0.0762	0.00
daf-16(mg54);daf-2	0.47	0.27	0.52	0.12	0.53	0.26	0.51	0.03	0.0029	0.83
daf-16(tm5030);daf-2	0.25	0.26	0.36	0.24	0.30	0.17	0.30	0.06	0.2873	0.49
daf-16(tm5032);daf-2	0.00	0.08	0.00	0.42	0.00	0.31	0.00	0.00	0.0106	0.00
daf-16(tm6659);daf-2	0.38	0.22	0.69	0.32	0.71	0.16	0.60	0.18	0.0015	0.98
N2 wild-type	1.00	0.25	1.00	0.21	1.00	0.21	1.00	0.00	0.5286	1.63
***daf-16a(tm5032*-specific)**										
daf-2(e1370)	0.32	0.32	0.85	0.47	0.66	0.44	0.61	0.27	control	control
daf-16(mu86);daf-2	0.00	0.27	0.00	0.18	0.00	0.05	0.00	0.00	0.0459	0.00
daf-16(mg54);daf-2	0.34	0.45	0.57	0.50	0.43	0.45	0.45	0.12	0.2537	0.73
daf-16(tm5030);daf-2	0.00	0.49	0.00	0.16	0.00	0.27	0.00	0.00	0.0039	0.00
daf-16(tm5032);daf-2	0.16	0.21	0.40	0.49	0.15	0.55	0.24	0.14	0.0586	0.39
daf-16(tm6659);daf-2	0.26	0.13	0.77	0.44	0.45	0.44	0.50	0.25	0.1107	0.81
N2 wild-type	1.00	0.27	1.00	0.45	1.00	0.45	1.00	0.00	0.1926	1.63
daf-16b										
daf-2(e1370)	0.81	0.32	0.88	0.08	0.82	0.11	0.84	0.04	control	control
daf-16(mu86);daf-2	0.00	0.27	0.00	1.24	0.00	0.19	0.00	0.00	0.0017	0.00
daf-16(mg54);daf-2	1.15	0.26	0.86	0.13	0.93	0.11	0.98	0.15	0.3017	1.17
daf-16(tm5030);daf-2	1.06	0.14	0.99	0.11	0.63	0.28	0.89	0.23	0.8191	1.07
daf-16(tm5032);daf-2	0.97	0.26	0.92	0.12	0.60	0.32	0.83	0.20	0.8654	0.99
daf-16(tm6659);daf-2	0.67	0.20	0.49	0.06	0.55	0.33	0.57	0.09	0.0790	0.68
N2 wild-type	1.00	0.29	1.00	0.07	1.00	0.17	1.00	0.00	0.0233	1.20
daf-16f										
daf-2(e1370)	0.80	0.10	1.03	0.09	1.04	0.16	0.96	0.14	control	control
daf-16(mu86);daf-2	0.00	0.03	0.00	0.07	0.00	0.14	0.00	0.00	0.0036	0.00
daf-16(mg54);daf-2	0.16	0.09	0.17	0.08	0.13	0.14	0.16	0.02	0.0063	0.16
daf-16(tm5030);daf-2	0.65	0.09	0.92	0.08	0.64	0.13	0.74	0.16	0.1419	0.77
daf-16(tm5032);daf-2	0.73	0.09	0.79	0.11	0.63	0.19	0.72	0.08	0.1386	0.75
daf-16(tm6659);daf-2	0.00	0.15	0.00	0.14	0.00	0.02	0.00	0.00	<0.0001	0.00
N2 wild-type	1.00	0.25	1.00	0.12	1.00	0.23	1.00	0.00	0.6128	1.04
pan-daf-16 #1										
daf-2(e1370)	0.72	0.06	0.95	0.07	0.96	0.10	0.88	0.14	control	control
daf-16(mu86);daf-2	0.00	0.03	0.00	0.07	0.00	0.14	0.00	0.00	0.0159	0.00
daf-16(mg54);daf-2	0.31	0.09	0.33	0.07	0.28	0.11	0.31	0.03	0.0120	0.35
daf-16(tm5030);daf-2	0.68	0.08	0.88	0.06	0.62	0.09	0.72	0.13	0.2511	0.83
daf-16(tm5032);daf-2	0.78	0.08	0.89	0.11	0.68	0.14	0.78	0.11	0.4710	0.89
daf-16(tm6659);daf-2	0.23	0.15	0.23	0.15	0.31	0.03	0.26	0.04	0.0054	0.29
N2 wild-type	1.00	0.16	1.00	0.26	1.00	0.15	1.00	0.00	0.2767	1.14
pan-daf-16 #2										
daf-2(e1370)	0.63	0.20	0.99	0.04	1.05	0.06	0.89	0.22	control	control
daf-16(mu86);daf-2	0.76	0.13	0.54	0.08	0.78	0.08	0.69	0.13	0.0377	0.78
daf-16(mg54);daf-2	0.30	0.14	0.33	0.05	0.30	0.09	0.31	0.01	0.0014	0.35
daf-16(tm5030);daf-2	0.62	0.23	0.90	0.07	0.57	0.27	0.70	0.18	0.2679	0.79
daf-16(tm5032);daf-2	0.66	0.21	0.86	0.12	0.56	0.35	0.69	0.15	0.2132	0.78
daf-16(tm6659);daf-2	0.22	0.20	0.24	0.07	0.30	0.32	0.26	0.04	0.0012	0.29
N2 wild-type	1.00	0.19	1.00	0.16	1.00	0.10	1.00	0.00	0.7706	1.12

Supplemental Table 4.2. Summary of dauer data and statistics

Column statistics are calculated from multiple replicates.

		dauer				adult		non-dauer larvae		
# replicates	genotype	***p*-value** *vs* control (unpaired *t*-test with Welch's)	statistically significant	mean	SD	mean	SD	mean	SD	N
3	*daf-2(e1368)*	control	control	92.8	3.1	0.8	1.4	6.4	3.7	807
3	*daf-16(mu86);daf-2*	0.0004	yes	0.0	0.0	100.0	0.0	0.0	0.0	965
6	*daf-16(mg54);daf-2*	0.0004	yes	0.0	0.0	100.0	0.0	0.0	0.0	2318
3	*daf-16(tm5030);daf-2*	0.0004	yes	0.0	0.0	100.0	0.0	0.0	0.0	1280
3	*daf-16(tm5032);daf-2*	0.0004	yes	0.0	0.0	100.0	0.0	0.0	0.0	1338
5	*daf-16(tm6659);daf-2*	0.4342	no	95.3	5.6	1.5	2.6	3.2	3.5	1403
4	N2 wild-type	0.0004	yes	0.0	0.0	100.0	0.0	0.0	0.0	950
2	wild-type sib of *daf-16(tm6659);daf-2*	0.0004	yes	0.0	0.0	100.0	0.0	0.0	0.0	793
2	*daf-16(tm6659)* sib of *daf-16(tm6659);daf-2*	0.0004	yes	0.0	0.0	100.0	0.0	0.0	0.0	448
3	*daf-2* sib of *daf-16(tm6659);daf-2*	0.6300	no	95.1	6.7	4.6	6.2	0.3	0.6	844

		dauer				adult		non-dauer larvae		
# replicates	genotype	***p*-value** *vs* control (unpaired *t*-test with Welch's)	statistically significant	mean	SD	mean	SD	mean	SD	N
6	*daf-2(e1370)*	control	control	100.0	0.0	0.0	0.0	0.0	0.0	1584
5	*daf-16(mu86);daf-2*	***	yes	0.0	0.0	100.0	0.0	0.0	0.0	1469
6	*daf-16(mg54);daf-2*	***	yes	0.0	0.0	100.0	0.0	0.0	0.0	1655
6	*daf-16(tm5030);daf-2* #	0.0204	yes	78.6	15.7	0.1	0.2	21.4	15.7	1339
5	*daf-16(tm5032);daf-2* #	0.0408	yes	76.5	17.6	0.0	0.0	23.5	17.6	1047
3	*daf-16(tm6659);daf-2*	0.4226	no	99.7	0.5	0.0	0.0	0.3	0.5	648
3	N2 wild-type	***	yes	0.0	0.0	100.0	0.0	0.0	0.0	752
2	wild-type sib of *daf-16(tm6659);daf-2*	***	yes	0.0	0.0	100.0	0.0	0.0	0.0	699
1	*daf-16(tm6659)* sib of *daf-16(tm6659);daf-2*	***	yes	0.0	0.0	100.0	0.0	0.0	0.0	289
2	*daf-2* sib of *daf-16(tm6659);daf-2*	^	no	100.0	0.0	0.0	0.0	0.0	0.0	396

*** *p*-value cannot be calculated because SD = 0, but effectively $p < 0.0001$

^ *p*-value cannot be calculated because SD = 0, but effectively $p = 1$

daf-16(tm5030);daf-2(e1370) and *daf-2(tm5032);daf-2(e1370)* non-dauer larvae developed into sterile adults after an additional 48 hours at 25°C

Supplemental Table 4.3. *daf-2(e1368)* dauer arrest raw data.

Column statistics are calculated from measurements from three plates per genotype for each replicate.

		dauer			adult		non-dauer larvae		
Replicate	**genotype**	***p*-value** (unpaired, two-tailed *t*-test with Welch's)	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**N**
Replicate 1	*daf-2(e1368)*	control	95.1	2.5	0.0	0.0	4.9	2.5	175
***shown figure**	*daf-16(mu86);daf-2*	0.0002	0.0	0.0	100.0	0.0	0.0	0.0	244
Both A and F	*daf-16(mg54);daf-2*	0.0002	0.0	0.0	100.0	0.0	0.0	0.0	450
	daf-16(tm5030);daf-2	0.0002	0.0	0.0	100.0	0.0	0.0	0.0	314
	daf-16(tm5032);daf-2	0.0002	0.0	0.0	100.0	0.0	0.0	0.0	370
	daf-16(tm6659);daf-2	0.3347	97.4	2.7	0.0	0.0	2.6	2.7	184
	N2 wild-type	0.0002	0.0	0.0	100.0	0.0	0.0	0.0	391
Replicate 2	*daf-2(e1368)*	control	89.3	4.1	0.0	0.0	10.7	4.1	289
Both A and F	*daf-16(mu86);daf-2*	0.0007	0.0	0.0	100.0	0.0	0.0	0.0	425
	daf-16(mg54);daf-2	0.0007	0.0	0.0	100.0	0.0	0.0	0.0	397
	daf-16(tm5030);daf-2	0.0007	0.0	0.0	100.0	0.0	0.0	0.0	318
	daf-16(tm5032);daf-2	0.0007	0.0	0.0	100.0	0.0	0.0	0.0	350
	daf-16(tm6659);daf-2	0.1255	95.0	2.2	0.0	0.0	5.0	2.2	287
	N2 wild-type	0.0007	0.0	0.0	100.0	0.0	0.0	0.0	372
Replicate 3	*daf-2(e1368)*	control	93.9	1.7	2.5	0.9	3.7	2.4	343
A only	*daf-16(mu86);daf-2*	0.0001	0.0	0.0	100.0	0.0	0.0	0.0	296
	daf-16(mg54);daf-2	0.0001	0.0	0.0	100.0	0.0	0.0	0.0	464
	daf-16(tm5030);daf-2	0.0001	0.0	0.0	100.0	0.0	0.0	0.0	648
	daf-16(tm5032);daf-2	0.0001	0.0	0.0	100.0	0.0	0.0	0.0	618
Replicate 4	*daf-2(e1368)* sib of *daf-16(tm6659);daf-2*	control	100.0	0.0	0.0	0.0	0.0	0.0	207
F only	*daf-16(mg54);daf-2*	***	0.0	0.0	100.0	0.0	0.0	0.0	282
	daf-16(tm6659);daf-2	0.4226	99.6	0.8	0.4	0.8	0.0	0.0	252
	daf-16(tm6659) sib of *daf-16(tm6659);daf-2*	***	0.0	0.0	100.0	0.0	0.0	0.0	300
	N2 wild-type	***	0.0	0.0	100.0	0.0	0.0	0.0	98
Replicate 5	*daf-2(e1368)* sib of *daf-16(tm6659);daf-2*	control	97.8	2.9	2.2	2.9	0.0	0.0	224
F only	*daf-16(mg54);daf-2*	0.0003	0.0	0.0	100.0	0.0	0.0	0.0	229
	daf-16(tm6659);daf-2	0.5633	98.9	0.9	1.1	0.9	0.0	0.0	222
	wild-type sib of *daf-16(tm6659);daf-2*	0.0003	0.0	0.0	100.0	0.0	0.0	0.0	354
	N2 wild-type	0.0003	0.0	0.0	100.0	0.0	0.0	0.0	89
Replicate 6	*daf-2(e1368)* sib of *daf-16(tm6659);daf-2*	control	87.4	2.8	11.6	3.2	1.0	0.5	413
F only	*daf-16(mg54);daf-2*	0.0003	0.0	0.0	100.0	0.0	0.0	0.0	496
	daf-16(tm6659);daf-2	0.5099	85.8	2.7	6.0	1.5	8.2	3.9	458
	wild-type sib of *daf-16(tm6659);daf-2*	0.0003	0.0	0.0	100.0	0.0	0.0	0.0	439
	daf-16(tm6659) sib of *daf-16(tm6659);daf-2*	0.0003	0.0	0.0	100.0	0.0	0.0	0.0	418

*** *p*-value cannot be calculated because SD = 0, but effectively $p < 0.0001$

Supplemental Table 4.4. *daf-2(e1370)* dauer arrest raw data.

Column statistics are calculated from measurements from three plates per genotype for each replicate.

		dauer			adult		non-dauer larvae		
Replicate	**genotype**	***p*-value** *vs* control (two-tailed, unpaired *t*-test with Welch's)	**mean**	**SD**	**mean**	**SD**	**mean**	**SD**	**N**
Replicate 1	*daf-2(e1370)*	control	100.0	0.0	0.0	0.0	0.0	0.0	321
Both A and F	*daf-16(mu86);daf-2*	***	0.0	0.0	100.0	0.0	0.0	0.0	419
	daf-16(mg54);daf-2	***	0.0	0.0	100.0	0.0	0.0	0.0	465
	daf-16(tm5030);daf-2 #	0.0102	65.4	6.1	0.0	0.0	34.6	6.1	446
	daf-16(tm5032);daf-2 #	0.0032	46.5	5.3	0.0	0.0	53.5	5.3	270
	daf-16(tm6659);daf-2	^	100.0	0.0	0.0	0.0	0.0	0.0	265
	N2 wild-type	***	0.0	0.0	100.0	0.0	0.0	0.0	527
Replicate 2	*daf-2(e1370)*	control	100.0	0.0	0.0	0.0	0.0	0.0	176
Both A and F	*daf-16(mu86);daf-2*	***	0.0	0.0	100.0	0.0	0.0	0.0	173
	daf-16(mg54);daf-2	***	0.0	0.0	100.0	0.0	0.0	0.0	133
	daf-16(tm5030);daf-2 #	0.1884	94.3	5.0	0.0	0.0	5.7	5.0	194
	daf-16(tm5032);daf-2 #	0.1441	93.2	5.1	0.0	0.0	6.8	5.1	186
	daf-16(tm6659);daf-2	^	100.0	0.0	0.0	0.0	0.0	0.0	150
	N2 wild-type	***	0.0	0.0	100.0	0.0	0.0	0.0	120
	wild-type sib of *daf-16(tm6659);daf-2*	***	0.0	0.0	100.0	0.0	0.0	0.0	351
	daf-2 sib of *daf-16(tm6659);daf-2*	^	100.0	0.0	0.0	0.0	0.0	0.0	160
Replicate 3	*daf-2(e1370)*	control	100.0	0.0	0.0	0.0	0.0	0.0	267
Both A and F	*daf-16(mg54);daf-2*	***	0.0	0.0	100.0	0.0	0.0	0.0	171
	daf-16(tm5030);daf-2 #	0.2697	97.3	3.1	0.0	0.0	2.7	3.1	135
	daf-16(tm6659);daf-2	0.1840	99.1	0.8	0.0	0.0	0.9	0.8	233
	N2 wild-type	***	0.0	0.0	100.0	0.0	0.0	0.0	105
	wild-type sib of *daf-16(tm6659);daf-2*	***	0.0	0.0	100.0	0.0	0.0	0.0	348
	daf-16(tm6659) sib of *daf-16(tm6659);daf-2*	***	0.0	0.0	100.0	0.0	0.0	0.0	289
	daf-2 sib of *daf-16(tm6659);daf-2*	^	100.0	0.0	0.0	0.0	0.0	0.0	236
Replicate 4	*daf-2(e1370)*		100.0	0.0	0.0	0.0	0.0	0.0	209
A only	*daf-16(mu86);daf-2*	***	0.0	0.0	100.0	0.0	0.0	0.0	168
	daf-16(mg54);daf-2	***	0.0	0.0	100.0	0.0	0.0	0.0	137
	daf-16(tm5030);daf-2 #	0.0240	59.3	11.1	0.0	0.0	40.7	11.1	87
	daf-16(tm5032);daf-2 #	0.0375	79.5	7.0	0.0	0.0	20.5	7.0	97
Replicate 5	*daf-2(e1370)*	control	100.0	0.0	0.0	0.0	0.0	0.0	286
A ony	*daf-16(mu86);daf-2*	***	0.0	0.0	100.0	0.0	0.0	0.0	264
	daf-16(mg54);daf-2	***	0.0	0.0	100.0	0.0	0.0	0.0	297
	daf-16(tm5030);daf-2 #	0.0130	71.2	5.7	0.0	0.0	28.8	5.7	167
	daf-16(tm5032);daf-2 #	0.0202	82.2	4.4	0.0	0.0	17.8	4.4	185
Replicate 6	*daf-2(e1370)*	control	100.0	0.0	0.0	0.0	0.0	0.0	325
A ony	*daf-16(mu86);daf-2*	***	0.0	0.0	100.0	0.0	0.0	0.0	445
	daf-16(mg54);daf-2	***	0.0	0.0	100.0	0.0	0.0	0.0	488
	daf-16(tm5030);daf-2 #	0.0556	84.0	6.8	0.4	0.6	15.7	6.3	310
	daf-16(tm5032);daf-2 #	<0.0001	81.2	0.1	0.0	0.0	18.8	0.1	309

*** *p*-value cannot be calculated because SD = 0, but effectively $p < 0.0001$

^ *p*-value cannot be calculated because SD = 0, but effectively $p = 1$

daf-16(tm5030);daf-2(e1370) and *daf-2(tm5032);daf-2(e1370)* non-dauer larvae developed into sterile adults after an additional 48 hours at 25°C

Supplemental Table 4.5. *daf-2(e1368)* life span data and statistics.

relevant figures	genotype	deaths (censored)	mean survival (days)	SD	median survival (days)	75th %ile (days)	P value (Log-rank)	P value compared to	% change mean survival	% change 75th %ile
Figure 2C	*daf-2(e1368)*	94 (6)	22.0	4.6	22	26				
Replicate 1	*daf-16(mu86);daf-2*	54 (50)	13.1	1.9	13	14	<0.0001	*daf-2(e1368)*	-40	-46
***shown figure**	*daf-16(mg54);daf-2*	84 (15)	14.6	1.6	14	15	<0.0001	*daf-2(e1368)*	-34	-42
	daf-16(tm5030);daf-2	91 (9)	17.7	3.4	17	20	<0.0001	*daf-2(e1368)*	-20	-23
	daf-16(tm5032);daf-2	89 (11)	18.8	3.4	18	21	<0.0001	*daf-2(e1368)*	-15	-19
Figure 2C	*daf-2(e1368)*	44 (48)	26.5	4.9	27	31				
Replicate 2	*daf-16(mg54);daf-2*	59 (46)	16.4	3.4	17	19	<0.0001	*daf-2(e1368)*	-38	-39
	daf-16(tm5030);daf-2	58 (48)	20.8	3.9	21	23	<0.0001	*daf-2(e1368)*	-22	-26
	daf-16(tm5032);daf-2	80 (25)	19.9	4.1	21	23	<0.0001	*daf-2(e1368)*	-25	-26
	N2 wild-type	22 (28)	18.5	2.7	19	21	<0.0001	*daf-2(e1368)*	-30	-32
Figure 2C	*daf-2(e1368)*	90 (10)	20.9	4.1	21	24				
Replicate 3	*daf-16(mu86);daf-2*	88 (12)	13.1	2.0	13	14	<0.0001	*daf-2(e1368)*	-37	-42
	daf-16(mg54);daf-2	75 (25)	12.8	1.7	12	14	<0.0001	*daf-2(e1368)*	-39	-42
	daf-16(tm5030);daf-2	98 (2)	17.5	3.2	18	20	<0.0001	*daf-2(e1368)*	-16	-17
	daf-16(tm5032);daf-2	90 (10)	18.3	2.7	18	20	<0.0001	*daf-2(e1368)*	-12	-17
Figure 2D	*daf-2(e1368)* - sib of *daf-16(tm6659);daf-2*	94 (10)	26.0	6.7	27	32				
Replicate 1	*daf-16(mg54);daf-2*	46 (4)	16.7	3.8	13	15	<0.0001	*daf-2(e1368)*	-36	-53
	daf-16(tm6659);daf-2	100 (7)	24.8	5.7	25	29	0.0356	*daf-2(e1368)*	-5	-9
	daf-16(tm6659) sib of *daf-16(tm6659);daf-2*	51 (0)	16.9	5.0	18	20	<0.0001	*daf-2(e1368)*	-35	-38
	wild-type sib of *daf-16(tm6659);daf-2*	48 (3)	17.0	5.2	18	21	<0.0001	*daf-2(e1368)*	-35	-34
	N2 wild-type	49 (0)	16.5	5.3	18	20	<0.0001	*daf-2(e1368)*	-37	-38
Figure 2D	*daf-2(e1368)* - sib of *daf-16(tm6659);daf-2*	86 (16)	24.8	3.7	25	27				
Replicate 2	*daf-16(mg54);daf-2*	35 (25)	14.5	1.7	15	15	<0.0001	*daf-2(e1368)*	-42	-44
	daf-16(tm6659);daf-2	88 (13)	22.6	4.0	23	25	0.0013	*daf-2(e1368)*	-9	-7
	wild-type sib of *daf-16(tm6659);daf-2*	44 (16)	16.4	2.5	17	19	<0.0001	*daf-2(e1368)*	-34	-30
	N2 wild-type	52 (8)	17.9	2.9	19	19	<0.0001	*daf-2(e1368)*	-28	-30
Figure 2D	*daf-2(e1368)* - sib of *daf-16(tm6659);daf-2*	70 (35)	23.1	7.5	21	28				
Replicate 3	*daf-16(mg54);daf-2*	45 (59)	12.1	2.4	12	14	<0.0001	*daf-2(e1368)*	-48	-50
***shown figure**	*daf-16(tm6659);daf-2*	70 (35)	23.7	5.8	23	28	0.8216	*daf-2(e1368)*	NS	NS
	wild-type sib of *daf-16(tm6659);daf-2*	63 (43)	16.8	3.1	18	19	<0.0001	*daf-2(e1368)*	-27	-32

