Functional Characterization of a FoxO Target Gene

Winter 2014 University of Michigan Honors Thesis - MCDB

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Abstract

FoxO transcription factors play important roles in many cellular processes, including regulation of lifespan in invertebrates and potentially in vertebrates. Our understanding of FoxO target genes that play a role in lifespan control is incomplete and *C. elegans* is a useful system in which to approach this problem since it has a single FoxO ortholog (DAF-16) and a relatively short lifespan. Using multiple unique genetic filters and RNA-sequencing, we have previously identified a list of 47 putative *daf-16/FoxO* target genes that are likely to play a role in lifespan extension. *Y39G8B.7* is one of these genes and showed a significant shortening of lifespan in a long-lived background during a pilot lifespan assay. I have validated *Y39G8B.7* as a *bona fide daf-16/FoxO* target gene and investigated its functional role in longevity.

Introduction

Forkhead box, class O (FoxO) transcription factors (TFs) are a group of proteins that play a complex role in development, longevity, and homeostatic maintenance in a wide variety of species (Arden, 2008). In the context of aging, FoxO TFs promote lifespan extension in numerous organisms, and their misregulation may be of importance in age associated diseases in humans, such as cancer, type II diabetes, and osteoporosis (Arden, 2008; van der Horst & Burgering, 2007). Furthermore, genetic variation in the *FOXO3A* gene in humans is associated with extreme longevity and healthy aging (Willcox et al., 2008). Despite the important role they play in aging, the group of FoxO target genes that mediate this process is poorly defined and requires more investigation.

Our current understanding of the regulation and effects of FoxO factors has been largely elucidated using the nematode *Caenorhabditis elegans* and fruit fly *Drosophila melanogaster*

model systems. It was in *C. elegans* that a forward genetic screen first implicated a FoxO ortholog, *daf-16*, as an important independent phenotypic mediator. The study demonstrated that DAF-16/FoxO played a critical role in formation of dauer larvae (Swanson & Riddle, 1981). Dauer is a reversible developmental arrest stage that can be induced by stressful environmental conditions such as overcrowding, lack of food, or elevated temperature. Various genetic screens utilized this developmental feature to identify modifiers of the dauer phenotype, a few of which would be found to be the foundation of an insulin-like signaling pathway in *C. elegans* that regulates the activity of DAF-16/FoxO. The *C. elegans* insulin receptor, *daf-2/InsR* and *daf-23*, which would later be identified as *age-1* (PI3K), were isolated as genes whose mutation caused constitutive dauer arrest (Gottlieb & Ruvkun, 1994; Vowels & Thomas, 1992). On the other hand, *daf-16/FoxO* was identified as a gene that, when mutated, caused defective dauer formation in normally constitutive strains (Gottlieb & Ruvkun, 1994; Vowels & Thomas, 1992). The data from these dauer screens suggested a pathway in which *daf-2/InsR*, *daf-23*, and *daf-18/PTEN* interact to regulate the function of *daf-16/FoxO.*

In addition to being implicated in the process of dauer formation, *daf-16/FoxO* and other components of the insulin/insulin-like growth factor signaling (IIS) pathway that regulate it were found to play a significant role in longevity of *C. elegans*. *age-1*/*PI3K* was the first long-lived single mutant identified (Friedman & Thomas, 1987; Klass, 1983), and individual mutation of *daf-2/InsR* was later found to nearly double lifespan of worms (Kenyon, 1993). These large extensions were both found to be dependent on the proper function of DAF-16/FoxO, as a single additional mutation in *daf-16/FoxO* in both the *age-1/PI3K* and *daf-2/InsR* backgrounds suppressed the long-lived phenotypes (Kenyon, 1993; Murakami & Johnson, 1996). Beyond its requirement for IIS lifespan extension, *daf-16/FoxO* was also found to be required for longevity

induced by IIS independent process of germline stem cell ablation (Hsin & Kenyon, 1999). The regulation of DAF-16/FoxO is still an active area of research today, but the transcription factor's importance in *C.elegans* stress resistance and longevity, as well as its conservation in a wide variety of species spurred further investigation in other model organisms.

In *D. melanogaster*, the well-conserved DAF-16 homolog, dFoxO appears to play a role that is very similar to its *C. elegans* counterpart. dFoxO overexpression causes a phenotype similar to starvation that can be rescued by co-expression of upstream IIS components, suggesting a nutrient sensing role not unlike that of DAF-16 in the dauer decision of worms (Kramer, Davidge, Lockyer, & Staveley, 2003). Flies that are lack dFoxO are viable, but are more sensitive to oxidative stress (Jünger et al., 2003). Furthermore, dFoxO expression in the fly pericerebral fat body leads to a large extension of lifespan that is mediated through the conserved components of the IIS pathway (Hwangbo, Gersham, Tu, & Palmer, 2004). Like in *C. elegans*, the DAF-16 ortholog in *D. melanogaster* promotes lifespan extension and increased stress resistance.

Due to the critical role that FoxO transcription factors play in worms and flies, as well as the highly conserved nature of the IIS pathway that regulates them, deciphering the biology of mammalian FoxOs has been of great interest. In mammals, the FoxO subfamily has four known members - FoxO1, 3, 4, and 6. All four share a DNA-binding domain that binds to the same consensus motif as DAF-16/FoxO and are regulated by conserved components of the IIS pathway (Furuyama, Nakazawa, Nakano, & Mori, 2000). The FoxO proteins have been implicated as potential and confirmed players in mammalian aging and age-associated diseases.

FoxO1 and FoxO6 both appear to be involved in the development and progression of diabetes in the *Mus musculus* model system. FoxO1, acts as a negative regulator of insulin sensitivity through its control of β-cell proliferation (Kitamura et al., 2002; Nakae et al., 2002). Impaired insulin suppression leads to abnormally high levels of FoxO6 in the liver, which serve as a causative factor for uninhibited gluconeogenesis. This increase in circulating glucose leads to fasting hyperglycemia in morbid obesity and type II diabetes (Dae Hyun Kim, Zhang, Lee, & Dong, 2013). FoxO3 does not appear to play a direct role in mammalian diabetes, but it is involved in diabetic nephropathy and renal disease (Kato et al., 2006).

In addition to diabetes, FoxOs have been implicated in two other age-associated diseases as well: osteoporosis and cancer. FoxO1, 3, and 4 are expressed at similar levels in bones and bone cells and play important roles in the maintenance of bone homeostasis, which goes awry during osteoporosis. Namely, FoxOs regulate both osteoblastogenesis and osteoclastogenesis through both direct and indirect mechanisms (Almeida, 2011). In the context of cancer, FoxOs have been shown to be tumor suppressors (Paik et al., 2007) and to be involved in a complex interplay with p53 to regulate disease and aging (van der Horst & Burgering, 2007).

The importance of DAF-16, IIS, and their fly counterparts in longevity and healthy aging of their respective organisms is irrefutable, and more data is emerging that suggests a similar role for mammalian FoxOs. Studying the role of these transcription factors is more difficult in murine models, as null mutations in IIS components like FoxO1 and insulin-like growth factor type I receptor (IGF-1R) are lethal. However, mice that are heterozygous for *Igf1r* have been shown to live significantly longer than their litter-mates and have increased resistance to oxidative stress (Holzenberger et al., 2003). Additionally, unlike FoxO1 nulls, FoxO3 null mice are viable, but show age-dependent infertility, accelerated ovarian follicular development and ultimate follicular

depletion that may be similar to the premature ovarian failure that is a common cause of human premature aging in women (Castrillon, Miao, Kollipara, Horner, & DePinho, 2003). In humans genetic variation in the *FOXO3A* gene is strongly associated with longevity and healthy aging (Willcox et al., 2008). It is clear that FoxO transcription factors play a definitive or likely role in the pathobiology of a host of complex, diverse age-associated diseases, as well as longevity itself. Deciphering the regulation and mechanisms of action of these proteins should prove to be important for understanding these processes.