Supplemental Table 4.6. *daf-2(1370)* life span data and statistics.

relevant figures	genotype	deaths (censored)	mean survival (days)	SD	median survival (days)	75th %ile (days)	P value (Log-rank)	P value compared to	% change mean survival	% change 75th %ile
Figure 2A	*daf-2(e1370)*	100 (0)	43.7	10.6	43.5	53				
Replicate 1	*daf-16(tm5030);daf-2*	97 (2)	32.4	8.3	35	38	<0.0001	*daf-2(e1370)*	-26	-28
***shown figure**	*daf-16(tm5032);daf-2*	94 (6)	31.8	6.7	33	36	<0.0001	*daf-2(e1370)*	-27	-32
	daf-16(mu86);daf-2	79 (22)	13.9	2.1	14	15	<0.0001	*daf-2(e1370)*	-68	-72
	daf-16(mg54);daf-2	75 (23)	15.0	3.6	14	16	<0.0001	*daf-2(e1370)*	-66	-70
Figure 2A	*daf-2(e1370)*	98 (0)	46.5	8.6	47	52				
Replicate 2	*daf-16(tm5030);daf-2*	91 (7)	26.5	5.9	27	31	<0.0001	*daf-2(e1370)*	-43	-40
	daf-16(tm5032);daf-2	91 (7)	31.6	7.5	31	36	<0.0001	*daf-2(e1370)*	-32	-31
	daf-16(mu86);daf-2	60 (32)	19.4	5.1	18	24	<0.0001	*daf-2(e1370)*	-58	-54
	daf-16(mg54);daf-2	84 (10)	16.0	2.0	16	18	<0.0001	*daf-2(e1370)*	-66	-65
Figure 2A	*daf-2(e1370)*	200 (0)	48.5	10.8	51	56				
Replicate 3	*daf-16(tm5030);daf-2*	96 (4)	29.3	11.0	30.5	38	<0.0001	*daf-2(e1370)*	-40	-32
	daf-16(tm5032);daf-2	99 (1)	31.1	8.6	31	38	<0.0001	*daf-2(e1370)*	-39	-32
Figure 2A	***daf-2(e1370)*	48 (0)	41.6	6.7	43	48				
Replicate 4	***daf-16(mu86);daf-2*	44 (6)	14.0	1.9	14	15	<0.0001	*daf-2(e1370)*	-66	-69
Figure 2B	***daf-16(mg54);daf-2*	35 (14)	14.5	3.1	15	16	<0.0001	*daf-2(e1370)*	-65	-67
Replicate 1	***daf-16(tm5030);daf-2*	43 (7)	34.2	6.8	36	38	<0.0001	*daf-2(e1370)*	-18	-21
****RNA-seq**	***daf-16(tm5032);daf-2*	45 (3)	34.6	5.6	36	38	<0.0001	*daf-2(e1370)*	-17	-21
Replicate 1	***daf-16(tm6659);daf-2*	105 (0)	40.5	7.5	43	45	0.4489	*daf-2(e1370)*	NS	NS
	daf-2 sib of *daf-16(tm6659);daf-2*	45 (0)	38.9	9.1	42	45	0.1648	*daf-2(e1370)*	NS	NS
	wild-type sib of *daf-16(tm6659);daf-2*	37 (5)	16.3	3.0	16	18	<0.0001	*daf-2(e1370)*	-61	-63
	N2 wild-type	40 (5)	16.1	2.1	16	17	<0.0001	*daf-2(e1370)*	-61	-65
Figure 2A	***daf-2(e1370)*	60 (0)	46.2	9.7	48	53				
Replicate 5	***daf-16(mu86);daf-2*	40 (10)	14.7	2.6	15	17	<0.0001	*daf-2(e1370)*	-68	-68
Figure 2B	***daf-16(mg54);daf-2*	41 (9)	15.3	3.2	17	17	<0.0001	*daf-2(e1370)*	-67	-68
Replicate 2	***daf-16(tm5030);daf-2*	40 (10)	38.2	6.3	38	43	<0.0001	*daf-2(e1370)*	-17	-19
***shown figure**	***daf-16(tm5032);daf-2*	47 (3)	36.1	7.4	37	43	<0.0001	*daf-2(e1370)*	-22	-19
****RNA-seq**	***daf-16(tm6659);daf-2*	104 (1)	46.2	7.6	51	55	0.2284	*daf-2(e1370)*	NS	NS
Replicate 2	*daf-2* sib of *daf-16(tm6659);daf-2*	51 (1)	47.1	10.4	51	53	0.2618	*daf-2(e1370)*	NS	NS
	wild-type sib of *daf-16(tm6659);daf-2*	37 (13)	16.5	2.8	17	17	<0.0001	*daf-2(e1370)*	-64	-68
	N2 wild-type	32 (18)	17.0	2.0	17	17	<0.0001	*daf-2(e1370)*	-63	-68
Figure 2A	***daf-2(e1370)*	50 (0)	46.0	8.9	47.5	54				
Replicate 6	***daf-16(mu86);daf-2*	39 (9)	15.2	2.2	16	17	<0.0001	*daf-2(e1370)*	-67	-69
Figure 2B	***daf-16(mg54);daf-2*	37 (12)	16.7	2.8	16	19	<0.0001	*daf-2(e1370)*	-64	-65
Replicate 3	***daf-16(tm5030);daf-2*	47 (3)	39.6	7.9	41	45	<0.0001	*daf-2(e1370)*	-14	-17
****RNA-seq**	***daf-16(tm5032);daf-2*	50 (2)	35.6	7.9	37	41	<0.0001	*daf-2(e1370)*	-23	-24
Replicate 3	***daf-16(tm6659);daf-2*	98 (3)	46.9	7.8	51	54	0.0503	*daf-2(e1370)*	NS	NS
	daf-2 sib of *daf-16(tm6659);daf-2*	47 (3)	46.0	7.9	47	52	0.7780	*daf-2(e1370)*	NS	NS
	wild-type sib of *daf-16(tm6659);daf-2*	32 (19)	19.0	3.2	18	20	<0.0001	*daf-2(e1370)*	-59	-63
	N2 wild-type	37 (13)	17.6	2.3	17	19	<0.0001	daf-2(e1370)	-62	-65

Supplemental Table 4.7. Mutant-RNAi combination life span data and statistics.

relevant figures	genotype	summary of genotype	deaths (censored)	mean survival (days)	SD	P value (Log-rank)	P value compared to	% change mean survival
Replicate 1	*daf-2(e1370)*;control RNAi	control RNAi	99 (5)	46.0	11.2			
Figure 4A	*daf-2(e1370);daf-16a* RNAi	A RNAi	104 (1)	33.0	6.0	<0.0001	control RNAi	-28
Figure 4B	*daf-2(e1370);daf-16f* RNAi	F RNAi	102 (1)	40.9	7.8	<0.0001	control RNAi	-11
Figure 4C	*daf-2(e1370);pan-daf-16* RNAi	A/F/B RNAi	59 (0)	20.6	3.1	<0.0001	control RNAi	-55
	daf-16(tm5032);daf-2(e1370);control RNAi	A mutant + control RNAi	98 (6)	32.0	5.4	<0.0001	control RNAi	-30
	daf-16(tm5032);daf-2(e1370);daf-16a RNAi	A mutant + A RNAi	84 (19)	28.0	3.4	<0.0001	A mutant + control RNAi	-13
	daf-16(tm5032);daf-2(e1370);daf-16f RNAi	A mutant + F RNAi	81 (24)	18.3	2.2	<0.0001	A mutant + control RNAi	-43
	daf-16(tm5032);daf-2(e1370);pan-daf-16 RNAi	A mutant + A/F/B RNAi	50 (10)	17.5	1.3	<0.0001	A mutant + control RNAi	-45
	daf-16(tm6659);daf-2(e1370);control RNAi	F mutant + control RNAi	99 (7)	48.2	11.7	0.2284	control RNAi	NS
	daf-16(tm6659);daf-16a RNAi	F mutant + A RNAi	97 (7)	22.9	4.1	<0.0001	F mutant + control RNAi	-52
	daf-16(tm6659);daf-2(e1370);daf-16f RNAi	F mutant + F RNAi	99 (6)	47.1	9.4	0.7294	F mutant + control RNAi	NS
	daf-16(tm6659);daf-2(e1370);pan-daf-16 RNAi	F mutant + A/F/B RNAi	57 (3)	19.9	2.7	<0.0001	F mutant + control RNAi	-59
Replicate 2	*daf-2(e1370)*;control RNAi	control RNAi	106 (1)	50.9	10.5			
Figure 4A	*daf-2(e1370);daf-16a* RNAi	A RNAi	101 (4)	36.8	4.9	<0.0001	control RNAi	-28
Figure 4B	*daf-2(e1370);daf-16f* RNAi	F RNAi	104 (2)	39.2	4.6	<0.0001	control RNAi	-23
Figure 4C	*daf-2(e1370);pan-daf-16* RNAi	A/F/B RNAi	57 (3)	22.1	2.2	<0.0001	control RNAi	-57
	daf-16(tm5032);daf-2(e1370);control RNAi	A mutant + control RNAi	88 (21)	34.5	5.9	<0.0001	control RNAi	-32
	daf-16(tm5032);daf-2(e1370);daf-16a RNAi	A mutant + A RNAi	65 (41)	32.1	1.8	0.0015	A mutant + control RNAi	-7
	daf-16(tm5032);daf-2(e1370);daf-16f RNAi	A mutant + F RNAi	60 (46)	18.7	2.0	<0.0001	A mutant + control RNAi	-46
	daf-16(tm5032);daf-2(e1370);pan-daf-16 RNAi	A mutant + A/F/B RNAi	42 (18)	17.7	1.5	<0.0001	A mutant + control RNAi	-49
	daf-16(tm6659);daf-2(e1370);control RNAi	F mutant + control RNAi	92 (4)	55.4	10.1	0.0094	control RNAi	+9
	daf-16(tm6659);daf-16a RNAi	F mutant + A RNAi	94 (11)	23.6	2.9	<0.0001	F mutant + control RNAi	-55
	daf-16(tm6659);daf-2(e1370);daf-16f RNAi	F mutant + F RNAi	99 (6)	58.3	9.9	0.0922	F mutant + control RNAi	NS
	daf-16(tm6659);daf-2(e1370);pan-daf-16 RNAi	F mutant + A/F/B RNAi	59 (1)	21.2	2.1	<0.0001	F mutant + control RNAi	-62
Replicate 3	*daf-2(e1370)*;control RNAi	control RNAi	88 (2)	54.0	7.7			
Figure 4A	*daf-2(e1370);daf-16a* RNAi	A RNAi	74 (1)	43.9	7.1	<0.0001	control RNAi	-19
Figure 4B	*daf-2(e1370);daf-16f* RNAi	F RNAi	75 (0)	45.4	5.6	<0.0001	control RNAi	-16
Figure 4C	*daf-2(e1370);pan-daf-16* RNAi	A/F/B RNAi	56 (5)	23.2	3.9	<0.0001	control RNAi	-57
	daf-2(e1370);daf-16a/f RNAi	A/F RNAi	73 (2)	23.9	4.4	<0.0001	control RNAi	-56
	daf-16(tm5030);daf-2(e1370);control RNAi	A mutant + control RNAi	72 (17)	41.0	4.8	<0.0001	control RNAi	-24
	daf-16(tm5030);daf-2(e1370);daf-16a RNAi	A mutant + A RNAi	55 (21)	39.5	6.7	0.9948	A mutant + control RNAi	NS
	daf-16(tm5030);daf-2(e1370);daf-16f RNAi	A mutant + F RNAi	50 (25)	20.4	2.4	<0.0001	A mutant + control RNAi	-50
	daf-16(tm5030);daf-2(e1370);pan-daf-16 RNAi	A mutant + A/F/B RNAi	40 (20)	18.7	2.0	<0.0001	A mutant + control RNAi	-54
	daf-16(tm5030);daf-2(e1370);daf-16a/f RNAi	A mutant + A/F RNAi	41 (36)	20.0	1.8	<0.0001	A mutant + control RNAi	-51
	daf-16(tm6659);daf-2(e1370);control RNAi	F mutant + control RNAi	86 (3)	55.7	5.5	0.4591	control RNAi	NS
	daf-16(tm6659);daf-16a RNAi	F mutant + A RNAi	75 (1)	25.8	4.4	<0.0001	F mutant + control RNAi	-54
	daf-16(tm6659);daf-2(e1370);daf-16f RNAi	F mutant + F RNAi	73 (2)	56.0	7.4	0.3320	F mutant + control RNAi	NS
	daf-16(tm6659);daf-2(e1370);pan-daf-16 RNAi	F mutant + A/F RNAi	58 (3)	22.6	3.7	<0.0001	F mutant + control RNAi	-59
	daf-16(tm6659);daf-2(e1370);daf-16a/f RNAi	F mutant + A/F/B RNAi	73 (1)	25.3	4.1	<0.0001	F mutant + control RNAi	-55
Replicate 4	*daf-16(tm5030);daf-2(e1370)*;control RNAi	A mutant + control RNAi	98 (2)	34.2	5.8			
Figure 4B	*daf-16(tm5030);daf-2(e1370);daf-16a* RNAi	A mutant + A RNAi	96 (4)	27.8	5.3	<0.0001	A mutant + control RNAi	-19
	daf-16(tm5030);daf-2(e1370);daf-16f RNAi	A mutant + F RNAi	88 (12)	18.8	3.2	<0.0001	A mutant + control RNAi	-45
	daf-16(tm5030);daf-2(e1370);pan-daf-16 RNAi	A mutant + A/F/B RNAi	94 (6)	17.0	2.2	<0.0001	A mutant + control RNAi	-50

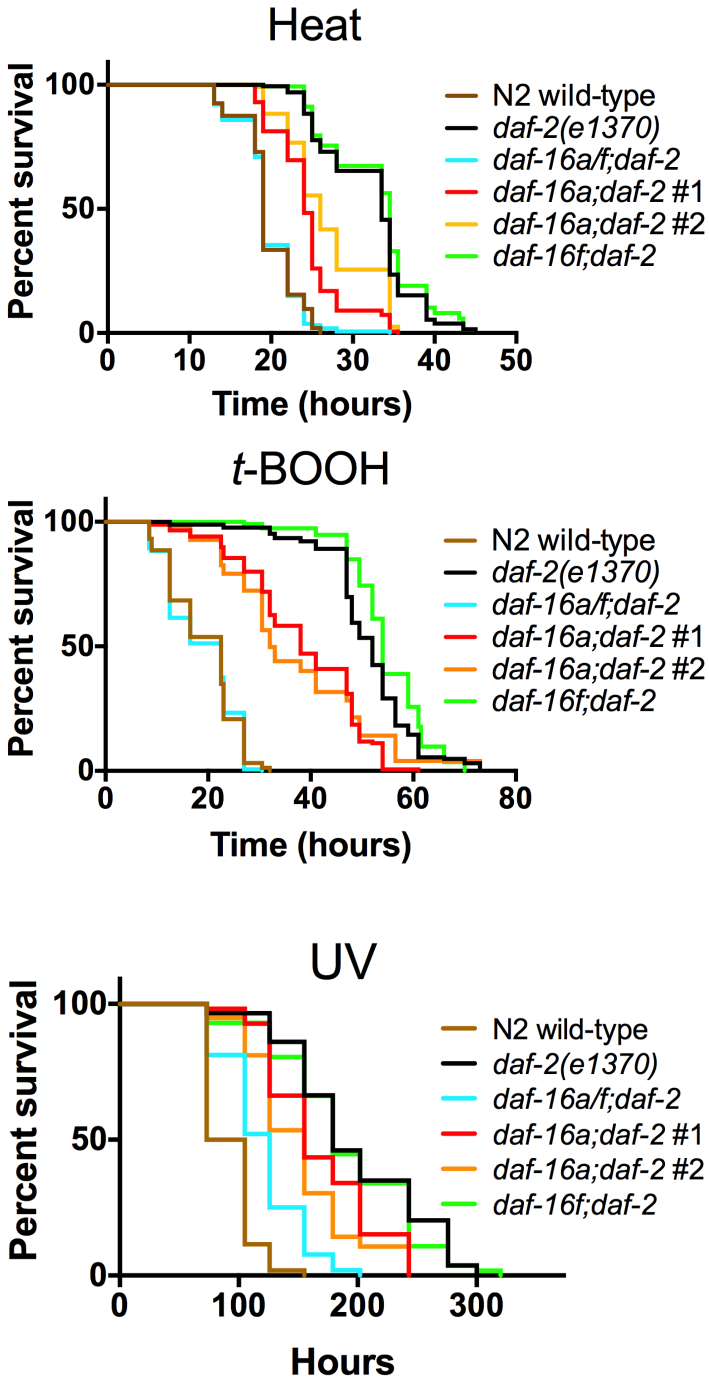
Supplemental Table 4.8. *glp-1(e2141)* life span data and statistics.

relevant figures	genotype	deaths (censored)	mean survival (days)	SD	median survival (days)	75th %ile (days)	P value (Log-rank)	P value compared to	% change mean survival	% change 75th %ile
Figure 2E	*glp-1(e2141)*	98 (1)	24.8	8.4	26	32				
Replicate 1	*daf-16(mu86);glp-1*	89 (5)	13.0	2.0	13	14	<0.0001	*glp-1(e2141)*	-48	-56
***shown figure**	*daf-16(mg54);glp-1*	95 (1)	12.7	1.9	13	14	<0.0001	*glp-1(e2141)*	49	-56
	daf-16(tm5030);glp-1	91 (6)	19.3	6.6	18	24	<0.0001	*glp-1(e2141)*	-22	-25
	daf-16(tm5032);glp-1	102 (0)	20.5	7.0	21	25	<0.0001	*glp-1(e2141)*	-17	-22
Figure 2E	*glp-1(e2141)*	96 (2)	22.1	8.1	21	28				
Replicate 2	*daf-16(mu86);glp-1*	91 (9)	13.0	2.9	12	16	<0.0001	*glp-1(e2141)*	-41	-43
	daf-16(mg54);glp-1	98 (1)	13.0	3.0	12	16	<0.0001	*glp-1(e2141)*	-41	-43
	daf-16(tm5030);glp-1	103 (2)	18.4	6.0	18	23	<0.0001	*glp-1(e2141)*	-17	-18
	daf-16(tm5032);glp-1	97 (1)	17.8	6.4	18	23	<0.0001	*glp-1(e2141)*	-19	-18
Figure 2E	*glp-1(e2141)*	84 (10)	22.8	10.0	25	33				
Replicate 3	*daf-16(mu86);glp-1*	73 (22)	11.8	2.5	12	13	<0.0001	*glp-1(e2141)*	-48	-61
	daf-16(mg54);glp-1	91 (0)	10.7	2.1	11	13	<0.0001	*glp-1(e2141)*	-53	-61
	daf-16(tm5030);glp-1	96 (4)	19.4	7.4	22	25	<0.0001	*glp-1(e2141)*	-15	-24
	daf-16(tm5032);glp-1	94 (4)	18.0	7.3	20	25	<0.0001	*glp-1(e2141)*	-21	-24
Figure 2E	*glp-1(e2141)*	58 (1)	26.3	4.0	27	28				
Replicate 4	*daf-16(mu86);glp-1*	56 (4)	16.0	2.4	16	18	<0.0001	*glp-1(e2141)*	-39	-36
	daf-16(mg54);glp-1	49 (1)	16.4	1.6	16	17	<0.0001	*glp-1(e2141)*	-38	-39
	daf-16(tm5030);glp-1	60 (0)	22.2	2.9	22	24	<0.0001	*glp-1(e2141)*	-16	-14
	daf-16(tm5032);glp-1	60 (0)	22.2	3.4	22	24	<0.0001	*glp-1(e2141)*	-16	-14
Figure 2F	*glp-1(e2141)* sib of *daf-16(tm6659);glp-1*	99 (6)	22.1	12.5	19	35				
Replicate 1	*daf-16(mu86);glp-1*	102 (3)	12.9	3.6	13	16	<0.0001	*glp-1(e2141)*	-42	-54
***shown figure**	*daf-16(tm6659);glp-1*	102 (3)	24.6	14.8	19	37	0.0454	*glp-1(e2141)*	+11	+6
Figure 2F	*glp-1(e2141)* sib of *daf-16(tm6659);glp-1*	106 (1)	19.3	11.0	13	28				
Replicate 2	*daf-16(mu86);glp-1*	105 (0)	13.6	3.4	13	16	<0.0001	*glp-1(e2141)*	-30	-43
	daf-16(tm6659);glp-1	108 (0)	19.8	11.1	14	28	0.9139	*glp-1(e2141)*	NS	NS
Figure 2F	*glp-1(e2141)* sib of *daf-16(tm6659);glp-1*	91 (0)	22.1	11.6	18	33				
Replicate 3	*daf-16(mu86);glp-1*	89 (1)	11.5	3.7	11	15	<0.0001	*glp-1(e2141)*	-48	-55
	daf-16(tm6659);glp-1	90 (0)	20.4	10.2	18	29	0.2159	*glp-1(e2141)*	NS	NS

Supplemental Table 4.9. Mutant-RNAi life span data and statistics.

relevant figures	genotype	summary of genotype	deaths (censored)	mean survival (days)	SD	P value (Log-rank)	P value compared to	% change mean survival
Replicate 1	*daf-2(e1370)*;control RNAi	control RNAi	94 (11)	25.7	6.4			
Figure 4D	*daf-2(e1370);daf-16a* RNAi	A RNAi	48 (57)	23.9	3.4	0.0003	control RNAi	-7
Figure 4E	*daf-2(e1370);daf-16f* RNAi	F RNAi	81 (21)	24.0	4.6	<0.0001	control RNAi	-7
Figure 4F	*daf-2(e1370);pan-daf-16* RNAi	A/F/B RNAi	26 (33)	17.0	2.5	<0.0001	control RNAi	-34
	daf-16(tm5030);daf-2(e1370);control RNAi	A mutant + control RNAi	81 (21)	20.7	6.0	<0.0001	control RNAi	-20
	daf-16(tm5030);daf-2(e1370);daf-16a RNAi	A mutant + A RNAi	71 (32)	22.1	0.8	0.9582	A mutant + control RNAi	NS
	daf-16(tm5030);daf-2(e1370);daf-16f RNAi	A mutant + F RNAi	82 (23)	15.9	2.4	<0.0001	A mutant + control RNAi	-23
	daf-16(tm5030);daf-2(e1370);pan-daf-16 RNAi	A mutant + A/F/B RNAi	43 (17)	14.7	2.3	<0.0001	A mutant + control RNAi	-29
	daf-16(tm6659);daf-2(e1370);control RNAi	F mutant + control RNAi	100 (6)	23.9	6.8	0.0293	control RNAi	-7
	daf-16(tm6659);daf-16a RNAi	F mutant + A RNAi	61 (43)	18.9	2.3	<0.0001	F mutant + control RNAi	-21
	daf-16(tm6659);daf-2(e1370);daf-16f RNAi	F mutant + F RNAi	96 (9)	26.2	5.4	0.3143	F mutant + control RNAi	NS
	daf-16(tm6659);daf-2(e1370);pan-daf-16 RNAi	F mutant + A/F/B RNAi	38 (22)	16.8	2.0	<0.0001	F mutant + control RNAi	-30
Replicate 2	*daf-2(e1370)*;control RNAi	control RNAi	78 (11)	27.3	6.5			
Figure 4D	*daf-2(e1370);daf-16a* RNAi	A RNAi	30 (44)	21.7	3.7	<0.0001	control RNAi	-20
Figure 4E	*daf-2(e1370);daf-16f* RNAi	F RNAi	63 (11)	26.5	3.3	0.0003	control RNAi	-3
Figure 4F	*daf-2(e1370);pan-daf-16* RNAi	A/F/B RNAi	27 (32)	17.6	2.2	<0.0001	control RNAi	-36
	daf-2(e1370);daf-16a/f RNAi	A/F RNAi	23 (51)	19.3	2.1	<0.0001	control RNAi	-30
	daf-16(tm5032);daf-2(e1370);control RNAi	A mutant + control RNAi	75 (15)	20.1	5.4	<0.0001	control RNAi	-26
	daf-16(tm5032);daf-2(e1370);daf-16a RNAi	A mutant + A RNAi	60 (15)	22.0	4.5	0.3603	A mutant + control RNAi	NS
	daf-16(tm5032);daf-2(e1370);daf-16f RNAi	A mutant + F RNAi	53 (22)	16.0	2.1	<0.0001	A mutant + control RNAi	-20
	daf-16(tm5032);daf-2(e1370);pan-daf-16 RNAi	A mutant + A/F/B RNAi	46 (14)	15.5	2.0	<0.0001	A mutant + control RNAi	-23
	daf-16(tm5032);daf-2(e1370);daf-16a/f RNAi	A mutant + A/F RNAi	53 (20)	17.1	1.9	<0.0001	A mutant + control RNAi	-15
	daf-16(tm6659);daf-2(e1370);control RNAi	F mutant + control RNAi	83 (7)	26.3	7.1	0.5149	control RNAi	NS
	daf-16(tm6659);daf-16a RNAi	F mutant + A RNAi	30 (45)	18.5	2.7	<0.0001	F mutant + control RNAi	-30
	daf-16(tm6659);daf-2(e1370);daf-16f RNAi	F mutant + F RNAi	68 (7)	30.8	4.6	0.0004	F mutant + control RNAi	+17
	daf-16(tm6659);daf-2(e1370);pan-daf-16 RNAi	F mutant + A/F/B RNAi	30 (30)	18.0	2.0	<0.0001	F mutant + control RNAi	-32
	daf-16(tm6659);daf-2(e1370);daf-16a/f RNAi	F mutant + A/F RNAi	34 (42)	18.3	2.7	<0.0001	F mutant + control RNAi	-30

Supplemental Figure 4.7. Thermotolerance, oxidative stress resistance, and UV stress resistance of *daf-16* isoform-specific mutants in the *daf-2(e1370)* background. Survival data for animals exposed to 33°C heat, 7.5mM *t*-BOOH, or 1200 J/m^2 UV are shown. See Methods for more detail. For thermotolerance and oxidative stress assays, patterns and absolute values of survival were very similar for all 3 replicates, and therefore combined data for all replicates are presented. For UV stress resistance, one representative trial is shown, but note that *daf-16f* mutation reduced UV resistance in 1 of 3 trials. See Supplemental Table 4.10 for data and statistics.



Supplemental Table 4.10. Stress resistance data and statistics.

A. Thermotolerance (33°C)

genotype	deaths (cens.)	mean survival (hours)	SD	median survival (hours)	P value (Log-rank)	P value compared to	% change mean	% change median	P value (Log-rank)	P value compared to	% change mean	% change median
Replicate 1												
daf-2(e1370)	40 (22)	30.1	6.3	35.5								
daf-16(mg54);daf-2	57 (3)	19.5	4.4	19	<0.0001	*daf-2(e1370)*	-35	-46	0.0721	N2 wild-type	NS	NS
daf-16(tm5030);daf-2	58 (3)	24.4	3.1	24	<0.0001	*daf-2(e1370)*	-19	-32	0.5459	*daf-16(tm5032);daf-2*	NS	NS
daf-16(tm5032);daf-2	60 (1)	24.8	4.1	24	<0.0001	*daf-2(e1370)*	-18	-32				
daf-16(tm6659);daf-2	49 (8)	31.2	6.2	35.5	0.9993	*daf-2(e1370)*	NS	NS				
N2 wild-type	57 (3)	18.5	3.4	19	<0.0001	*daf-2(e1370)*	-39	-46				
Replicate 2												
daf-2(e1370)	55 (6)	34.0	5.4	34.5								
daf-16(mg54);daf-2	60 (0)	20.7	2.8	22	<0.0001	*daf-2(e1370)*	-39	-36	0.8421	N2 wild-type		
daf-16(tm5030);daf-2	58 (1)	24.6	5.6	22	<0.0001	*daf-2(e1370)*	-28	-36	0.0001	*daf-16(tm5032);daf-2*	-15	-15
daf-16(tm5032);daf-2	58 (2)	28.9	5.6	26	<0.0001	*daf-2(e1370)*	-15	-25				
daf-16(tm6659);daf-2	50 (1)	35.1	4.6	34.5	0.4300	*daf-2(e1370)*	NS	NS				
N2 wild-type	54 (2)	20.5	2.4	22	<0.0001	*daf-2(e1370)*	-40	-36				
Replicate 3												
daf-2(e1370)	44 (1)	30.2	4.6	33.5								
daf-16(mg54);daf-2	44 (0)	18.0	2.4	19	<0.0001	*daf-2(e1370)*	-40	-43	0.0118	N2 wild-type	-9	0
daf-16(tm5030);daf-2	52 (1)	24.1	3.5	25	<0.0001	*daf-2(e1370)*	-20	-25				
daf-16(tm6659);daf-2	41 (2)	31.5	6.1	33.5	0.1278	*daf-2(e1370)*	NS	NS				
N2 wild-type	45 (0)	19.7	3.7	19	<0.0001	*daf-2(e1370)*	-35	-43				

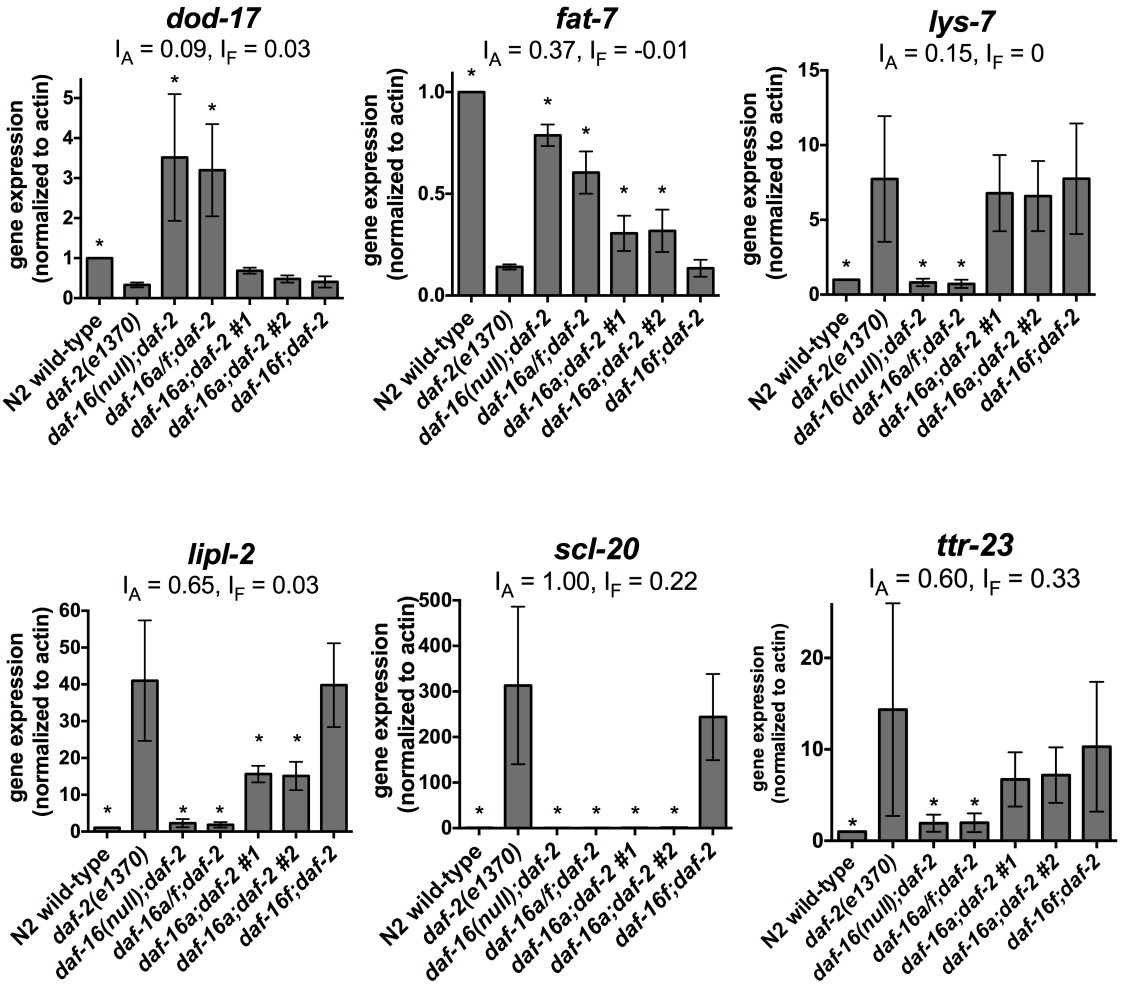
B. Oxidative stress (*t*-BOOH)

genotype	deaths (cens.)	mean survival (hours)	SD	median survival (hours)	P value (Log-rank)	P value compared to	% change mean	% change median	P value (Log-rank)	P value compared to	% change mean	% change median
Replicate 1												
daf-2(e1370)	58 (1)	53.7	8.2	54								
daf-16(mg54);daf-2	60 (0)	23.3	5.5	27	<0.0001	*daf-2(e1370)*	-57	-50	0.0163	N2 wild-type	+14	+64
daf-16(tm5030);daf-2	59 (1)	41.9	12.2	41	<0.0001	*daf-2(e1370)*	-22	-24	<0.0001	*daf-16(tm5032);daf-2*	+21	+24
daf-16(tm5032);daf-2	60 (0)	34.6	11.3	33	<0.0001	*daf-2(e1370)*	-36	-39				
daf-16(tm6659);daf-2	54 (3)	54.1	7.5	54	0.9578	*daf-2(e1370)*	NS	NS				
N2 wild-type	60 (0)	20.4	6.7	16.5	<0.0001	*daf-2(e1370)*	-62	-69				
Replicate 2												
daf-2(e1370)	59 (0)	49.8	4.9	47								
daf-16(mg54);daf-2	56 (2)	15.8	6.7	12.5	<0.0001	*daf-2(e1370)*	-68	-73	0.0705	N2 wild-type	NS	NS
daf-16(tm5030);daf-2	57 (1)	36.1	9.9	38	<0.0001	*daf-2(e1370)*	-28	-19	0.0001	*daf-16(tm5032);daf-2*	+19	+25
daf-16(tm5032);daf-2	60 (0)	30.3	7.9	30.5	<0.0001	*daf-2(e1370)*	-39	-35				
daf-16(tm6659);daf-2	59 (0)	55.2	7.8	59	<0.0001	*daf-2(e1370)*	+11	+26				
N2 wild-type	57 (2)	18.0	6.7	22.5	<0.0001	*daf-2(e1370)*	-64	-52				
Replicate 3												
daf-2(e1370)	48 (11)	49.9	13.9	48								
daf-16(mg54);daf-2	60 (0)	16.9	5.2	12.5	<0.0001	*daf-2(e1370)*	-66	-74	0.0289	N2 wild-type	-7	-46
daf-16(tm5030);daf-2	46 (10)	35.9	12.0	32	<0.0001	*daf-2(e1370)*	-28	-33				
daf-16(tm6659);daf-2	57 (3)	46.5	16.3	48	0.4865	*daf-2(e1370)*	NS	NS				
N2 wild-type	49 (8)	18.2	5.9	23	<0.0001	*daf-2(e1370)*	-64	-52				

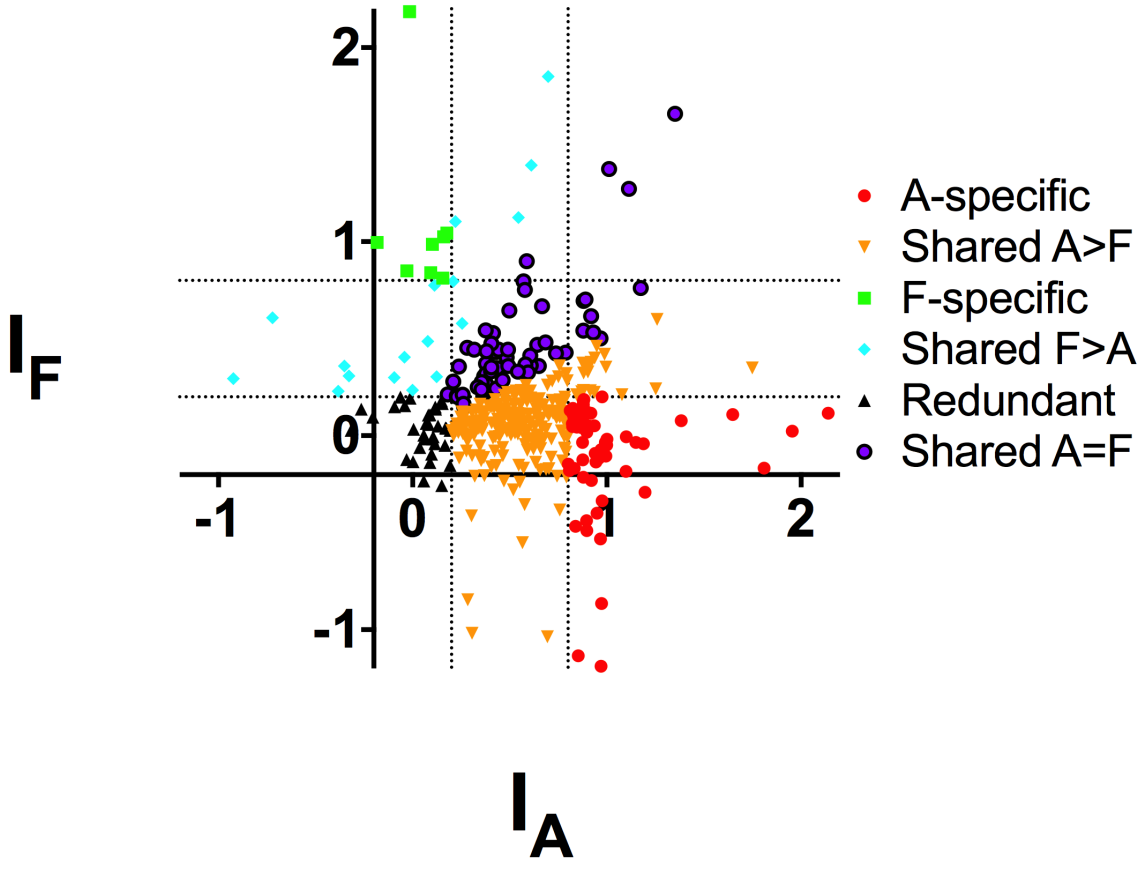
C. UV stress

genotype	deaths (cens.)	mean survival (hours)	SD	median survival (hours)	P value (Log-rank)	P value compared to	% change mean	% change median	P value (Log-rank)	P value compared to	% change mean	% change median
Replicate 1												
daf-2(e1370)	55 (2)	197.1	68.4	202								
daf-16(mg54);daf-2	60 (0)	116.2	39.3	105	<0.0001	*daf-2(e1370)*	-41	-48	<0.0001	N2 wild-type	+23	+28
daf-16(tm5030);daf-2	59 (0)	165.8	60.6	156	0.0069	*daf-2(e1370)*	-16	-23	0.0600	*daf-16(tm5032);daf-2*	NS	NS
daf-16(tm5032);daf-2	55 (1)	158.1	39.6	156	<0.0001	*daf-2(e1370)*	-20	-23				
daf-16(tm6659);daf-2	58 (0)	188.5	70.1	175	0.6215	*daf-2(e1370)*	NS	NS				
N2 wild-type	57 (0)	94.6	21.5	82	<0.0001	*daf-2(e1370)*	-52	-59				
Replicate 2												
daf-2(e1370)	55 (4)	196.7	57.5	179								
daf-16(mg54);daf-2	52 (1)	119.3	32.6	126	<0.0001	*daf-2(e1370)*	-39	-30	<0.0001	N2 wild-type	+31	+42
daf-16(tm5030);daf-2	53 (2)	167.4	44.9	155	0.0010	*daf-2(e1370)*	-15	-13	0.0620	*daf-16(tm5032);daf-2*	NS	NS
daf-16(tm5032);daf-2	57 (1)	150.2	44.0	155	<0.0001	*daf-2(e1370)*	-24	-13				
daf-16(tm6659);daf-2	56 (1)	190.3	58.9	179	0.5871	*daf-2(e1370)*	NS	NS				
N2 wild-type	54 (2)	91.3	20.9	89	<0.0001	*daf-2(e1370)*	-54	-50				
Replicate 3												
daf-2(e1370)	61 (0)	250.2	76.4	248								
daf-16(mg54);daf-2	59 (0)	126.9	36.6	128	<0.0001	*daf-2(e1370)*	-49	-48	<0.0001	N2 wild-type	+46	+38
daf-16(tm5030);daf-2	62 (0)	194.0	74.7	200	<0.0001	*daf-2(e1370)*	-22	-19				
daf-16(tm6659);daf-2	60 (0)	171.0	62.6	175	<0.0001	*daf-2(e1370)*	-32	-29				
N2 wild-type	58 (0)	86.9	36.6	93	<0.0001	*daf-2(e1370)*	-65	-63				

Supplemental Figure 4.8. qPCR validation of additional DAF-16A/F target genes
Mean and standard deviation are plotted for three biological replicates of young adults. Asterisks denote statistically significant changes compared to *daf-2(e1370)* control ($p < 0.05$, paired ratio *t*-test).



Supplemental Figure 4.9. Expanded scatterplot showing more DAF-16A/F targets.
Central box demarcated by intersection of dashed lines represents the scatterplot shown in Figure 5F. Three genes with extreme indices (>2.2 or <-1.2) are omitted for presentation purposes.



Supplemental Table 4.11. qPCR data and statistics for DAF-16A/F targets

Mean and standard deviation for each cohort is calculated based on triplicate measurements. Mean and standard deviation overall is calculated based on means of three biological replicates.