Despite the plethora of research on FoxOs, our knowledge of their transcriptional targets that are involved in lifespan extension is incomplete. In *C. elegans*, various microarray studies have been performed with the intent of identifying DAF-16/FoxO target genes involved in longevity. Surprisingly, there is relatively low overlap between the resulting gene sets (McCormick, Chen, Ramaswamy, & Kenyon, 2012; McElwee, Schuster, Blanc, Thomas, & Gems, 2004; Murphy, 2006). This low agreement makes it difficult to prioritize putative targets for functional testing, and as a result, proper evaluation of these potential target genes' role in lifespan extension is lacking.

Our lab addressed this problem using multiple genetic filters and RNA-sequencing (RNA-seq) to vet target genes for ones specifically involved in lifespan. The first filter took advantage of the fact that the *daf-16/FoxO* locus produces multiple protein isoforms, but only two of them, DAF-16A and F promote lifespan extension (Kwon & Narasimhan, 2011). Additionally, an isoform-specific mutant, (*mg54*), introduces stop codons into the A and F coding regions and suppresses extension to the same degree as *daf-16*(*mu86*) null mutation (unpublished). Therefore, in the contexts of longevity induced by reduced insulin signaling and germline ablation, genes whose

expression levels are changed by both *mg54* or *mu86* mutation are likely to be specifically regulated by DAF-16A or F and involved in lifespan extension.

The second filter involved looking for genes whose expression levels were altered in three different long-lived backgrounds in a *daf-16/FoxO* dependent manner. Gene expression levels were determined using RNA-seq, which has several advantages over traditional microarray technology. Namely, RNA-seq provides more accurate transcriptome information through better dynamic expression range and its improved ability to discern between isoforms and alleles (Wang, Gerstein, & Snyder, 2009). Two long-lived mutant *daf-2/InsR* alleles were used, *daf-2(e1368)* and *daf-2(e1370),* as well as a long-lived germline ablation mutant, *glp-1(e2141).* Whole transcriptome RNA-seq expression levels for the two *daf-2* backgrounds were compared to WT, *mg54;daf-2,* and *mu86;daf-2.* The *glp-1* background expression levels were also compared to *mg54;glp-1* and *mu86;glp-1,* but lacked the direct WT comparison due to large transcriptome size differences associated with germline ablation. By looking for genes whose expression levels were altered in both *mg54* and *mu86* mutants, as well as in three different long-lived backgrounds, 47 genes were identified that correlate with longevity in *C. elegans*.

In these 47 genes are multiple "gold-standard" DAF-16/FoxO targets involved in lifespan, such as *sod-3, mtl-1,* and *dod-3* are present. The list also includes several novel potential target genes, but most importantly, it provides a practical prioritization for evaluating each gene's functional role in lifespan. To begin to test these functional roles, we set up pilot lifespan assays using RNAi targeted for many of the 47 putative genes with *glp-1* animals. Of these, the poorly characterized gene, *Y39G8B.7* is of interest because of its up-regulation in both the *glp-1* and *daf-2* backgrounds and subsequent down-regulation with the addition of *mg54* or *mu86* (Table 1). Additionally, the pilot lifespan data suggested a significant reduction in lifespan using

Y39G8B.7 RNAi in the *glp-1* background (*Fig. 1*). *Y39G8B.7* is relatively small protein (179aa) with no obvious conservation. It contains a signal peptide domain and an ShK toxin domain and has been identified in previous microarray studies. However, its functional role in lifespan has yet to be tested. In order to further characterize *Y39G8B.7*, I validated its role as a target gene using qPCR and evaluated its affect on lifespan extension and dauer formation in a more rigorous fashion. Experiments were performed using *Y39G8B.7* RNAi, as there is not null mutant currently available.