	Independent cohorts						Statistical Analysis			
	1		2		3				P value (ratio paired t-test)	Fold change
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
dod-17										
daf-2(e1370)	0.40	0.57	0.31	0.23	0.28	0.18	0.33	0.06	control	control
daf-16(mu86);daf-2	2.01	0.44	3.36	0.20	5.17	0.17	3.52	1.58	0.0200	10.660
daf-16(mg54);daf-2	2.91	0.43	2.22	0.28	4.47	0.17	3.20	1.15	0.0092	9.699
daf-16(tm5030);daf-2	0.62	0.34	0.67	0.25	0.77	0.12	0.69	0.07	0.9712	2.084
daf-16(tm5032);daf-2	0.42	0.23	0.44	0.31	0.58	0.19	0.48	0.09	0.5286	1.453
daf-16(tm6659);daf-2	0.33	0.27	0.57	0.36	0.32	0.20	0.41	0.14	0.9818	1.232
N2 wild-type	1.00	0.51	1.00	0.19	1.00	0.23	1.00	0.00	0.0053	3.032
far-3										
daf-2(e1370)	2.16	0.13	5.28	0.23	4.20	0.21	3.88	1.58	control	control
daf-16(mu86);daf-2	0.44	0.15	0.35	0.18	0.41	0.23	0.40	0.05	0.0215	0.104
daf-16(mg54);daf-2	0.43	0.20	0.76	0.08	0.54	0.19	0.57	0.17	0.0050	0.148
daf-16(tm5030);daf-2	0.38	0.16	0.82	0.15	0.46	0.01	0.55	0.23	0.0055	0.143
daf-16(tm5032);daf-2	0.59	0.37	1.07	0.12	0.78	0.19	0.81	0.24	0.0056	0.209
daf-16(tm6659);daf-2	2.35	0.16	5.58	0.13	3.68	0.15	3.87	1.62	0.9758	0.997
N2 wild-type	1.00	0.22	1.00	0.24	1.00	0.28	1.00	0.00	0.0407	0.258
fat-7										
daf-2(e1370)	0.13	0.40	0.15	0.13	0.15	0.04	0.14	0.01	control	control
daf-16(mu86);daf-2	0.82	0.34	0.81	0.03	0.73	0.05	0.79	0.05	0.0023	5.597
daf-16(mg54);daf-2	0.63	0.38	0.69	0.03	0.49	0.07	0.60	0.10	0.0075	4.289
daf-16(tm5030);daf-2	0.23	0.37	0.29	0.23	0.40	0.01	0.31	0.09	0.0258	2.174
daf-16(tm5032);daf-2	0.26	0.38	0.26	0.10	0.44	0.09	0.32	0.10	0.0377	2.260
daf-16(tm6659);daf-2	0.09	0.27	0.17	0.13	0.14	0.11	0.13	0.04	0.6365	0.955
N2 wild-type	1.00	0.41	1.00	0.07	1.00	0.09	1.00	0.00	0.0008	7.107
hen-1										
daf-2(e1370)	14.72	0.28	23.26	0.17	32.22	0.30	23.40	8.75	control	control
daf-16(mu86);daf-2	1.06	0.27	0.43	0.18	2.22	0.27	1.24	0.91	0.0200	0.053
daf-16(mg54);daf-2	0.72	0.32	0.58	0.12	2.25	0.46	1.18	0.93	0.0092	0.051
daf-16(tm5030);daf-2	17.39	0.18	43.11	0.25	15.45	0.43	25.32	15.44	0.9712	1.082
daf-16(tm5032);daf-2	19.97	0.22	42.81	0.11	23.75	0.16	28.85	12.24	0.5286	1.233
daf-16(tm6659);daf-2	14.22	0.16	32.45	0.14	24.25	0.15	23.64	9.13	0.9818	1.010
N2 wild-type	1.00	0.20	1.00	0.24	1.00	0.43	1.00	0.00	0.0053	0.043
lea-1										
daf-2(e1370)	6.77	0.12	8.51	0.24	8.46	0.33	7.92	0.99	control	control
daf-16(mu86);daf-2	0.57	0.11	0.43	0.37	0.55	0.15	0.52	0.08	0.0029	0.066
daf-16(mg54);daf-2	0.54	0.11	0.54	0.30	0.56	0.23	0.54	0.02	0.0006	0.069
daf-16(tm5030);daf-2	6.50	0.09	10.34	0.33	6.02	0.09	7.62	2.37	0.7266	0.963
daf-16(tm5032);daf-2	7.01	0.13	8.88	0.28	6.96	0.15	7.62	1.09	0.6655	0.962
daf-16(tm6659);daf-2	0.68	0.11	0.70	0.40	0.87	0.28	0.75	0.11	0.0009	0.095
N2 wild-type	1.00	0.06	1.00	0.28	1.00	0.31	1.00	0.00	0.0013	0.126

Supplemental Table 11 (continued)

	Independent cohorts						Statistical Analysis			
	1		2		3				P value (ratio paired t-test)	Fold change
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
lipl-2										
daf-2(e1370)	58.49	0.16	38.59	0.28	25.99	0.50	41.02	16.38	control	control
daf-16(mu86);daf-2	3.53	0.24	1.69	0.29	1.55	0.18	2.26	1.11	0.0013	0.055
daf-16(mg54);daf-2	2.57	0.17	1.12	0.32	1.83	0.37	1.84	0.72	0.0067	0.045
daf-16(tm5030);daf-2	17.75	0.09	15.89	0.25	13.27	0.17	15.64	2.25	0.0260	0.381
daf-16(tm5032);daf-2	18.38	0.13	16.11	0.20	10.85	0.36	15.11	3.86	0.0094	0.368
daf-16(tm6659);daf-2	50.21	0.17	41.50	0.31	27.67	0.36	39.79	11.37	0.9442	0.970
N2 wild-type	1.00	0.12	1.00	0.53	1.00	0.28	1.00	0.00	0.0041	0.024
lys-7										
daf-2(e1370)	8.28	0.14	3.27	0.31	11.63	0.13	7.73	4.21	control	control
daf-16(mu86);daf-2	0.60	0.05	0.76	0.13	1.09	0.16	0.82	0.25	0.0262	0.106
daf-16(mg54);daf-2	0.50	0.15	0.63	0.36	1.03	0.23	0.72	0.27	0.0213	0.093
daf-16(tm5030);daf-2	3.89	0.04	8.69	0.26	7.78	0.17	6.79	2.55	0.9199	0.878
daf-16(tm5032);daf-2	4.03	0.13	7.11	0.22	8.63	0.14	6.59	2.35	0.8731	0.853
daf-16(tm6659);daf-2	4.26	0.17	11.63	0.43	7.36	0.18	7.75	3.70	0.9438	1.003
N2 wild-type	1.00	0.06	1.00	0.17	1.00	0.18	1.00	0.00	0.0369	0.129
mtl-1										
daf-2(e1370)	23.59	0.34	28.25	0.18	28.64	0.08	26.83	2.81	control	control
daf-16(mu86);daf-2	0.35	0.36	0.31	0.28	0.09	0.14	0.25	0.14	0.0092	0.009
daf-16(mg54);daf-2	0.28	0.34	0.68	0.25	0.14	0.16	0.37	0.28	0.0104	0.014
daf-16(tm5030);daf-2	4.89	0.26	15.03	0.23	5.62	0.11	8.51	5.66	0.0587	0.317
daf-16(tm5032);daf-2	5.31	0.28	12.64	0.18	5.86	0.13	7.94	4.08	0.0344	0.296
daf-16(tm6659);daf-2	12.73	0.24	27.28	0.10	13.83	0.13	17.95	8.10	0.1658	0.669
N2 wild-type	1.00	0.37	1.00	0.29	1.00	0.11	1.00	0.00	0.0004	0.037
sod-3										
daf-2(e1370)	22.94	0.25	14.52	0.50	24.08	0.14	20.51	5.22	control	control
daf-16(mu86);daf-2	0.47	0.24	0.55	0.26	0.65	0.15	0.56	0.09	0.0024	0.027
daf-16(mg54);daf-2	1.25	0.23	1.60	0.40	1.92	0.16	1.59	0.33	0.0064	0.078
daf-16(tm5030);daf-2	4.86	0.21	5.03	0.33	7.89	0.20	5.93	1.70	0.0153	0.289
daf-16(tm5032);daf-2	4.47	0.25	3.51	0.25	6.23	0.18	4.74	1.38	0.0034	0.231
daf-16(tm6659);daf-2	19.70	0.17	26.35	0.38	23.10	0.24	23.05	3.33	0.6236	1.124
N2 wild-type	1.00	0.26	1.00	0.57	1.00	0.18	1.00	0.00	0.0029	0.049
sams-5										
daf-2(e1370)	14.03	0.23	5.58	0.13	5.66	0.11	8.42	4.86	control	control
daf-16(mu86);daf-2	1.91	0.27	4.23	0.14	2.30	0.08	2.81	1.24	0.1698	0.334
daf-16(mg54);daf-2	2.62	0.25	2.38	0.19	2.27	0.10	2.42	0.18	0.0494	0.288
daf-16(tm5030);daf-2	6.28	0.21	3.36	0.27	7.84	0.08	5.83	2.27	0.4342	0.692
daf-16(tm5032);daf-2	5.90	0.27	1.64	0.14	6.06	0.11	4.53	2.51	0.2228	0.538
daf-16(tm6659);daf-2	2.14	0.20	2.55	0.19	1.38	0.26	2.02	0.60	0.0505	0.240
N2 wild-type	1.00	0.14	1.00	0.18	1.00	0.13	1.00	0.00	0.0218	0.119

Supplemental Table 11 (continued)

	Independent cohorts						Statistical Analysis			
	1		2		3				P value (ratio paired t-test)	Fold change
	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
scl-20										
daf-2(e1370)	512.00	0.25	229.13	0.17	198.09	0.16	313.07	172.97	control	control
daf-16(mu86);daf-2	0.85	0.21	0.17	1.64	0.28	0.06	0.43	0.36	0.0013	0.001
daf-16(mg54);daf-2	0.59	0.21	0.35	0.12	0.43	0.05	0.46	0.12	0.0008	0.001
daf-16(tm5030);daf-2	1.38	0.54	0.71	1.01	0.66	0.68	0.91	0.40	0.0001	0.003
daf-16(tm5032);daf-2	1.48	0.69	0.81	0.45	1.35	0.35	1.21	0.36	0.0022	0.004
daf-16(tm6659);daf-2	240.52	0.44	340.14	0.20	151.17	0.22	243.94	94.53	0.5928	0.779
N2 wild-type	1.00	0.12	1.00	0.35	1.00	0.03	1.00	0.00	0.0027	0.003
ttr-23										
daf-2(e1370)	27.28	0.12	11.08	0.22	4.72	0.38	14.36	11.63	control	control
daf-16(mu86);daf-2	2.99	0.39	1.49	0.22	1.26	0.24	1.91	0.94	0.0204	0.133
daf-16(mg54);daf-2	3.10	0.10	1.09	0.41	1.72	0.23	1.97	1.02	0.0472	0.137
daf-16(tm5030);daf-2	9.99	0.15	5.94	0.33	4.20	0.21	6.71	2.97	0.1517	0.467
daf-16(tm5032);daf-2	9.65	0.11	8.11	0.34	3.78	0.13	7.18	3.04	0.1801	0.500
daf-16(tm6659);daf-2	12.21	0.15	16.22	0.44	2.41	0.22	10.28	7.10	0.4331	0.716
N2 wild-type	1.00	0.62	1.00	0.25	1.00	0.41	1.00	0.00	0.0410	0.070

Supplemental Table 4.12. Comparison of A- and F-indices calculated from qPCR and RNA-seq data.

Indices are calculated from mean gene expression values (qPCR) or from FPKM gene expression values (RNA-seq) as described in Methods.

	A-Index		F-Index		
gene	qPCR	RNA-seq	qPCR	RNA-seq	Class
far-3	0.97	0.94	0.00	-0.14	A-specific
scl-20	1.00	0.99	0.22	-0.10	A-specific
lea-1	0.04	-0.18	0.97	1.00	F-specific
dod-17	0.09	0.13	0.03	0.05	Redundant
hen-1	-0.17	-0.21	-0.01	0.10	Redundant
lys-7	0.15	-0.03	0.00	-0.12	Redundant
mtl-1	0.70	0.61	0.34	0.23	Redundant
fat-7	0.37	0.36	-0.01	0.03	Shared A>F
lipl-2	0.65	0.58	0.03	-0.05	Shared A>F
sod-3	0.80	0.79	-0.13	-0.21	Shared A>F
sams-5	0.54	0.22	1.07	1.10	Shared F>A
ttr-23	0.60	0.49	0.33	0.36	Shared A=F

Supplemental Table 4.13. Strains used and generated in this study.
Double and triple mutant strains and siblings were constructed using standard genetic techniques.

Strain	Genotype	Outcrossed	Reference
N2 Bristol	wild-type		
DR1572	*daf-2(e1368)*	6X	Kimura *et al.* 1997
CB1370	*daf-2(e1370)*	6X	Kimura *et al.* 1997
CB4037	*glp-1(e2141)*	6X	Priess *et al.* 1987
CF1038	*daf-16(mu86)*	6X	Lin *et al.* 1997
GR1308	*daf-16(mg54)*	6X	Ogg *et al.* 1997
BQ63	*daf-16(tm5030)*	6X	This study
BQ64	*daf-16(tm5032)*	6X	This study
BQ65	*daf-16(tm6659)*	6X	This study
COP308	*Pdaf-16a::daf-16a + Cb unc-119(+)*	6X	This study
COP339	*Pdaf-16f::daf-16f + Cb unc-119(+)*	6X	This study

Supplemental Table 4.14. RACE, qPCR, and RNAi cloning primer sequences.
Continued on next page.

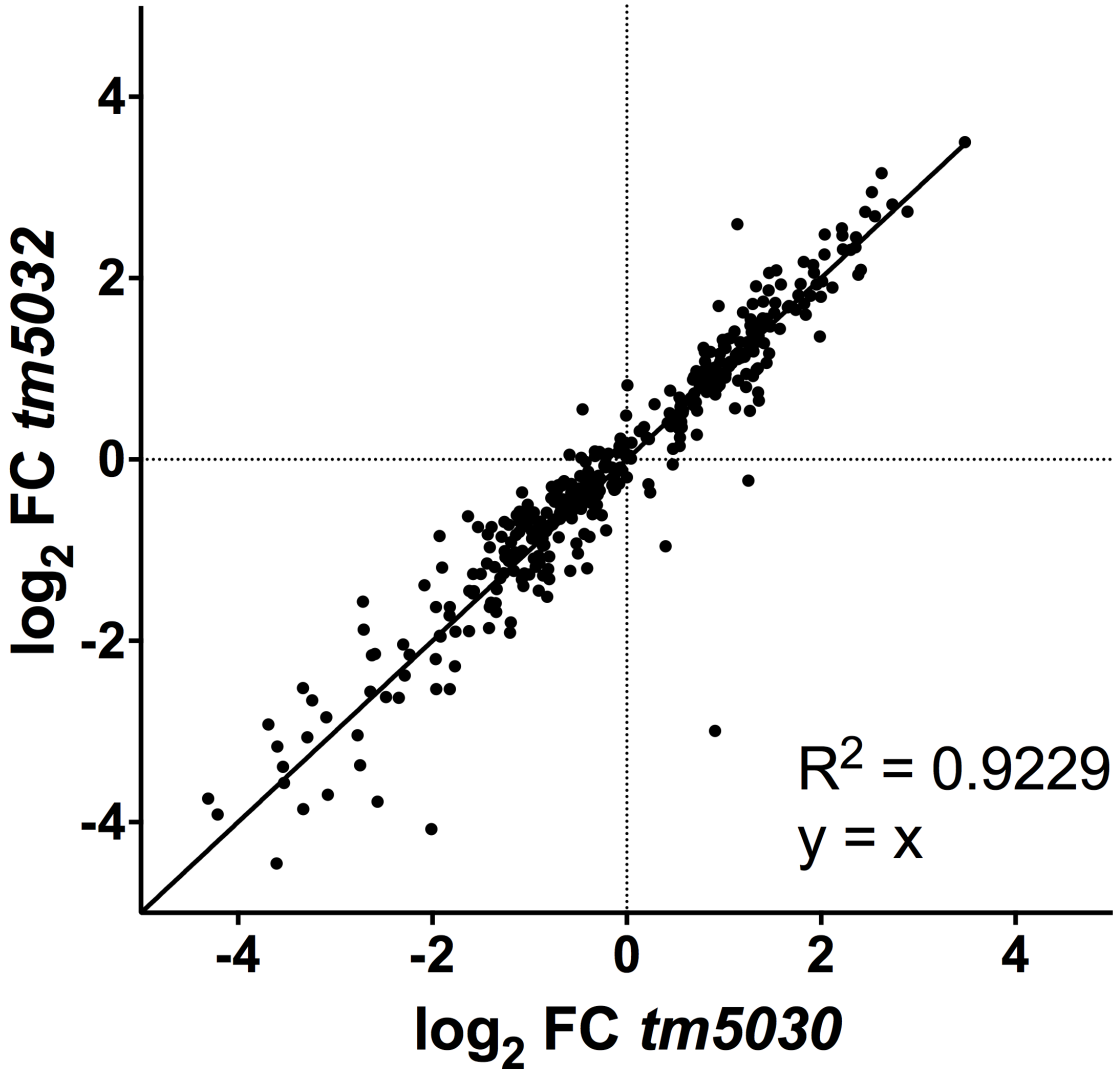
RACE primers			
Target	Primer name	Primer 1 sequence (5' to 3')	Reference
daf-16a/d/f/h	A/F-RACE	GCTGTCGACCCGTTTGAAGAT	This study
daf-16a	A-RACE	AGGTCAGCATCTTCTTCAGGAA	This study
daf-16d/f/h	F-RACE	TGTTGATGGAGGTCGAGATTGA	This study
daf-16a, daf-16d/f/h	Abridged Anchor	GGCCACGCGTCGACTAGTACGGGIIGG GIIGGGIIG	Invitrogen cat. #18374-058
qPCR primers for *daf-16/FoxO* isoforms			
Target	Primer 1 name	Primer 1 sequence (5' to 3')	Reference
daf-16	pan-qPCR1_For	AAAGAGCTCGTGGTGGGTTA	This study
daf-16	pan-qPCR1_Rev	TTCGAGTTGAGCTTTGTAGTCG	This study
daf-16	pan-qPCR2_For	AAGCCGATTAAGACGGAACC	Bansal *et al* 2014
daf-16	pan-qPCR2_Rev	GTAGTGGCATTGGCTTGAAG	Bansal *et al* 2014
daf-16a	A30-qPCR_For	TGAAGAAGATGCTGACCTA	This study
daf-16a	A32-qPCR_For	TGAATGATGATATGGAACCG	This study
daf-16d/f/h	F-qPCR_For	TTGACAGCGGAAGAACTA	This study
daf-16a, daf-16d/f/h	AF-qPCR_Rev	ATCTGGAGTATGAAGCATTG	This study
daf-16b	B-qPCR_For	TCGGATATCATTGCCAAAGC	This study
daf-16b	B-qPCR_Rev	TGACGGATCGAGTTCTTCCAT	This study

qPCR primers for *daf-16/FoxO* target genes			
Target	Primer 1 name	Primer 1 sequence (5' to 3')	Reference
act-1	act-1_Rev	TGGAGAGGGAAGCGAGGATAGA	Alam *et al.* 2010
act-1	act-1_For	CCAGGAATTGCTGATCGTATGCAGAA	Alam *et al.* 2010
dod-17	dod-17_Rev	GTTAGCGACAGTGAGTGTG	Gubelmann *et al* 2011
dod-17	dod-17_For	CAGGAAATCTTATTCGGACTACTC	Gubelmann *et al* 2011
far-3	far-3_Rev	AGCAACTTGGGTTTCAATGAG	Gubelmann *et al* 2011
far-3	far-3_For	ACGTGGTCTTTATGCTCGT	Gubelmann *et al* 2011
fat-7	fat-7_Rev	GGGAAATAGTGCTTTCTCTGG	Gubelmann *et al* 2011
fat-7	fat-7_For	AGTTAAGGAGCATGGAGGC	Gubelmann *et al* 2011
hen-1	hen-1_Rev	AATCAGCCAGTTTGATACATGG	Gubelmann *et al* 2011
hen-1	hen-1_For	GTCATGGCAACAAGTACATACC	Gubelmann *et al* 2011
lea-1	lea-1_Rev	CCTTGTCCTTGGTCTTGTC	Gubelmann *et al* 2011
lea-1	lea-1_For	ATGTAGAGAACAAAGCAGCAG	Gubelmann *et al* 2011
lipl-2	lipl-2_Rev	AAACGAAAGCTGCACTCTG	Gubelmann *et al* 2011
lipl-2	lipl-2_For	GTTACATGGCCAAATGGGA	Gubelmann *et al* 2011
lys-7	lys-7_Rev	TTAATCCGGATTGTCTGGC	Gubelmann *et al* 2011
lys-7	lys-7_For	CAACTAACTGGCCAAATAACG	Gubelmann *et al* 2011
mtl-1	mtl-1_For	ATGGCTTGCAAGTGTGACTG	Alam *et al.* 2010
mtl-1	mtl-1_Rev	CACATTTGTCTCCGCACTTG	Alam *et al.* 2010
sams-5	sams-5_Rev	CTTATCCACATGAACTCCAGC	Gubelmann *et al* 2011
sams-5	sams-5_For	CTCGAAAGGATTTGACTACAAGAC	Gubelmann *et al* 2011
scl-20	scl-20_Rev	ACTCTTGGTTCTTCCATCCG	Gubelmann *et al* 2011
scl-20	scl-20_For	GTTCGCTGGATAAATATGCCC	Gubelmann *et al* 2011
sod-3	sod-3_Rev	CGTGCTCCCAAACGTCAATTCCAA	Alam *et al.* 2010
sod-3	sod-3_For	TATTAAGCGCGACTTCGGTTCCCT	Alam *et al.* 2010
ttr-23	ttr-23_For	CTGCAATCATTACGGTATGTG	Gubelmann *et al* 2011
ttr-23	ttr-23_Rev	TCGTAGTTGTCTACTCCGA	Gubelmann *et al* 2011
RNAi cloning			
Target	Primer 1 name	Primer 1 sequence (5' to 3')	Reference
daf-16a	16C RNAi 5	AACTGAAGCTTCTGGACATCTAC	Kwon *et al* 2010
daf-16a	16C RNAi 3	TATAGGCTAGCATCTTCTTCAG	Kwon *et al* 2010
daf-16d/f/h	16D RNAi 5	AACTGAAGCTTGATTCGCCGCTACC	Kwon *et al* 2010
daf-16d/f/h	16D RNAi 3	CCCGTGCTAGCTAGTTCTTCCGC	Kwon *et al* 2010
daf-16	16ACD RNAi 5	ATCTGAAGCTTCATTCTCGTTTC	Kwon *et al* 2010
daf-16	16ACD RNAi 3	CTTGACTCGCTAGCTGTCTGATC	Kwon *et al* 2010

Supplemental Table 4.15. Number of 5' RACE clones sequenced for each strain.
A semi-synchronized population of young adults were harvested for RNA. RNA was purified and analyzed as described in Methods. The A/F-RACE primer was used for first-strand cDNA synthesis. PCR amplification was performed using either the A30-RACE primer (*daf-16a* RACE primer) or the F-RACE primer (*daf-16f* RACE primer), in combination with the Abridged Anchor Primer from the Invitrogen RACE kit. For *daf-16(tm5032)*, a different primer A32-RACE was used, indicated by asterisks. All indicated RACE clones were consistent with sequences presented in Supplemental Figure 4.2 and Supplemental Figure 4.4. Note that clones without SL1 trans-spliced leaders (w/o SL1) were partial fragments of the sequences.

	daf-16a RACE primer			*daf-16f* RACE primer		
	w/ SL1	w/o SL1	empty	w/ SL1	w/o SL1	empty
N2 wild-type	4	9	2	15	1	1
daf-16(tm5030)	10	3	6	7	0	0
daf-16(tm5032)	4*	5*	3*	7	3	0
daf-16(tm6659)	8	17	2	0	0	20

Supplemental Figure 4.10. Comparison of effects of two *daf-16a* alleles on expression of DAF-16A/F target genes.
Log_2 fold-change (FC) is shown for *daf-2(e1370)* vs. *daf-16a(tm5030);daf-2* on the X-axis, and log_2 FC is shown for *daf-2(e1370)* vs. *daf-16a(tm5032);daf-2* on the Y-axis.



Supplemental Table 4.16. Effect of more stringent cutoffs for selection of DAF-16A/F targets on downstream analysis of categories.

We selected DAF-16A/F targets primarily on the basis of fold-change. Genes must meet the appropriate fold-change threshold for all three comparisons: (1) *daf-2* vs. *daf-2;e1370*, *daf-2* vs. *daf-16(mg54);daf-2* and *daf-2 vs. daf-16(mu86);daf-2*.

We then required FDR < 0.05 for only one of the three comparisons for several reasons: (1) Nearly all genes showed concordance in fold-change for all three comparisons even if they did not satisfy FDR < 0.05 for all three. (2) Some known DAF-16/FoxO targets only satisfied FDR < 0.05 in one or two comparisons. (3) The choice of requiring FDR < 0.05 for one, two, or three comparisons did not affect downstream analysis, and therefore we list all genes for completeness.

See Materials and Methods for all criteria.

	FDR > 0.05 for at least one comparison		FDR > 0.05 for at least two comparisons		FDR > 0.05 for all three comparisons	
	Number	Percent	Number	Percent	Number	Percent
A-specific	57	14	48	16	33	18
F-specific	8	2	6	2	4	2
Redundant	35	9	28	9	15	8
Shared A>F	219	55	173	56	102	56
Shared A=F	60	15	41	13	22	12
Shared F>A	20	5	13	4	6	3
Total	399	100	309	100	182	100

Supplemental Discussion

The finding that DAF-16A plays a stronger role in life span control than DAF-16F was surprising, as previous experiments with transgenic strains suggested that DAF-16F was more important [35,42]. The previous study by Kwon *et al.* used transgenic strains harboring multiple cDNA copies of either *daf-16a* or *daf-16f* in a *daf-16* null background [35]. We identified two major technical differences in the study by Kwon *et al.*: (1) over-expression of DAF-16 isoforms and (2) expression from a non-endogenous genomic locus, and therefore may be missing native DNA regulatory elements. Thus, we reasoned that experiments employing single-copy transgenic strains might reconcile the two studies.

We generated single-copy transgenic lines expressing either *daf-16a* or *daf-16f* (Supplemental Figure 4.11) using the Mos1-mediated single-copy insertion (MosSCI) technique [86] and verified that the full-length sequences were integrated. Crossing the *daf-16f* transgene into *daf-16a/f;daf-2(e1370)* rescued life span extension and dauer arrest (Supplemental Figure 4.12A-B), indicating that the *daf-16f* transgene is functional. Importantly, these animals had the same phenotypes as *daf-16a;daf-2*, suggesting that this *daf-16f* transgene has the same activity as *daf-16f* expressed from the genomic locus. This equal activity is also illustrated by partial rescue of life span by the *daf-16f* transgene in *daf-16a/f;daf-2(e1368)*, consistent with *daf-16a;daf-2(e1368)* life span (Supplemental Figure 4.12C). However, the *daf-16f* transgene did not rescue dauer arrest (Supplemental Figure 4.12D). This is consistent with the requirement for *daf-16a* in *daf-2(e1368)* arrest (Figure 4.2A). Thus, *daf-16f*-transgene-rescued *daf-16a/f;daf-2* mutants exhibited the same phenotypes as *daf-16a;daf-2* mutants, providing a second, independent line of evidence that *daf-16a* is required for dauer arrest and life span extension.

Thus, single-copy *daf-16f* does not rescue life span to the same extent as multi-copy *daf-16f*, which can effectively replace the need for any *daf-16* expressed from the endogenous genomic locus [35,42]. We tested the hypothesis that copy number of *daf-16f* is the

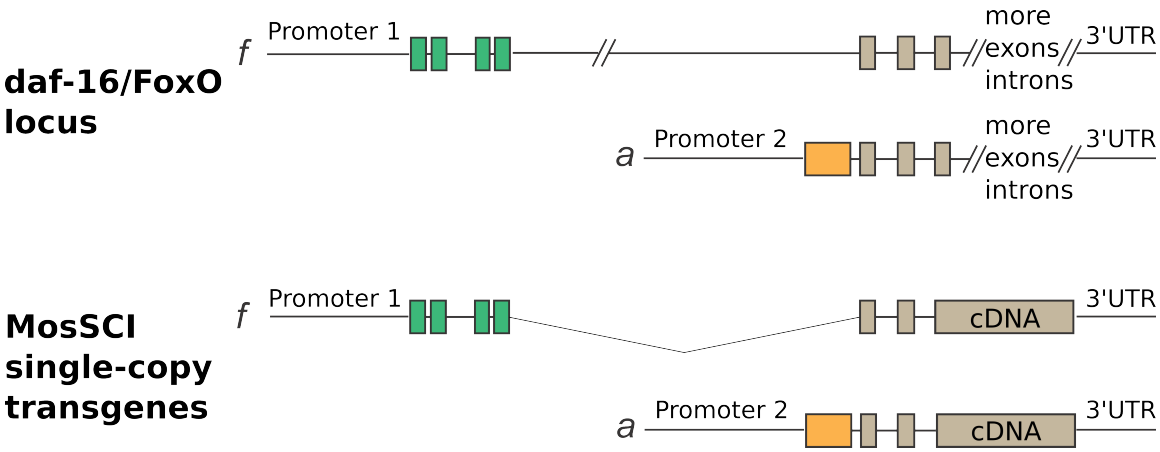
critical difference between our studies. Thus, we doubled the gene dosage of *daf-16f* in the absence of *daf-16a* by introducing the *daf-16f* transgene into *daf-16a;daf-2(e1370)*. This extended life span compared to the *daf-16a;daf-2* and *daf-16a/f;daf-2;daf-16f-* transgene strains (Figure 4.3G, Supplemental Figure 4.12A). Over-expression likely allowed the additional DAF-16F protein to assume some functions normally performed by DAF-16A, consistent with the expression profiling data that indicated partially overlapping roles for DAF-16A and DAF-16F (Figure 4.5). Taken together, the data strongly suggest that multi-copy expression of *daf-16f* in previous studies over-stated the role of *daf-16f* [35,42].

However, the single-copy *daf-16a* transgene had a more modest effect on dauer arrest and life span of *daf-16a/f;daf-2(e1370)* compared to single-copy *daf-16f* (Supplemental Figure 4.13A-B). Thus, both we and Kwon *et al.* [35] found stronger effects of *daf-16f* transgenes compared to *daf-16a* transgenes. Given that we found *daf-16a* expressed from the endogenous genomic locus to be sufficient for life span extension (Figure 4.3B,D,G), this raised the possibility that *daf-16a* transgenes are not fully functional. To test this hypothesis, we crossed our *daf-16a* single-copy transgene into *daf-16a;daf-2(e1368)*. The *daf-16a* transgene only rescued ~ 50% of the dauer arrest and life span extension of *daf-16(+);daf-2(e1368)* compared to *daf-16a;daf-2(e1368)* (Supplemental Figure 4.13C-D). To ensure this incomplete rescue was not specific to one strain, we tested two independently isolated lines expressing the *daf-16a* transgene and found they had the same incomplete dauer rescue (Supplemental Figure 4.13E). Therefore, the single-copy *daf-16a* transgenes are not fully functional. Given the similarity of our transgene construct compared to that of Kwon *et al.* [35], this raises the strong possibility that the Kwon *et al. daf-16a* transgene also does not recapitulate endogenous *daf-16a* function.

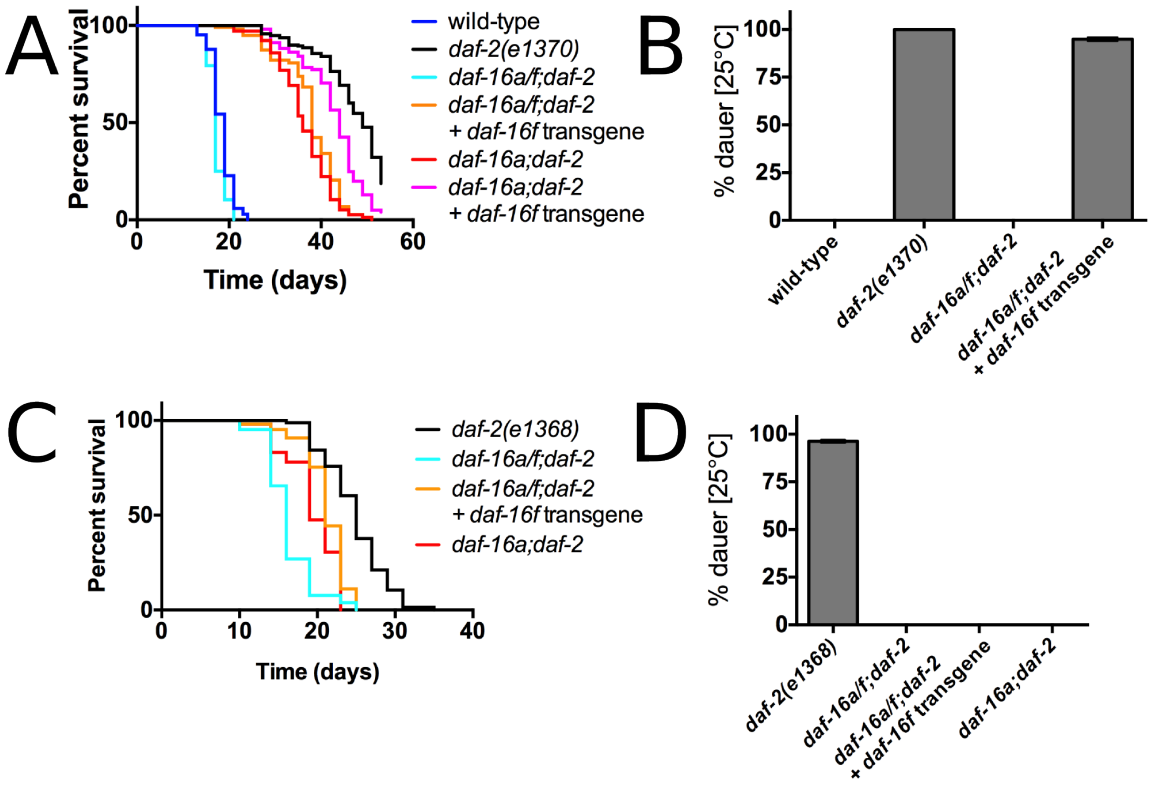
Taken together, these results reconcile the previous study by Kwon *et al.* [35] and our study. Multi-copy *daf-16f* over-states the role of *daf-16f*, while single-copy *daf-16f* supports our model (Figure 4.3G, Figure 4.7, Supplemental Figure 4.12A). *daf-16a* transgenes do not recapitulate endogenous functions of *daf-16a* (Supplemental Figure 4.13C-D). Further study is needed to identify the precise DNA regulatory elements

required for full activity of *daf-16a*. In contrast, our isoform-specific mutants express intact isoforms at endogenous levels (Supplemental Figure 4.6) from the native genomic locus. Given that the interpretation of the Kwon *et al.* over-expression transgenes is complicated by overlapping roles for DAF-16A and DAF-16F (Figure 4.5), our isoform-specific mutants newly elucidate the endogenous functions of DAF-16A and DAF-16F.

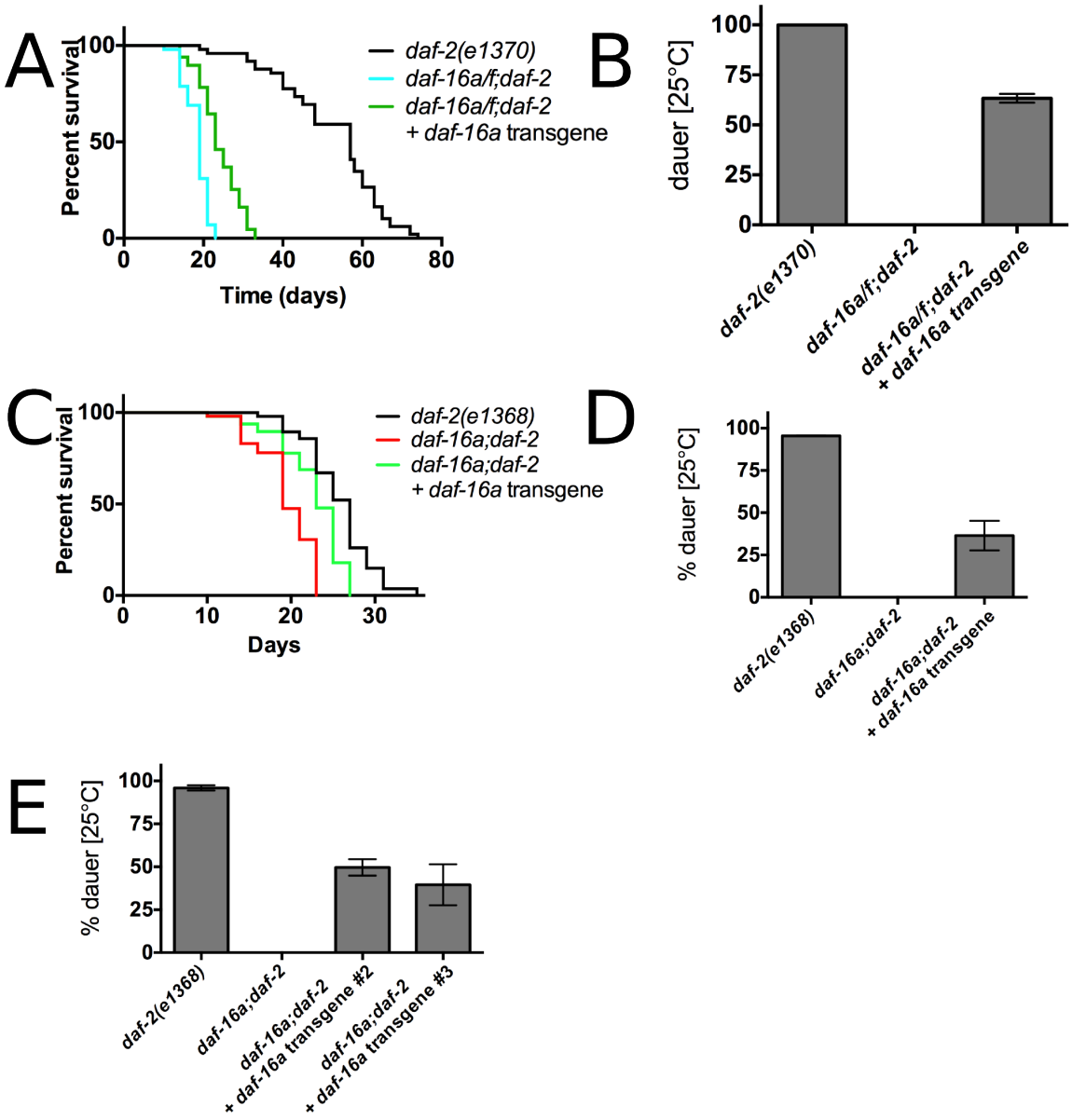
Supplemental Figure 4.11. Diagram of single-copy *daf-16a* and *daf-16* transgenes.
These transgenes are both integrated on Chr II at the *ttTi5605* locus. Promoter 1 is 3kb while Promoter 2 is 4kb. The complete 1.7kb 3'UTR was incorporated. Note that a CB-*unc-119* sequence is incorporated 5' upstream of both transgenes for strain generation and selection, similar to that of Kwon *et al.* [35].



Supplemental Figure 4.12. The *daf-16f* transgene is functional and is consistent with the *daf-16a* mutant.
(A-B) The *daf-16f* transgene is functional and rescues *daf-16a/f;daf-2(e1370)* life span and dauer arrest to the same levels as *daf-16a;daf-2*. Furthermore, the *daf-16f* transgene further extends *daf-16a;daf-2*, demonstrating that over-expression of *daf-16f* over-states the role of *daf-16f*.
(C-D) The *daf-16f* transgene is functional and rescues *daf-16a/f;daf-2(e1368)* life span and dauer arrest to the same levels as *daf-16a;daf-2*.



Supplemental Figure 4.13. The *daf-16a* transgene is partially functional.
(A-B) The *daf-16a* transgene has a very modest effect on *daf-16a/f;daf-2(e1370)* life span and dauer arrest, consistent with observations by Kwon *et al.* [35] that *daf-16f* transgenes promote longevity to a greater extent than *daf-16a* transgenes.
(C-E) The *daf-16a* transgene does not rescue the dauer arrest and life span phenotypes of *daf-16a;daf-2(e1368)*. Thus, the *daf-16a* transgene does not recapitulate endogenous *daf-16a* activity.



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Chapter 5 Identification of critical DAF-16/FoxO target genes required for longevity

Abstract

FoxO transcription factors (TFs) control life span and aging in invertebrates and may do so in humans. The *Caenorhabditis elegans* FoxO ortholog DAF-16 promotes longevity and controls the expression of thousands of genes, but it is unknown if there exist single genes controlled by DAF-16/FoxO that are essential for life span extension. We previously used isoform-specific *daf-16/FoxO* alleles to categorize DAF-16/FoxO target genes according to their regulation by distinct DAF-16 isoforms. Life span phenotypes of isoform-specific mutants suggested that DAF-16A-specific and DAF-16A/F shared targets are most likely to influence life span. Here, we screen loss-of-function mutants to identify 8 DAF-16A-specific and 10 shared targets that influence longevity. Strikingly, the DAF-16A-specific target genes *hsp-12.3* and *sodh-1* are fully required for life span extension in animals with reduced *daf-2/IGFR* signaling. To our knowledge this is the first use of genetic mutants for systematic functional testing of DAF-16/FoxO targets, and demonstrates that a small subset of DAF-16/FoxO targets plays a disproportionate role in life span control. This offers a framework to prioritize further study of mechanisms of life span extension downstream of DAF-16/FoxO.

Introduction

FoxO transcription factors (TFs) control aging, development, and metabolism in diverse species. FoxO TFs promote longevity in invertebrates and may do so in humans [1–3], and mouse models implicate FoxO dysregulation in the pathogenesis of age-related diseases such as cancer, Type 2 diabetes, and osteoporosis [4–9]. However, FoxO TFs are highly pleiotropic and their transcriptional outputs vary greatly dependent on context [10]. While this complexity may underlie FoxO's influence on aging, it also poses significant challenge to understanding mechanisms of FoxO-mediated life span extension. Identifying specific protective functions of FoxO is likely necessary to develop targeted treatments to ameliorate and prevent age-related disease.

In *Caenorhabditis elegans*, the FoxO ortholog DAF-16 promotes longevity in the contexts of reduced DAF-2 insulin-like signaling and germline ablation [11,12]. Expression profiling studies have identified thousands of genes regulated by DAF-16/FoxO in these contexts [13–16]. Studies utilizing RNA interference (RNAi) to knock down the expression of individual DAF-16/FoxO target genes suggest that many of these genes are likely to make minor contributions to life span extension [14,17,18]. This is consistent with a model where DAF-16/FoxO upregulates and downregulates numerous genes that act in a cumulative fashion to dramatically increase life span [17,19].

It is possible that DAF-16/FoxO targets essential for life span extension remain unidentified, as an exhaustive analysis of DAF-16/FoxO target gene contributions to longevity has not been experimentally feasible. At the present time, less than 10% of target genes have been tested by RNAi, and existing RNAi results have not yet been validated by genetic null mutations. In this study, we use prior insights from studies of DAF-16 isoforms to prioritize those genes most likely to influence life span for rigorous functional testing.

Results

Prioritization of genes for systematic mutant testing

Three DAF-16/FoxO isoforms are encoded by one genomic locus that share C-termini but diverge in their N-termini and regulatory DNA sequences [20,21]. We previously found in Chapters 3 and 4 that these three isoforms act in a hierarchy to control longevity, where DAF-16A is sufficient for full life span extension, DAF-16F is sufficient for partial life span extension in the absence of DAF-16A, and DAF-16B does not promote longevity. Importantly, the DAF-16/FoxO isoforms control distinct, though overlapping, sets of target genes. This allows us to correlate longevity with the expression of particular genes, and therefore we prioritized those genes for functional testing.

daf-16a/f mutation suppresses the longevity of *daf-2(e1370)* mutants to the same extent as *daf-16* null mutation, and therefore target genes controlled specifically by DAF-16A/F are most likely to influence life span. We further showed that *daf-16a* mutation partially suppresses longevity, while *daf-16f* mutation alone has no effect (Figure 5.1A-B), and used the gene expression profiles of these isoform-specific *daf-16* mutants to bin DAF-16A/F targets into discrete categories (Figure 5.1C).

Because DAF-16A-specific targets are the major difference between *daf-2(e1370)* and *daf-16a;daf-2*, we reasoned that some subset of this category is likely responsible for the partially reduced life span of *daf-16a;daf-2* animals. Therefore, we sought to test strains that harbor loss-of-function mutations in A-specific targets.

Conversely, because the expression of shared genes is largely maintained after individual *daf-16a* and *daf-16f* mutation, we reasoned that some subset of this category is responsible for the residual life span extension of *daf-16a;daf-2* mutants compared to *daf-16a/f;daf-2* mutants. However, the vast majority of genes (334/399) are shared DAF-16A/F targets, and this list is experimentally intractable. Therefore, we first selected those genes that are redundantly regulated, *i.e.* they show minimal change with either *daf-16a* or *daf-16f* mutation alone. These genes are the most likely to continue to play the

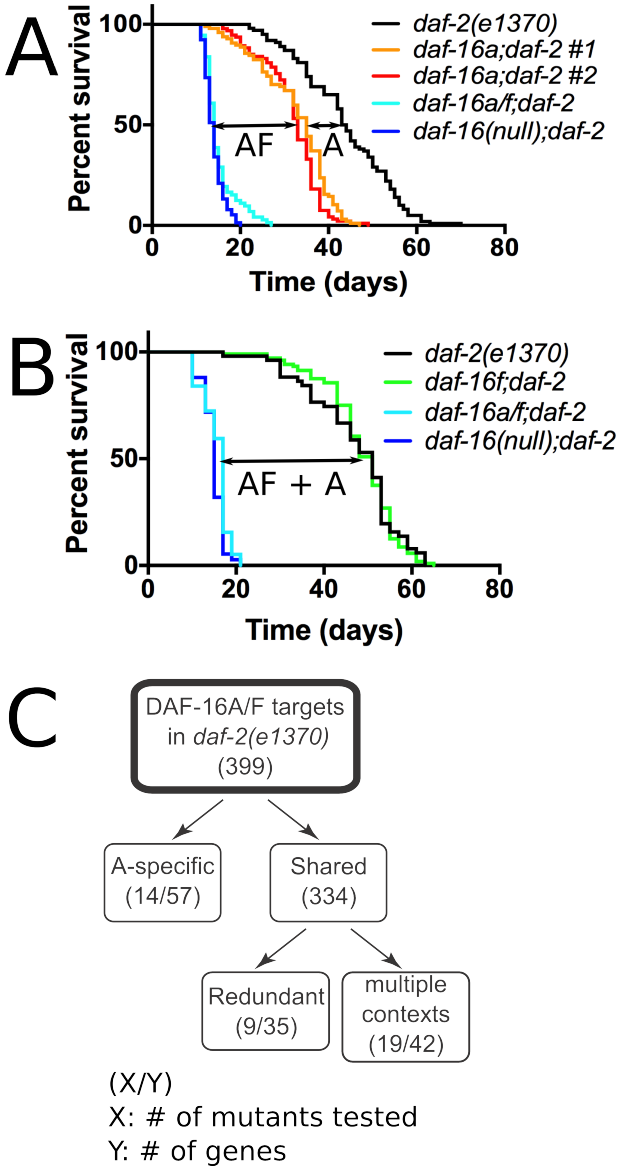
same roles they do in *daf-2(e1370)* mutants, following *daf-16a* or *daf-16f* mutation. Furthermore, in Chapter 3 we identified DAF-16A/F targets whose expression levels are altered in *daf-2(e1368)* mutants and germline-ablated animals. Therefore, we prioritized those genes that are jointly regulated by DAF-16A and DAF-16F in all three contexts, because these are highly associated with longevity.

This prioritization totals 134 genes that are likely to influence life span. We considered this a small enough set to proceed with functional analysis.

Figure 5.1. Rationale for prioritizing genes for functional testing by genetic mutation.

(A-B) Survival curves of (A) *daf-16a;daf-2* and (B) *daf-16f;daf-2* mutants. A and AF indicate the suspected roles of A-specific and A/F shared targets respectively.

(C) Flowchart indicating prioritization of DAF-16A/F targets for testing. For definitions of A-specific genes and redundantly regulated genes, see text and Chapter 4. The "multiple contexts" category encompasses DAF-16A/F targets that we identified in *daf-2(e1370)*, *daf-2(e1368),* and germline-ablated animals (see text and Chapter 3). Note that 4 of the 46 "multiple context" genes from Chapter 3 were identified as A-specific or redundantly regulated in Chapter 4. (X/Y) indicates X healthy, available mutants that we tested, and Y indicates the number of genes in that group.

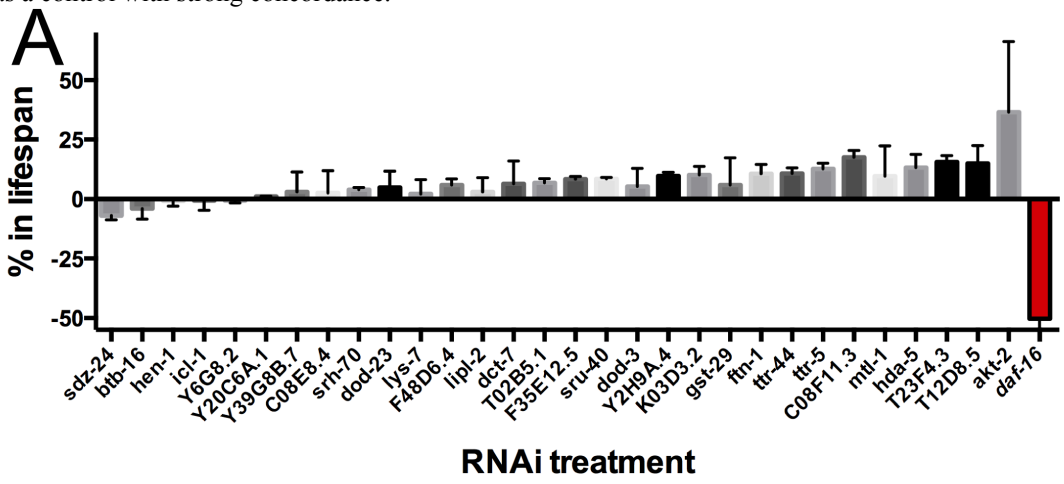


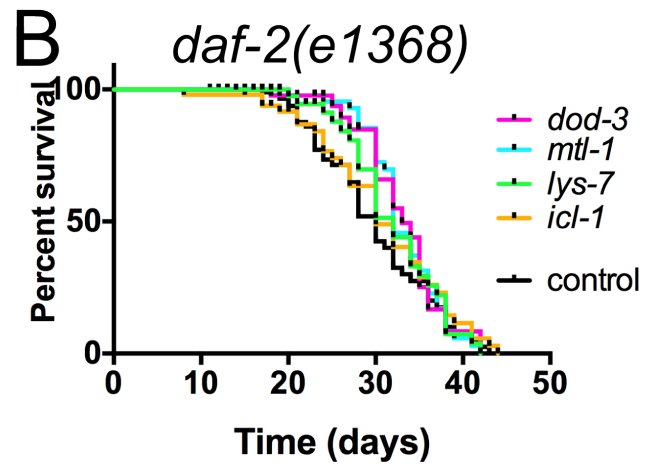
Poor correlation between RNAi and mutation screening

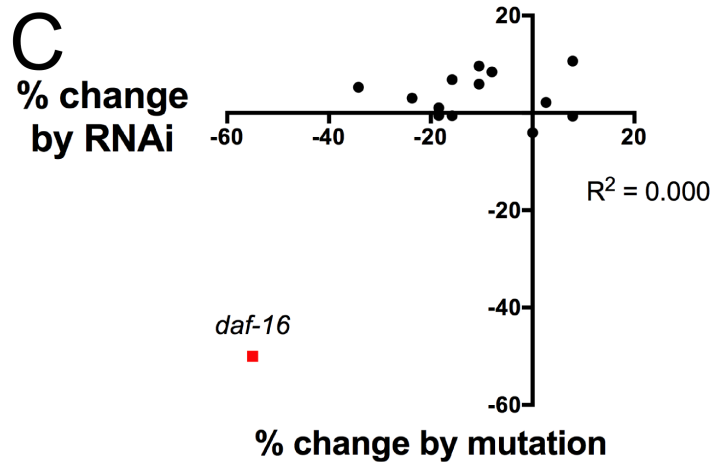
During the course of developing our overall strategy to identify longevity assurance mechanisms downstream of DAF-16/FoxO, we also employed RNA interference (RNAi) which has previously been used to test DAF-16/FoxO targets for contributions to life span extension [13,18]. However, we did not observe significant life span effects using RNAi (Figure 5.2A). In particular, RNAi treatments previously reported to reduce life span of *daf-2* mutants did not do so in our hands (Figure 5.2B), despite *daf-16* RNAi reducing life span robustly in the same experiment.

Interpretation of results based on RNAi is often complicated by off-target effects and incomplete knockdown, which could vary by laboratory conditions. Furthermore, when we compared treating *daf-2* mutants with target gene RNAi with the converse experiment of treating target gene mutants with *daf-2* RNAi, we did not observe any correlation (Figure 5.2C). This surprising result indicates that conclusions about the contributions of single target genes cannot be drawn from RNAi knockdown of those genes, without confirmation using a second method.

Figure 5.2. Poor correlation between RNAi and mutation screening
(A) Testing prioritized DAF-16A/F shared targets by RNAi did not result in substantial life span changes. Note that all except *F35E12.5* are Class 1 genes (*i.e.* upregulated by DAF-16/FoxO).
(B) *dod-3*, *mtl-1*, *lys-7*, and *icl-1* RNAi do not reduce *daf-2(e1368)* longevity in our hands. These were previously reported by Murphy *et al.* [17] reduce *daf-2* longevity (*dod-3* 0-22%, *mtl-1* 10-25%, *lys-7* 8-30%, *icl-1* 23%).
(C) RNAi and mutation loss-of-function of individual target genes do not correlate. *daf-16* (red) is shown as a control with strong concordance.







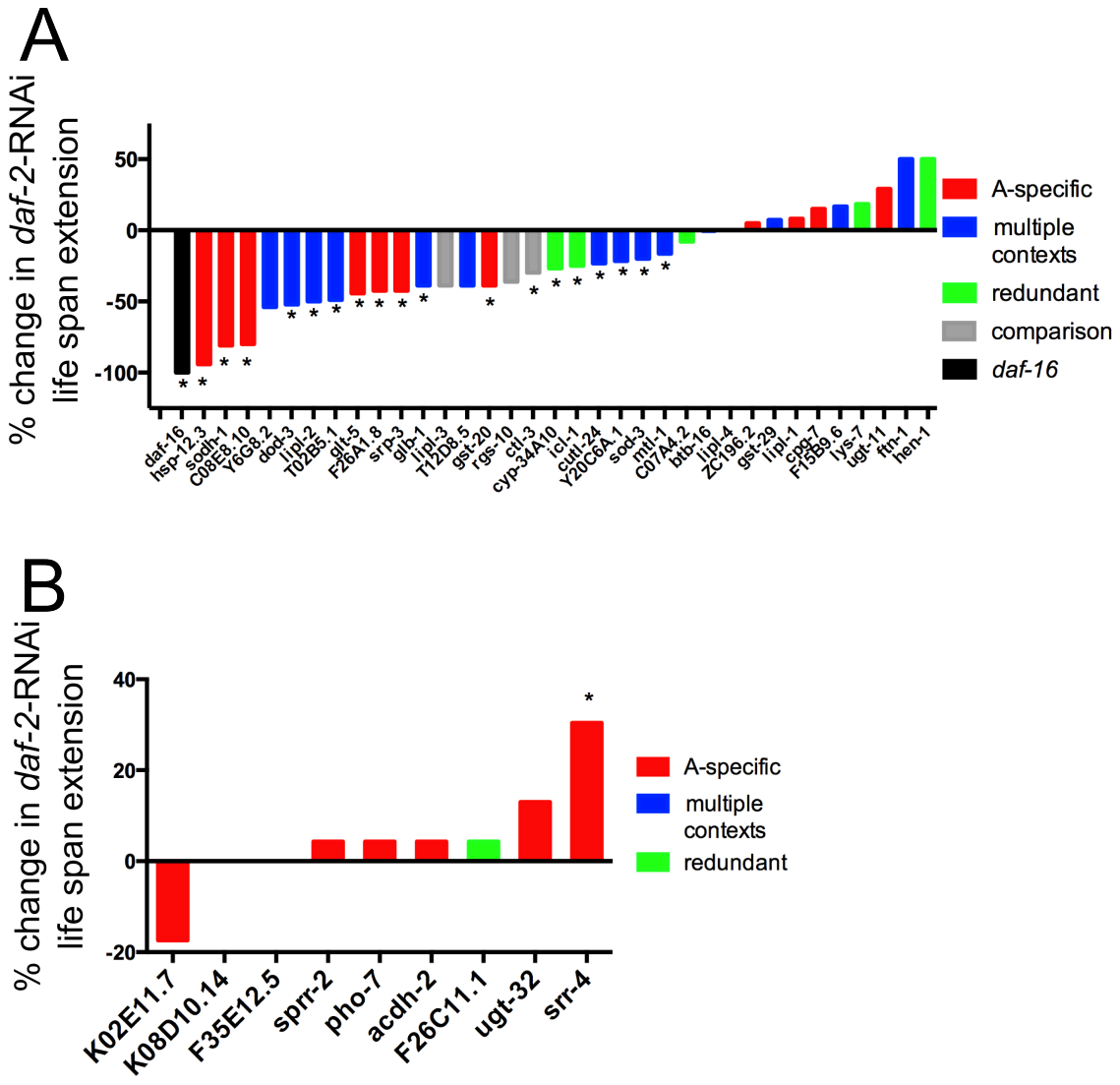
Mutant screening strategy

We developed the following strategy to test these genes: We first obtained mutants that were readily available and selected ones that were not obviously sick. This totaled 42 mutant strains, about one-third of the 134 prioritized genes (57 A-specific, 35 redundant, and 42 multi-context targets; Figure 5.1C). Furthermore, we also tested four DAF-16A/F target genes that would not be predicted to influence life span based on their pattern of regulation by DAF-16 isoforms.

For Class 1 genes, *i.e.* those upregulated by DAF-16/FoxO, we screened these strains for reduced *daf-2*-RNAi-mediated life span extension. We performed an initial screen at low numbers (~30 animals per strain per RNAi treatment), and then repeated this experiment with a larger number of animals for those mutations that had significant effects in the first experiment. To determine the effect of each mutation and correct for any changes in baseline mutant life span, we calculated the life span extension of each strain on *daf-2* RNAi relative to control RNAi treatment, and compared this to life span extension of N2 wild-type on *daf-2* RNAi relative to control RNAi in the same experimental replicate. Thus, as *daf-16* mutants do not show any life span extension by *daf-2* RNAi, *daf-16* mutation suppressed 100% of the *daf-2*-RNAi-mediated life span extension. In contrast, a *lipl-4* mutant shows the same percent life span extension on *daf-2* RNAi as wild-type, and thus *lipl-4* mutation suppresses 0% of the *daf-2*-RNAi-mediated life span extension.

For Class 2 genes, *i.e.* those downregulated by DAF-16/FoxO, we screened these strains for increased life span under control conditions. We also screened them for increased life span relative to wild-type following *daf-2*-RNAi treatment, similar to Class 1 genes.

Figure 5.3. Results of screening mutations in DAF-16A/F targets
(A) Effect of Class 1 targets (upregulated by DAF-16/FoxO) on *daf-2* RNAi-mediated longevity. (B) Effect of Class 2 targets (downregulated by DAF-16/FoxO) on life span compared to wild-type. * indicates statistically significant changes ($p < 0.05$ by log-rank test). See text and Figure 5.1 for details.



Genes regulated primarily by DAF-16A are major determinants of life span

Of all the genes we tested, the two genes with the greatest effects were the A-specific targets *hsp-12.3* and *sodh-1* (Figure 5.3A). Strikingly, *hsp-12.3* and *sodh-1* mutation caused 94% and 81% suppression of *daf-2*-RNAi mediated life span extension, respectively (Figure 5.4A-B). This was nearly the same effect as *daf-16* null mutation (100%; Figure 5.3A and Figure 5.5E). *sodh-1* encodes an enzyme similar to human alcohol dehydrogenases and sorbitol dehydrogenases (BLAST, WormBase). *sodh-1* mutants are reported to have reduced alcohol metabolism *in vivo* [22]. *hsp-12.3* encodes a heat shock protein similar to human alpha-crystallin, but lacks activity in classic chaperone assays [23]. Importantly, these genes suppressed longevity of *daf-2*-RNAi longevity without significantly reducing life span on control RNAi. The *sodh-1* mutant was 13% shorter lived than wild-type on control RNAi; by comparison, *daf-16* is 26% shorter-lived. The *hsp-12.3* mutant was 13% longer-lived on control RNAi. Therefore, it is likely that the animals are not sick, and *hsp-12.3* and *sodh-1* are major determinants of *daf-2/IGFR* longevity.

Mutations in another five upregulated DAF-16A-specific targets also significantly suppressed longevity: *C08E8.10, srp-3, F26A1.8, gst-20*, and *glt-5* (Figure 5.3A and Figure 5.4). *C08E8.10* encodes a short 93 amino acid protein of unknown function (WormBase). *srp-3* encodes a conserved, functional member of the serpin family, acting to inhibit the serine peptidases cathepsin G and chymotrypsin *in vitro* [24]. Translational fusion reporters indicate *srp-3* is found intracellularly in *C. elegans* muscle [24]. *F26A1.8* is similar to the TauE family of integral membrane proteins that are involved in anion transport during taurine metabolism (BLAST, WormBase). *gst-20* is a glutathione-S-transferase family member that is most similar to human hematopoietic prostaglandin D synthase (WormBase). *glt-5* is one of six conserved glutamate transporters in *C. elegans*, where it may function to prevent neurotoxic accumulation of glutamate [25].

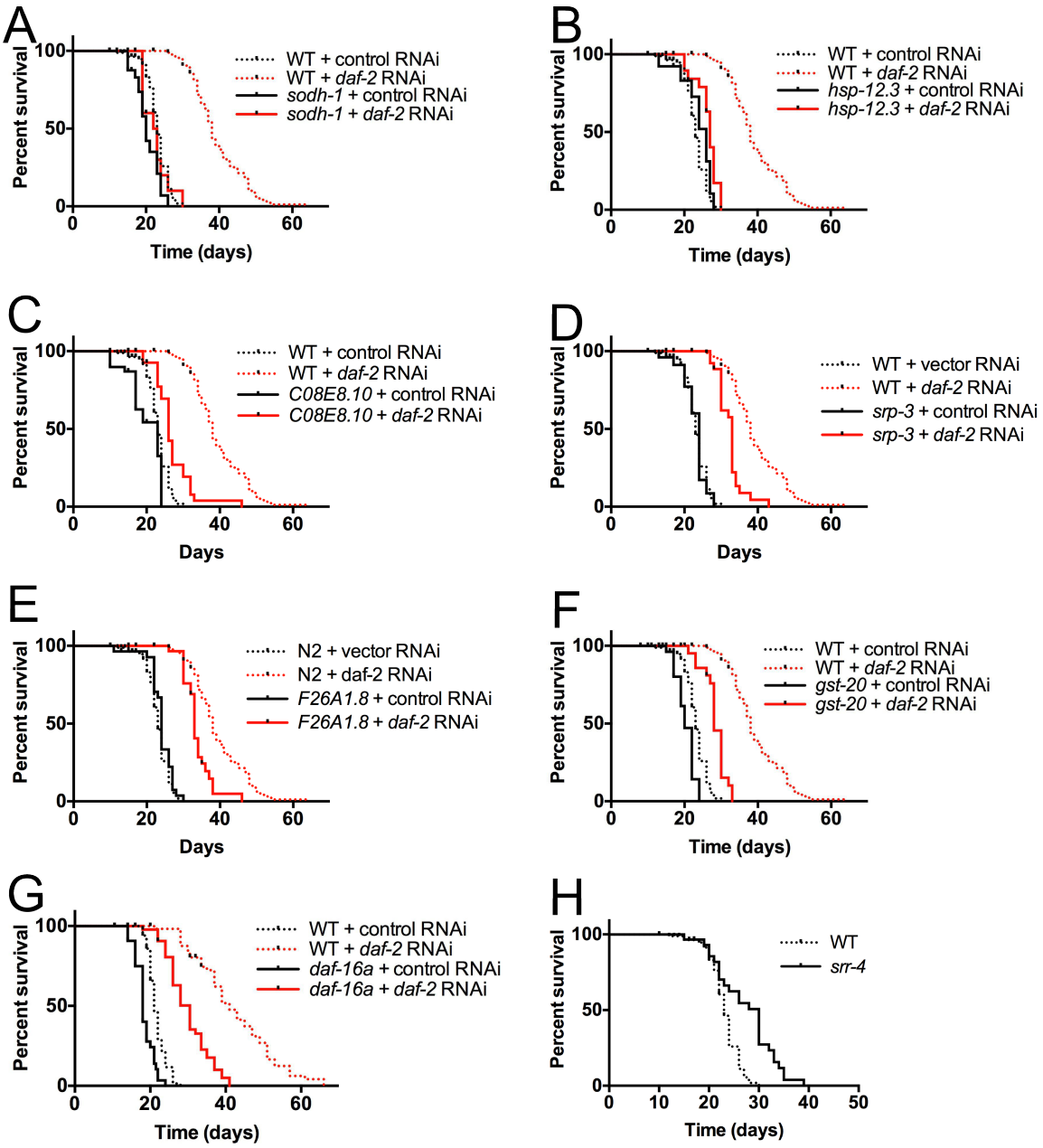
For comparison to A-specific targets, we tested two upregulated F-specific targets (out of 8) for which loss-of-function mutations were available (Figure 5.3A). Interestingly, these did reduce life span extension slightly. However, they had far less significant effects than

DAF-16A targets, and their pattern of regulation suggests that their upregulation is not important for life span extension.

srr-4, an A-specific target, was the only Class 2 gene that extended life span compared to wild-type when mutated (30% extension; Figure 5.3B and Figure 5.4H). *srr-4* is downregulated 3-fold in *daf-2* mutants dependent on *daf-16a*, and it was not previously identified by any microarray studies. *srr-4* is a 7 transmembrane-pass G-protein coupled receptor of unknown function (www.wormbase.org). Interestingly, *srr-4* mutants treated with *daf-2* RNAi are shorter-lived than wild-type animals treated with *daf-2* RNAi (data not shown), suggesting *srr-4* may have context-specific effects.

In sum, out of 14 DAF-16A-specific targets functionally tested, we identified 8 that influence longevity (Figure 5.3). Remarkably, two of them suppress longevity when mutated to almost the same extent as *daf-16* mutation itself. This is consistent with hierarchical DAF-16 isoform action in life span control, where DAF-16A plays the dominant role.

Figure 5.4. Survival curves of A-specific genes with the largest effects on life span. (A-F) Life span curves for six DAF-16A-specific upregulated genes with major effects on *daf-2*-RNAi-mediated life span extension are shown. (G) *daf-16a(tm5030)* mutant treated with *daf-2* RNAi or vector is displayed for comparison. (H) The only DAF-16/FoxO-downregulated target gene that influenced life span is shown.



Genes regulated jointly by DAF-16A/F are major determinants of life span

After the three DAF-16A targets, the Class 1 genes with the next strongest, statistically significant effects were three genes that we identified as DAF-16A/F targets in multiple contexts (Figure 5.3A). These were all more strongly affected by *daf-16a* mutation than *daf-16f* mutation. The first one is *dod-3* (Figure 5.5A), a short 106 amino acid protein with unknown function that has been validated in numerous studies of DAF-16 targets [13].

lipl-2 is a shared DAF-16A/F target in multiple contexts, is upregulated 32-fold in *daf-2(e1370)* compared to wild-type, and reduces life span extension by 50% when mutated ($p < 0.0001$; Figure 5.5B). *lipl-2* is one of five triglyceride lipases induced by fasting in *C. elegans* that are similar to human lysosomal lipase [26]. Intriguingly, of these five lipases, *lipl-1*, *lipl-3*, and *lipl-4* are also upregulated in *daf-2(e1370)* mutants according to our profiling. *lipl-1* is an A-specific target upregulated 100-fold in *daf-2(e1370)*, but *lipl-1* mutation did not have any effect on longevity. For comparison to *lipl-2*, we noted that *lipl-3* and *lipl-4* are shared DAF-16A/F targets, and thus we tested *lipl-3* and *lipl-4* but found no effect. The lipases *lipl-3* and *lipl-4* are only DAF-16A/F targets in two contexts, and are shared more equally between DAF-16A and DAF-16F compared to *lipl-2*, both of which we had reasoned would make them less likely to influence longevity. *lipl-2*'s stronger influence on longevity supports the notion that our two strategies helped to identify the specific lipase that promotes longevity in animals with reduced IIS.

T02B5.1, another shared DAF-16A/F target that reduces life span extension by 49% when mutated ($p < 0.0001$; Figure 5.5C), is most similar to human cocaine esterase and acetylcholinesterase. *T02B5.1* possesses a carboxylesterase domain that is 24% identical to the acetylcholinesterase of Pacific electric ray *Torpedo californica,* including conserved catalytic residues [27]. We also utilized a second allele of *T02B5.1* that truncates the C-terminus and likely causes weaker loss-of-function, and this reduced life span extension 16%, adding further evidence that *T02B5.1* promotes longevity. While it is a shared target of DAF-16A/F, *daf-16a* mutation has a significantly stronger effect on its expression, and *daf-16f;daf-2* still expresses *T02B5.1* at nearly 100-fold greater levels

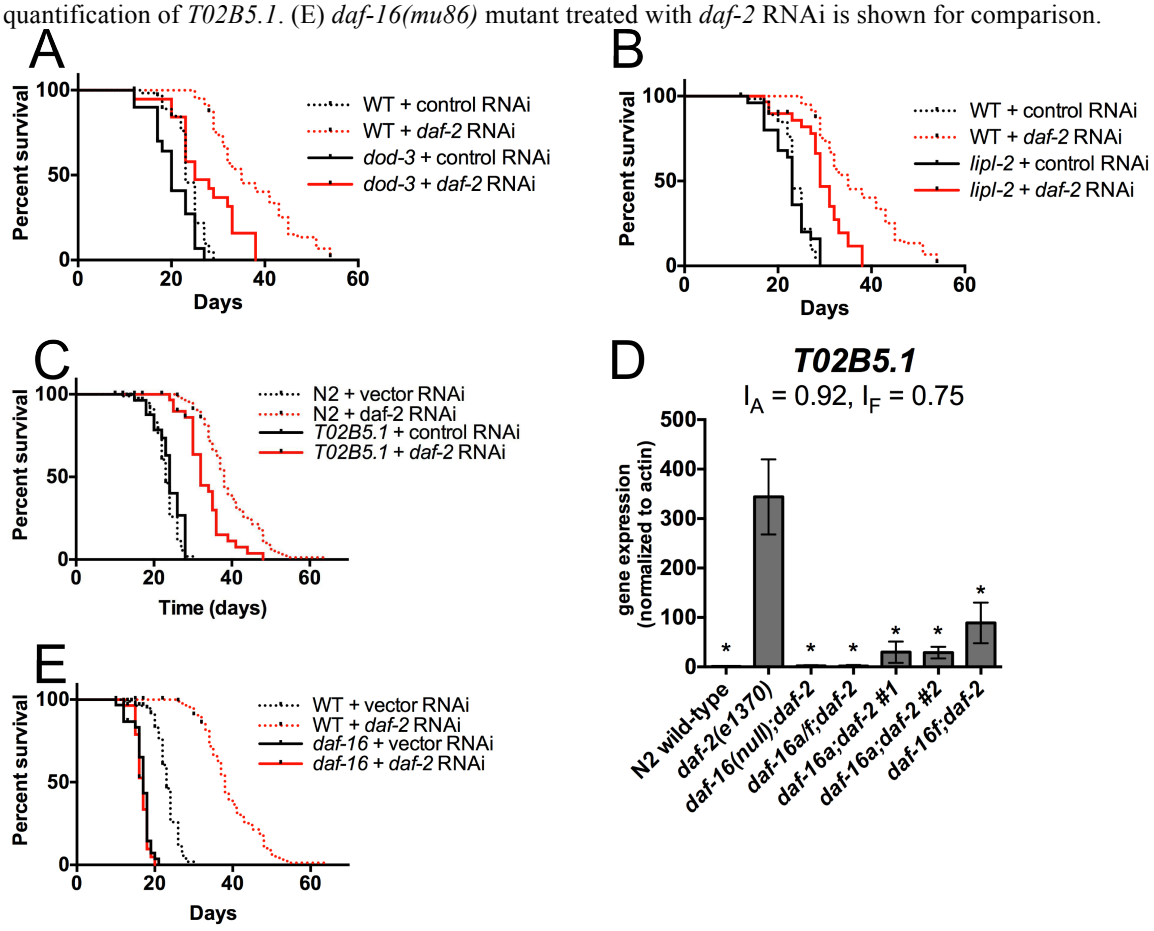
than wild-type (Figure 5.5D). Therefore, *T02B5.1*'s effect on longevity is consistent with the pattern that stronger regulation by DAF-16A is a predictor of a gene's role in life span control.

Some DAF-16A/F shared target genes such as *sod-3*, *mtl-1*, *icl-1*, and *glb-1* previously identified as having a role in life span control [17] had minor effects in our experiments (Figure 5.3A). Another three shared genes, *Y6G8.2*, *cyp-34A10*, and *C07A4.2* also had minor effects (Figure 5.3A). Their effects were much less significant than those of A-specific target genes and other A/F shared targets identified by our strategy based on hierarchical DAF-16 isoform action.

In sum, we identified 10 shared DAF-16A/F targets (out of 28 tested) that influence life span, including two novel longevity genes *lipl-2* and *T02B5.1* with major effects. Taken together with our A-specific targets (Figure 5.4), many genes are likely to contribute to *daf-2/IGFR* longevity, but a few play a disproportionate role.

Figure 5.5. Survival curves of shared DAF-16A/F target genes with the largest effects on life span.

(A-C) Effects of the three DAF-16A/F shared targets with the largest influences on life span. (D) qPCR quantification of *T02B5.1*. (E) *daf-16(mu86)* mutant treated with *daf-2* RNAi is shown for comparison.



Discussion

In this study, we employed a logical strategy to prioritize specific DAF-16/FoxO target genes for functional analysis. The results broadly suggest that regulation by DAF-16A is a major predictor of a gene's role in life span extension, and shared regulation by DAF-16A and DAF-16F is a secondary predictor (Figure 5.3, Figure 5.4, Figure 5.5). This is consistent with the life span phenotypes of isoform-specific *daf-16/FoxO* mutants, where DAF-16A is the dominant isoform, but DAF-16F is a secondary isoform that promotes longevity in the absence of DAF-16A. In particular, we discovered that the DAF-16A-specific target genes *hsp-12.3* and *sodh-1* are fully required for the longevity of *daf-2*-RNAi-treated animals (Figure 5.4A-B).

We also found a surprising lack of correlation between RNAi and mutation under similar laboratory conditions (Figure 5.2C). Our result is similar to a study in zebrafish concerning morpholinos, analogous to RNAi, that showed that mutation failed to validate 80% of morpholino-induced phenotypes [28]. This has major implications for understanding the contribution of individual DAF-16/FoxO target genes to longevity. RNAi is prone to off-target effects that could lead to false positives (*i.e.*. RNAi alters life span but mutation does not). Furthermore, RNAi may result in incomplete knockdown of mRNA and protein levels, especially in neurons and pharynx that are refractory to RNAi, resulting in false negatives (*i.e.* mutation alters life span but RNAi does not). Thus, critical longevity genes may be missed by RNAi.

sodh-1, *hsp-12.3*, and *T02B5.1* illustrate the potential advantages of mutation compared to RNAi. Previous studies report that *sodh-1* RNAi causes a 5-10% reduction in *daf-2* mutant, while *hsp-12.3* RNAi does not affect *daf-2* longevity [17,18]. We found an ~ 5% increase in life span following *T02B5.1* RNAi treatment in *daf-2* and germlineless mutants (Figure 5.2A). However, promoter reporters indicate that *sodh-1* is expressed in *daf-2* neurons [29] which is refractory to RNAi. qPCR indicates that *hsp-12.3* is upregulated 50-fold [18] and *T02B5.1* is upregulated 200-fold in *daf-2(e1370)* (Figure 5.5D), but the efficiency of *hsp-12.3* and *T02B5.1* RNAi is not known. Therefore, our

loss-of-function mutations likely reduced function of *sodh*-1, *hsp-12.3*, and *T02B5.1* far more than RNAi is capable of, and therefore had a much stronger effect on life span.

RNAi may also have led to false positives. *lipl-4* RNAi was previously shown to partially reduce the life span extension of *daf-2* mutants [30], but we did not observe any effect by mutation (Figure 5.3A). The *lipl* family lipases are likely to influence longevity, as *lipl-4* over-expression extends wild-type life span and *lipl-4* RNAi eliminates the longevity of animals lacking a germline [30,31]. However, we note that there are multiple *lipl* genes upregulated by *daf-2*. Thus, it is plausible that *lipl-4* RNAi actually reduces the levels expression of multiple *lipl* genes, which explains why *lipl-4* mutation has no effect on the longevity of *daf-2*-RNAi-treated animals (Figure 5.3A). Given that *lipl-2* is a shared DAF-16A/F target gene that contributes to *daf-2*-RNAi-mediated longevity (Figure 5.5B), previously observed effects of *lipl-4* RNAi may instead by attributable to *lipl-2*.

One question is why mutations in single upregulated DAF-16A-specific targets, such as *sodh-1* and *hsp-12.3*, appear to reduce longevity more than *daf-16a* mutation itself (Figure 5.4A,B,G). First, DAF-16A-specific targets were chosen based on an arbitrary cutoff whereby *daf-16a* mutation has at least 80% of the effect of *daf-16a/f* combined mutation, and *daf-16f* mutation has less than 20% of the effect (see Chapter 4). Second, neither *daf-16* null nor *daf-16a* mutation fully eliminates expression of these genes. Therefore, many of the DAF-16A targets are still expressed and may be slightly upregulated in *daf-16a;daf-2* mutants compared to wild-type. This maintained expression is likely sufficient to mediate some life span extension in *daf-16a;daf-2* mutants.

Our results are still consistent with the idea that many gene expression changes exert cumulative effects on life span to produce the dramatic longevity of *daf-2/IGFR* mutants [3,17]. In particular, previously identified genes such as *sod-3*, *mtl-1*, and *icl-1* involved in processes such as stress resistance and metabolism do indeed have small effects on life span (Figure 5.3A). However, our results indicate that among all DAF-16/FoxO target genes with roles in life span control, a small fraction are particularly important. It will be

interesting to determine to what extent those few gene expression changes alone can promote longevity.

It is highly likely that critical genes still remain to be identified. We have tested about one-third of genes suggested by our DAF-16 isoform analysis, because strong loss-of-function mutants are not available for the majority of our genes. Nevertheless, our analysis indicates that DAF-16/FoxO target genes absolutely required for *daf-2/IGFR* longevity exist, and that further testing and detailed functional analysis of genes that are regulated primarily by DAF-16A or jointly by DAF-16A/F should be prioritized. The gene regulatory events we have identified constitute protective functions of FoxO transcription factors. As many of the genes are conserved and have major effects on life span, they may be used as part of a strategy to manipulate FoxO outputs to prevent or ameliorate age-related disease.

Materials and Methods

C. elegans strains and maintenance

A complete list of strains used is found in Table 5.1. Animals were maintained at 15°C on nematode growth media (NGM) plates seeded with *Escherichia coli* OP50. Mutations with effects on life span were confirmed by PCR amplification to detect restriction fragment length or PCR polymorphisms. Percival I-36NL incubators (Percival Scientific, Inc., Perry, IA) were used for maintenance and life span assays.

Life span assays

Life span assays were performed as previously described [79], with minor modifications. Animals derived from a synchronized 4 hr egglay were grown at 15°C until the L4 larval stage and then shifted to 20°C. Plates harboring any males were discarded. Animals were grown for an additional 20-24 hr to day 1 of adulthood and then placed on life span plates containing 25μg/mL 5-fluoro-2'-deoxyuridine (FUDR; Sigma-Aldrich, St. Louis, MO) to prevent progeny growth. *glp-1* mutant animals were raised at the restrictive temperature to ablate the germline. Statistical significance was assessed using the standard chi-square-based log-rank test in GraphPad Prism.

RNAi

All RNAi NGM plates contained 5mM IPTG and 25μg/mL carbenicillin. NGM plates were seeded with a 5X concentrated overnight culture of *E. coli* HT115 with either control L4440 vector or RNAi plasmid. Plasmids from *E. coli* clones were sequenced for every experimental replicate to confirm their identity.

Table 5.1. List of loss-of-function mutants used in Chapter 5.

gene	allele
daf-2	*e1370*
daf-2	*e1368*
glp-1	*e2141*
daf-16	*mu86*
daf-16	*mg54*
daf-16	*tm5030*
daf-16	*tm5032*
daf-16	*tm6659*
acdh-2	*gk143151*
btb-16	*gk470241*
C07A4.2	*gk820370*
C08E8.10	*gk356583*
clec-190	*gk746445*
cpg-7	*ok3141*
cth-1	*ok3319*
ctl-3	*ok2042*
cutl-24	*gk191483*
cyp-34A10	*gk761632*
dod-3	*gk909808*
F15B9.6	*gk675436*
F26A1.8	*gk639772*
F26C11.1	*gk635549*
F35E12.5	*ok3418*
ftn-1	*ok3625*
glb-1	*ok2747*
glt-5	*bz70*
gst-20	*gk604858*
gst-29	*gk500211*
hen-1	*tm501*
hsp-12.3	*ok3095*

gene	allele
icl-1	*gk225172*
K02E11.7	*ok3588*
K08D10.14	*ok2976*
lipl-1	*gk832360*
lipl-2	*tm4324*
lipl-3	*gk224717*
lipl-4	*gk237517*
lips-5	*gk793539*
lys-7	*gk230857*
lys-7	*ok1384*
lys-7	*ok1385*
mtl-1	*tm1770*
oac-5	*gk398429*
pho-7	*gk658979*
R06F6.7	*gk153756*
rgs-10	*ok1039*
sod-3	*tm760*
sodh-1	*ok2799*
sprr-2	*ok3290*
srp-3	*ok1433*
srr-4	*gk779731*
T02B5.1	*tm5130*
T02B5.1	*gk854495*
T12D8.5	*gk829473*
ugt-11	*gk497718*
ugt-32	*gk231667*
Y20C6A.1	*gk476820*
Y6G8.2	*gk707403*
ZC196.2	*gk242000*

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Chapter 6 Conclusions

Overview

The goal of this dissertation was to understand the mechanisms by which DAF-16/FoxO influences the aging process and extends life span in *C. elegans*. While much is known about the numerous upstream pathways that regulate FoxO transcription factors, the downstream effects appear to be even more complex, making it difficult to correlate FoxO activation with phenotypic outputs. DAF-16/FoxO regulates other phenotypes that can be fully or partially uncoupled from longevity [1], implying that many DAF-16/FoxO target genes may be unrelated to longevity. Given that DAF-16/FoxO regulates thousands of genes, a coherent conceptual framework predicting how these genes relate to life span is needed.

In Chapters 2 through 4 of this dissertation, we employed different strategies to develop this conceptual framework and parse DAF-16/FoxO action into distinct transcriptional programs. Importantly, some of these programs promote longevity while others do not. In Chapter 5, we rigorously tested the genes found in the pro-longevity modules and discovered critical targets.

SGK-1 and AKT-1 act in opposition to control longevity

Sgk and Akt are similar kinases that both capable of phosphorylating three conserved FoxO protein RxRxxS/T motifs that determine FoxO subcellular localization and therefore activity. Some observational data in the literature suggested that *C. elegans* SGK-1 and AKT-1 have opposing effects on life span. This offered an opportunity to

discover FoxO transcriptional programs that diverge under Sgk and Akt signaling regimes in a conserved manner.

We sought to clarify the relationship between SGK-1, DAF-16/FoxO, and longevity. Using mutations in *sgk*-1 we found that SGK-1 promotes longevity in a DAF-16/FoxO-dependent manner. Hypothesizing that SGK-1 activates DAF-16/FoxO, we examined known indicators of DAF-16/FoxO activation. In contrast to AKT-1 which inhibits DAF-16/FoxO in a straightforward manner, we found mixed results with SGK-1 and discovered important SGK-1 outputs independent of DAF-16/FoxO. Thus, while SGK-1 promotes longevity, its relationship with DAF-16/FoxO is far more complex.

Future studies can further clarify SGK-1's influence on DAF-16/FoxO by focusing on the molecular mechanism by which SGK-1 and AKT-1 differentially regulate DAF-16/FoxO activity. Potentially, the divergence could be explained by the affinities of SGK-1 and AKT-1 for distinct RxRxxS/T motifs on DAF-16/FoxO. This would require refinement of the current model where phosphorylation of these sites inhibits DAF-16/FoxO transcriptional activity. Alternatively, SGK-1 and AKT-1 could differentially influence other transcriptional regulators that collaborate with DAF-16/FoxO.

For our purposes, the discovery that SGK-1 has DAF-16-independent effects on outputs normally attributed to DAF-16/FoxO activation greatly complicates the interpretation of DAF-16/FoxO transcriptional activity under AKT-1 and SGK-1 signaling regimes. Furthermore, it was later discovered by the Blackwell group that SGK-1 acts in two opposing pathways to control longevity, the balance of which depends on temperature and other environmental factors [2]. SGK-1 inhibits SKN-1/Nrf, another DAF-2-responsive transcription factor, at high temperatures to limit longevity [2]. However, at lower temperatures SGK-1 activates DAF-16/FoxO to promote longevity [3]. For future studies, it is an important goal to elucidate how SGK-1 and DAF-16/FoxO influence longevity in different environmental contexts and in collaboration with other transcription factors. However, the problem remains that it is still unclear how DAF-16/FoxO itself promotes longevity in a single context.

These novel insights into SGK-1 unnecessarily complicate efforts to identify DAF-16/FoxO target genes that influence life span. Therefore, we pursued other lines of inquiry that could untangle DAF-16/FoxO's complex mechanism of action.

DAF-16/FoxO isoforms control distinct target genes

In Chapters 3 and 4, we tested the hypothesis that DAF-16/FoxO isoforms control distinct sets of target genes and make differential contributions to longevity. After discovering that this is true, we used DAF-16/FoxO isoform-specificity as the basis for our strategy to prioritize target genes for rigorous functional testing in Chapter 5.

Isoform-specific alleles: Novel genetic tools to study longevity

Much of the work in this dissertation relied on our isolation of the first isoform-specific alleles of DAF-16/FoxO that specifically eliminate the function of one or more isoforms encoded by the *daf-16* genomic locus. In theory, these alleles could be used to uncouple different outputs of this pleiotropic transcription factor. However, it was initially unknown if cross-isoform interactions, genomic locus complexities, isoform redundancy or other unforeseen caveats would prevent useful interpretation.

For example, the *mg54* allele of *daf-16* was predicted to specifically eliminate DAF-16A and DAF-16F function, and DAF-16B does not promote longevity. Therefore, comparing *mg54* to *daf-16* null mutation could theoretically isolate DAF-16B functions that could then be ignored in rigorous testing of target genes.

Chapter 3 provided the required proof-of-principle that different genetic alleles of *daf-16* could be used in a coherent fashion to dissect DAF-16/FoxO actions. When introduced into long-lived strains with active DAF-16/FoxO, *mg54* alters gene expression in a qualitatively distinct manner compared to the null allele *mu86*. In particular, many Class 2 (downregulated by DAF-16) and some Class 1 (upregulated by DAF-16) genes were affected by *mu86* but not *mg54* mutation. This result held true in three separate strains,

each with unique signaling regimes and genetic backgrounds. Therefore, *mg54* serves as a robust genetic filter.

Chapter 4 introduced isoform-specific alleles that specifically eliminated either DAF-16A or DAF-16F. We characterized all the alleles in detail. Importantly, transcript levels and sequences of intact isoforms remained the same, mitigating concerns that interactions between isoforms would complicate life span and gene expression data. Furthermore, two separate *daf-16a* alleles produced identical effects on phenotypic outputs and gene expression, demonstrating reproducibility. Finally, a separate analysis of DAF-16A-specific targets identified in the context of *daf-2/IGFR* mutation demonstrated that they are also depend on DAF-16A in the context of germline ablation. Taken together, these isoform-specific alleles are highly valuable tools to understand how DAF-16/FoxO promotes longevity.

We also note that isoform-specific alleles have many advantages, and few disadvantages, compared to previous methods to study DAF-16 isoforms. Previous studies primarily relied on transgenic animals over-expressing individual DAF-16 isoforms [4,5]. Using single-copy transgenes, we found that simply doubling the gene dosage of DAF-16F exaggerates its role. Therefore, quantitative assessments of isoform contributions to longevity cannot be made using multi-copy transgenic animals. Isoform-specific mutations maintain all of the DNA regulatory elements that normally function to control synthesis of *daf-16* transcripts. We found that *daf-16a* transgenes do not rescue *daf-16a* isoform-specific mutation, suggesting that those transgenes are missing key regulatory elements. Transgenes do have the advantage of fluorescent tags allowing for visualization of tissue expression, but if they are over-expressed or missing key regulatory elements then the interpretation is limited. These differences likely explain why our results differ from the previously published finding that DAF-16F plays a stronger role than DAF-16A in life span control.

In summary, we characterized the first truly isoform-specific alleles of DAF-16/FoxO and demonstrated their utility. With the advent of facile CRISPR genome engineering in

C. elegans [6], many more useful *daf-16* alleles can be created that alter specific sequence elements of each isoform or introduce experimentally useful elements such as fluorescent tags. We used the isoform-specific alleles to dissect the mechanism of life span extension, but these reagents will also be useful for studying other processes modulated by DAF-16/FoxO, including development, reproduction, behavior, cell fate plasticity, and tumor growth [7–10]. It will be interesting to exploit these alleles to prioritize testing of critical targets in these contexts, similar to our strategy for life span control.

Hierarchical action of DAF-16/FoxO isoforms in longevity control

In Chapters 3 and 4, we employed isoform-specific *daf-16* alleles to reveal hierarchical action of DAF-16 isoforms in life span control, in this order: DAF-16A > DAF-16F > DAF-16B. This pattern was robust across three separate contexts where DAF-16/FoxO promotes longevity: *daf-2/IGFR* ligand-binding domain mutation, *daf-2/IGFR* tyrosine kinase domain mutation, and germline ablation. Our findings are consistent with previous work showing that DAF-16A and DAF-16F are both capable of promoting longevity, while DAF-16B is not [4,5]. However, the isoform-specific alleles made it possible to assess the endogenous contributions of *daf-16* isoforms to longevity and determine that DAF-16A plays a stronger role than DAF-16F.

We used whole-transcriptome profiling (RNA-seq) to elucidate the basis for hierarchical action of isoforms. We made conventional comparisons between transcriptomes to define general DAF-16 targets, and employed the *daf-16a/f* allele to filter out DAF-16B targets. We then categorized the genes using A-indices and F-indices calculated *in silico* that reflect each gene's relative regulation by DAF-16A and DAF-16F. We discovered that there are far more A-specific genes than F-specific genes, and the majority of shared genes show stronger regulation by DAF-16A. Importantly, the fact that DAF-16A exerts a stronger effect on global transcriptional regulation correlates with the isoforms' relative contributions to longevity.

It is important to note that our A-specific, shared, and F-specific targets are actually part of the same continuous spectrum, and the precise criteria for classifications are necessarily arbitrary. We clearly ruled out the possibility that DAF-16A and DAF-16F regulate fully distinct sets of genes. More intriguingly, we shed light on overlapping functions of DAF-16A and DAF-16F. When expression changes of all target genes are plotted according to regulation by DAF-16A or DAF-16F relative to DAF-16A/F together, the genes span the full spectrum from 0% to 100% regulation by each isoform. There is no straightforward relationship between a given gene's regulation by DAF-16A and its regulation by DAF-16F. This has implications for interpreting experiments in mammalian systems where FoxO isoforms are knocked out. It will be necessary to compare all possible combinations of single and multiple mammalian FoxO gene knockouts to determine which isoforms preferentially regulate which genes.

Open questions concerning hierarchical action by DAF-16/FoxO isoforms

Many questions remain concerning the molecular basis for hierarchical *daf-16* isoform action. Why do target genes vary in their regulation by DAF-16A and DAF-16F? It is unlikely that differences in direct DNA binding are responsible, because the DAF-16A and DAF-16F forkhead DNA binding domains are identical. Furthermore, DAF-16A, FoxO1, FoxO3, and FoxO4 all vary in their forkhead domains, yet bind the same DNA motif *in vitro* [11]. Another factor could be distinct spatial or temporal expression, partially explained by distinct promoters. However, RNA-sequencing reads suggest that production of some *daf-16a* transcripts may be controlled by the canonical *daf-16f* promoter, and some *daf-16b* transcripts by the *daf-16a* promoter, suggesting the overlap in isoform expression may be greater than originally thought. DAF-16A and DAF-16F are expressed in nearly all the same tissues [4], especially the neurons, intestine and hypodermis which are critical for DAF-16/FoxO-mediated longevity [12,13], thus arguing against tissue-specificity as a differentiating factor.

A promising unexplored hypothesis is that the unique N-termini of DAF-16A and DAF-16F confer association with distinct binding partners. Numerous transcriptional co-

regulators work in collaboration with FoxO transcription factors [1,14,15]. One potential experiment is to perform an RNAi screen of all transcriptional regulators, and identify those genes whose knockdown affects phenotypes only in the *daf-16a* mutant or only the *daf-16f* mutant. DAF-16A-specific and DAF-16F-specific genes may also be measured, and the A-index and F-index can be exploited for investigating any determinant of isoform-specific action. Those regulators that influence critical DAF-16A and shared DAF-16A/F life span targets should be prioritized for further study.

Interestingly, there are hints that DAF-16 isoforms compete with or antagonize each other, consistent with having both unique and overlapping roles. We have separately found that a *daf-16b*-specific mutant is long-lived compared to wild-type, suggesting that DAF-16B may actually oppose life span. In Chapter 3, we noticed that DAF-16B appears to downregulate Class 2 genes, raising the possibility that these Class 2 genes normally function to promote longevity, which is contrary to the model that Class 2 genes inhibit longevity [16]. Alternatively, DAF-16B may inhibit other isoforms, either through direct competition or by modulating signaling feedback loops. Transcriptional profiling of the *daf-16b* mutant would be an appropriate starting point for further experiments. Also, a life span assay performed at a lower temperature (15°C) revealed that *daf-16f* mutation extends *daf-2* longevity under those conditions. In contrast, *daf-16a* mutation has no effect, and *daf-16a/f* combined mutation fully suppresses longevity. The simplest explanation is that DAF-16F acts as a partial agonist that is sufficient for life span extension but antagonizes DAF-16A when both isoforms are present. Observing these potential interactions was made possible by the isoform-specific alleles. Future studies may clarify the nature of cross-isoform interactions and determine whether or not mammalian FoxO1, FoxO3, FoxO4, and FoxO6 share these mechanisms.

In summary, the isoform-specific alleles of *daf-16/FoxO* have opened up many new lines of inquiry. Ultimately, the hierarchical action of DAF-16/FoxO isoforms should be integrated with other known aspects of DAF-16/FoxO biology.

Identification of critical DAF-16/FoxO longevity targets

In Chapters 3 and 4, we identified DAF-16A/F target genes in multiple long-lived strains and further dissected those genes into A-specific, shared, and F-specific genes. With this information in hand, in Chapter 5 we proceeded to the practical consideration of how to prioritize and perform functional testing of these genes. To assess the role for a given gene in longevity, we disrupt the gene's function and determine its effect on life span.

Method of functional testing: Mutation versus RNAi

One of the most important reasons why we need rational prioritization of target genes is that null mutation, the "gold standard" method of functional analysis in *C. elegans*, is more laborious and less readily available than RNA interference (RNAi) [17]. Less than 10% of target genes have been tested by RNAi for effects on life span, and only a handful have been tested by mutation.

All published experiments that functionally tested significant numbers (dozens) of targets utilized RNAi [16,18–20]. This was a practical necessity, as loss-of-function alleles of most genes had not yet been isolated. However, the percentage of RNAi clones altering life span by off-target effects is unknown (false positives). In theory, this could be mitigated by using multiple RNAi clones, or by directly comparing RNAi clones knocking down DAF-16/FoxO targets with random RNAi clones. Furthermore, incomplete knockdown of mRNA and protein levels by RNAi, especially in neurons and pharynx which are refractory to RNAi, means that critical longevity genes may be missed (false negatives).

We screened a subset of genes by RNAi before all transcriptome profiling was completed. When we later compared these results to mutation of the same genes, we found very little correlation between RNAi and mutation. This result is reminiscent of a study in zebrafish concerning morpholinos, analogous to RNAi, that showed that 80% of morpholino-induced phenotypes were not recapitulated by mutation [21]. Previous studies have shown discordant results between RNAi and mutation for the superoxide

dismutase *sod-3* and the fatty acid desaturase *fat-7* [18,22,23]. These results suggest that the number of false positives by RNAi is very high, and testing many genes may only exacerbate the problem of off-target effects.

At the time of this writing, isolating loss- and gain-of-function mutations is becoming far less time-consuming and laborious. Initiatives such as the Million Mutation project, the *C. elegans* Knockout Consortium, and the National Bioresource Project are concerted efforts to ultimately generate alleles of every single gene [24–26]. This greatly reduces the need for individual research groups to generate alleles. Furthermore, custom mutations can now be made using the CRISPR technique [6]. Mutation analysis is still more laborious because of the genetic crosses required to reduce background mutations and obtain combination mutants, but the advantages relative to RNAi-based analysis are worth the effort.

To test our genes, we circumvented the labor involved in constructing combination mutants by subjecting strains with DAF-16 target gene mutations to *daf-2* RNAi. *daf-2* RNAi is robust, validated, and used by many research groups, whereas many other RNAi clones have only been tested as part of genome-wide screens and have never been validated. When possible, we utilized multiple alleles from different genetic backgrounds to reduce the chance of false positives due to background mutations. To reduce the chances that the animals are short-lived because they are simply sick, we measured animal life span on control RNAi and observed the animals for obvious developmental effects. At the time of this writing, we are in the process of even more rigorous life span experiments utilizing combination mutants with *daf-2* loss-of-function alleles. Thus, it is feasible to obtain interpretable data on the life spans of large numbers of mutants.

Now that isolation of *C. elegans* mutants is commonplace and straightforward, it will be interesting to see how many phenotypes attributed to RNAi gene knockdown will be confirmed by mutation. Drawing on our work in Chapter 4 using isoform-specific RNAi to treat isoform-specific mutants, we note that RNAi and mutation can and should be combined as part of a concerted strategy, especially when genes have overlapping roles.

However, mutation is the "gold standard" to assess gene function by reverse genetics. Given that the current model that DAF-16 controls many genes to influence longevity is based on RNAi data, the fact that mutation produces different results for many genes may lead to revision of this model.

Prioritization of target genes according to DAF-16/FoxO isoform regulation

We developed a rational approach to prioritizing target genes for functional testing based on hierarchical action of DAF-16 isoforms in longevity control. Given that *daf-16a* mutation shortens life span while *daf-16f* mutation does not, it follows that DAF-16A-specific targets are very likely to influence longevity. Thus, we chose to test all DAF-16A targets where reagents were readily available. The life span data also strongly suggests that shared targets contribute to longevity, and we prioritized those shared genes that are redundantly regulated by DAF-16A and DAF-16F, and those that were also identified as DAF-16A/F targets in the multiple contexts (Chapter 3).

The results of testing individual genes by mutation broadly validated our strategy. A-specific genes had the greatest effect on life span, while shared genes also contributed. This implies that the reason *daf-16a* is required for maximal life span extension by *daf-2/IGFR* is because certain A-specific genes are required (*hsp-12.3* and *sodh-1*; Figure 6.1). On the other hand, intact *daf-16f* can still partially extend life span in the absence of *daf-16a* because some shared *daf-16a/f* targets promote longevity (*T02B5.1* and *lipl-2*; Figure 6.1). Given that DAF-16A and DAF-16F may compete, it will be interesting to determine if *daf-16f*-regulated targets become more important for the longevity of *daf-16a* mutants, and vice versa.

However, we do not yet know if we enriched for longevity genes by prioritizing those shared genes that are DAF-16A/F targets in three contexts (two separate *daf-2* mutations and germline ablation). This would require comparing targets regulated by DAF-16A/F in only one or two context with those regulated in all three contexts.

It does appear that prioritizing genes redundantly regulated by DAF-16A/F did not enrich for longevity genes, as we only identified one redundant gene with a minor effect. By definition, redundant genes are those do not change with *daf-16a* and *daf-16f* mutation, but do change significantly with combined *daf-16a/f* mutation. Indeed, a separate analysis of germline-ablated animals showed that expression of these genes do change with *daf-16a* mutation alone in that context, suggesting that redundantly regulated genes do not necessarily comprise a biologically significant class. In contrast, A-specific genes were equally affected by *daf-16a* and *daf-16a/f* mutation in germline-ablated animals, as they were in *daf-2* mutants. This suggests there exists a unique mechanism to regulate A-specific genes, but not redundant genes.

The most limiting criteria for our analysis was availability of mutants. We only tested loss-of-function mutants that already exist, a criterion that applied to approximately one-third of the genes we identified. It is almost certain that we have missed critical longevity genes. Therefore, the most obvious next experiment is to utilize CRISPR [6] to generate loss-of-function mutations for the rest, prioritizing those genes with high degree of regulation by DAF-16A.

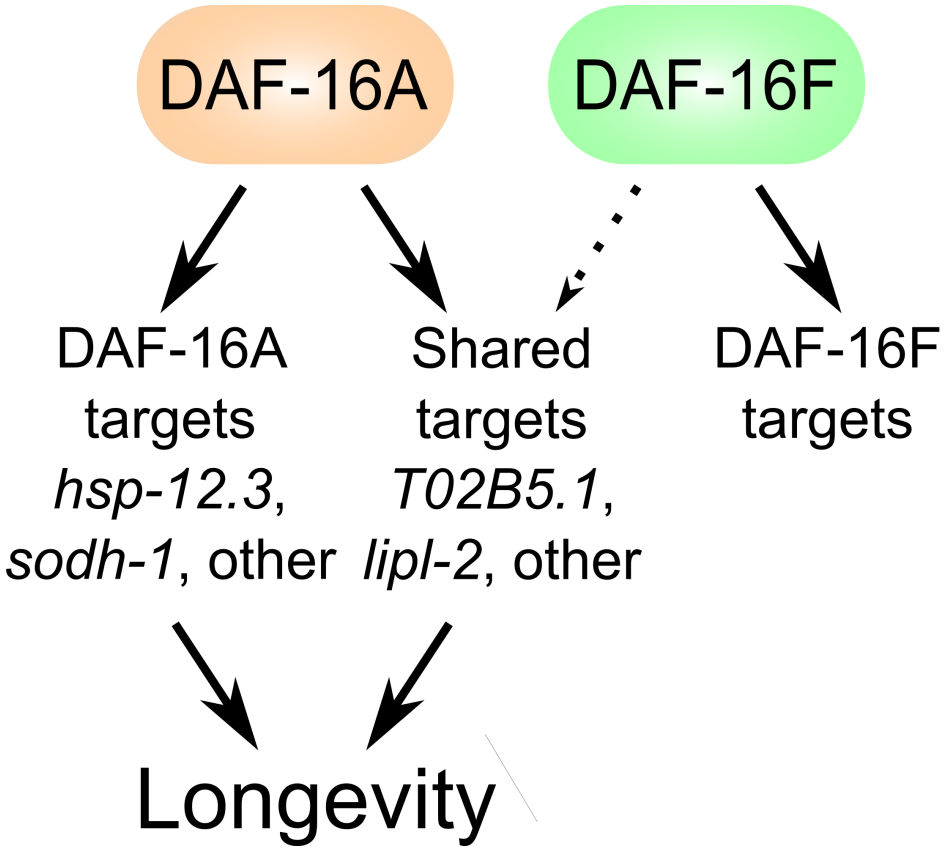


Figure 6.1. Model: Role of DAF-16A/F target genes in life span extension.

Open Questions

This dissertation is a step forward in deciphering the mechanism of DAF-16/FoxO-mediated life span extension. We identified hierarchical action by DAF-16 isoforms as a conceptual framework that can predict whether a given target gene is likely to influence longevity or not. We discovered that DAF-16/FoxO does regulate a small set of genes with disproportionate effects on longevity, including a handful that are almost fully required for life span extension. Future studies will shed light on the mechanism of life span extension by these genes, as well as the mechanisms of aging itself.

Future experiments to define the mechanism of DAF-16/FoxO-mediated longevity

We demonstrated that DAF-16/FoxO isoforms make differential contributions to longevity because they regulate distinct target genes. The next major question is: how are each of these genes acting? Many *C. elegans* gene functions have been predicted simply by homology to more well-studied genes. This previously proved useful when studying broad processes influenced by DAF-16/FoxO, such as metabolism or oxidative stress resistance. However, understanding the mechanism of life span extension by individual genes will require a more detailed analysis of function.

Hopefully, elucidating basic characteristics of these genes such as their molecular functions and expression patterns will yield novel lines of inquiry into the mechanisms of aging. Those experiments are highly gene-dependent and will not be discussed in detail here. However, some experiments apply to all the genes.

First, the role of individual target genes in control of aging and longevity should be further defined. Most of the genes we identified are Class 1 genes that upregulated by DAF-16/FoxO and are required for full life span extension due to reduced DAF-2/IGFR signaling, so this discussion will focus on those genes. It should be determined whether artificially increasing the function of these genes is sufficient for life span extension. This experiment would also provide additional evidence that mutation does not simply prevent life span extension due to general frailty or sickness. Furthermore, the role of the genes in

general longevity assurance can be assessed by mutating the genes in other longevity models, including altered mitochondrial function, dietary restriction, and reduced TOR (target of rapamycin) signaling [27–29].

Second, it will be interesting to understand how these individual genes might work together to extend life span. Combination mutants could reveal if the genes work in common or independent pathways. Ultimately, a set of DAF-16 target genes can be artificially manipulated in *daf-16* null mutants to match their expression in *daf-16* wild-type animals. If those animals are long-lived as if DAF-16 were activated, then that set of genes may be the most important for DAF-16/FoxO-mediated life span extension.

Third, the effect of these genes on markers of age-related decline should be assessed, as we have only measured life span in our studies. Analysis of aged *C. elegans* has revealed numerous changes that are reminiscent to those in elderly humans, including reduced movement, sarcopenia (muscle loss), immunosenescence (reduced resistance to infection), reduced fertility, pathogenicity caused by commensal or symbiotic bacteria, and reduced stress resistance [9,30–34]. Importantly, many of these changes are greatly delayed or attenuated by *daf-2/IGFR* mutation. This maintained youthfulness in aged *daf-2* mutants is often termed extended "health span." As the causes of death in *C. elegans* are not well-studied, it is possible that many of the changes that occur in aged *C. elegans* might not contribute to mortality, at least under laboratory conditions. In that case, many of the gene regulatory events that we identified may promote extended health span, but not longevity. It will be interesting to determine the extent of overlap between DAF-16/FoxO target genes that promote health span and those that promote longevity.

Implications for aging and longevity

Given that aging does cause numerous changes in an organism, how can a single DAF-16/FoxO target gene such as *sodh-1* or *hsp-12.3* have a major effect on longevity? It is more intuitive that dramatic life span extension requires alterations in many biological

processes each involving many genes. There are several possible explanations for how a small set of genes can have a disproportionate effect on longevity (Figure 6.2).

A straightforward explanation is that these genes might be involved in the regulation of many other genes, a model that applies to the *daf-2* and *daf-16* genes themselves. Indeed, it has been hypothesized that DAF-16/FoxO acts as a "regulator of regulators" [35], and secondary regulators may play a disproportionate role in life span control. Another explanation is that target genes might be required for full DAF-16/FoxO function, acting in a positive feedback loop. Previous experiments indicate that DAF-16/FoxO targets *aakg-4* and *tcer-1* bolster DAF-16/FoxO transcriptional activity [36,37]. However, these explanations are not readily suggested by annotated functions of *sodh-1* and *hsp-12.3*.

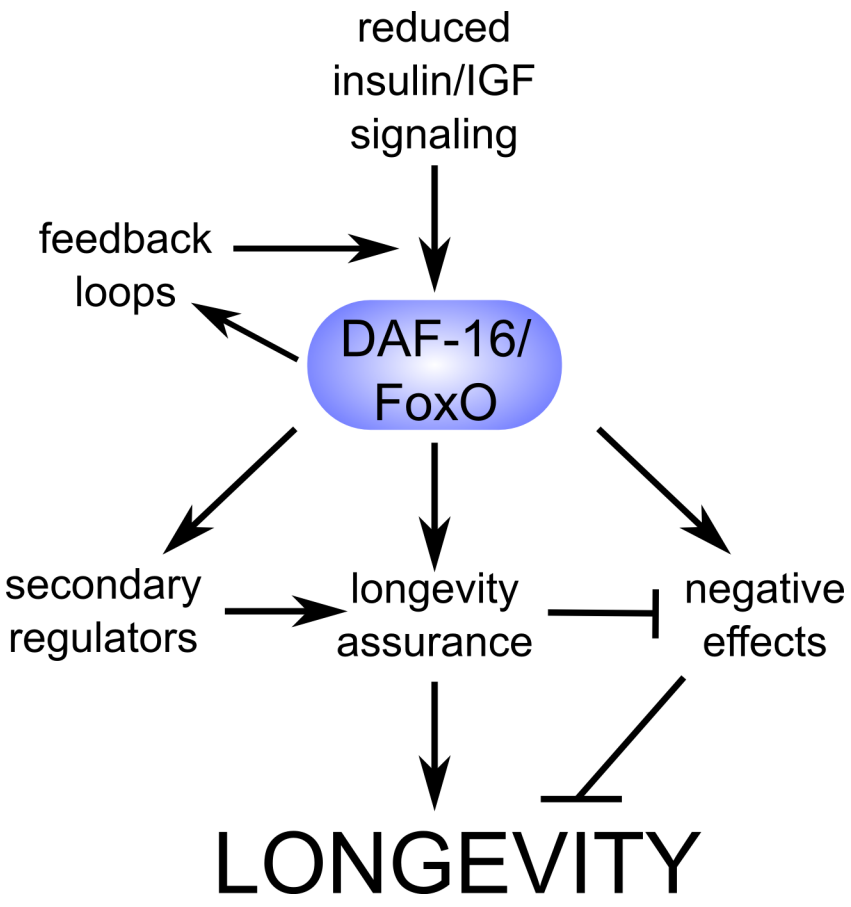
Another possible explanation is that genes like *sodh-1* and *hsp-12.3* are directly affecting the major causes of mortality. It is not clear what aged worms die of, and it is possible that only a few causes of death are relevant in the laboratory setting. In that case, the majority of DAF-16/FoxO target genes might instead function to delay death in the wild where animals more frequently die of external threats such as predators and extreme environmental conditions. In contrast, in the laboratory it is plausible that a handful of target genes protect against a handful of causes of death, and therefore those are the genes that are strongly required for longevity.

Perhaps the most interesting (though speculative) possibility is that *sodh-1* and *hsp-12.3* protect against the negative consequences of DAF-16/FoxO activation. It seems unlikely that all changes effected by DAF-16/FoxO are neutral or beneficial for longevity assurance. One open question in the aging field is why "reduced IIS" (insulin/IGF signaling), for example by *daf-2/IGFR* mutation in *C. elegans* or by fat-specific insulin receptor knockout in mice [38], greatly improves longevity and health span. If these mutations truly cause "reduced IIS" then they should cause insulin resistance, a defining feature of type 2 diabetes which reduces longevity and health span in humans. Indeed, reduced IIS in pancreatic beta cells and liver cause a diabetes-like phenotype in mice, which is reversed by FoxO1 haploinsufficiency [39,40]. This suggests that FoxO and

reduced IIS promote diabetes and therefore have negative effects on health, yet they promote longevity and health span in a conserved manner.

hsp-12.3 and *sodh-1* offer a potential solution to this apparent paradox. The human orthologs alpha-crystallin and sorbitol dehydrogenase are involved in cellular responses to type 2 diabetes and hyperglycemia [41,42]. If *daf-2* mutation and DAF-16/FoxO activation causes negative effects reminiscent of diabetes in *C. elegans*, but simultaneously upregulate *hsp-12.3* and *sodh-1*, then the negative effects may be counteracted. In this case, perhaps many genes involved in other mechanisms act in a cumulative manner to extend life span, but a few genes are permissive for life span extension because they protect against the negative effects of DAF-16/FoxO activation.

Figure 6.2. Hypothetical model of how single DAF-16/FoxO target genes might play a disproportionate role in life span control.
See text for details. Four categories of DAF-16/FoxO longevity targets are shown.



However, we should be cautious and agnostic about the functions of these genes until more definitive analysis is carried out. For example, the free radical theory of aging, where aging is the result of accumulated damage from free radicals, predicts that genes that defend against oxidative stress will extend life span and delay aging [43]. However, many studies reviewed in [44–46] call the role of free radicals in aging into question. One study found that superoxide dismutases can promote longevity, but they do so not by protecting against oxidative stress but rather by activating transcription factors including DAF-16/FoxO [47]. Therefore, traditional ideas about how specific processes influence life span are in question. Now that we have identified a handful of DAF-16/FoxO target genes that are strongly required for life span extension, it is now feasible to perform detailed studies of gene function, rather than inferring function from gene ontology annotations. As IIS reduction and DAF-16/FoxO activation dramatically increase life span and slow aging, the critical DAF-16/FoxO target genes identified in this dissertation may shed light on the fundamental nature of aging and suggest strategies to prevent and reverse age-related disease in humans.

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