RNA source comparison	*Y39G8B.7* expression change
WT vs. *daf-2(e1370)*	79.1924
daf-2(e1370) vs. *daf-16(mg54);daf-2(e1370)*	0.0073
daf-2(e1370) vs. *daf-16(mu86);daf-2(e1370)*	0.0115
WT vs. *daf-2(e1368)*	4.2188
daf-2(e1368) vs. daf-16(mg54);daf-2(e1368)	0.1346
daf-2(e1368) vs. daf-16(mu86);daf-2(e1368)	0.0654
glp-1 vs. daf-16(mg54);glp-1	0.3904
glp-1 vs. daf-16(mu86);glp-1	0.3329

Table 1: RNA-seq expression level comparisons for WT, *daf-2(e1370)*, *daf-16(mg54);daf-2(e1370)*, *daf-16(mu86);daf-2(e1370)*, *daf-2(e1368)*, *daf-16(mg54);daf-2(e1368)*, *daf-16(mu86);daf-2(e1368)*, *glp-1*, *daf-16(mg54);glp-1*, and *daf-16(mu86);glp-1*

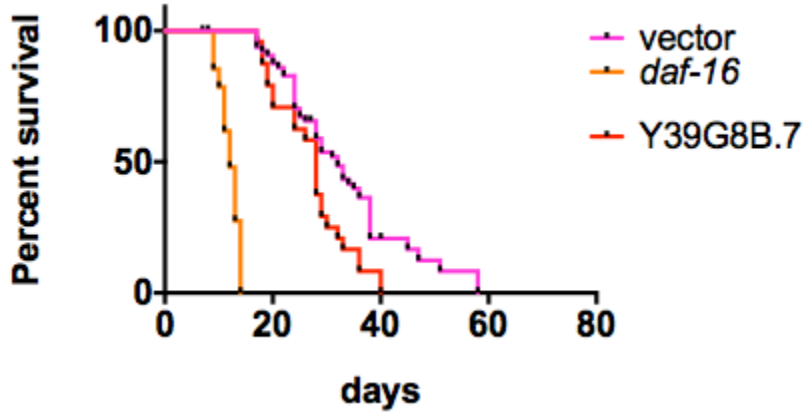

Figure 1: survival curves for *glp-1* animals grown on *daf-16* RNAi, *Y39G8B.7* RNAi, and vector. *Y39G8B.7* RNAi causes statistically significant (p=0.05) reduction in lifespan

Materials and Methods

Strains and Reagents

The following strains were used: N2 Bristol (wild-type), *daf-2(e1370* (Kimura, 1997)*), daf-16(mg54)*(Ogg et al., 1997) , *daf-16(mu86)*(Lin, Hsin, Libina, & Kenyon, 2001) , *daf-2(e1368)* (Hsin & Kenyon, 1999), and *glp-1(e2141)*(Priess, Schnabel, & Schnabel, 1987).

Double mutants were generated using standard genetic crossing techniques. Animals were grown and maintained in Percival I-30NL or I-36NL incubators (Percival Scientific, Inc., Perry, IA, USA)

Quantitative Real-Time PCR (qPCR)

RNA was extracted from 5 cohorts of young-adult animals for each strain as described in (Chen, Guo, Dumas, Ashrafi, & Hu, 2013). cDNA was synthesized using Superscript III Reverse Transcriptase kit (Invitrogen) and SYBR Green (Applied Biosystems, Warrington, UK) Real-Time PCR was performed using primers for *sod-3, Y39G8B.7,* and *act-1* as an internal control. Paired one-tailed t tests were performed using Excel.

RNAi

Frozen glycerol stocks of HT115 *E. coli* expressing dsRNA from an L4440 vector were obtained from the Ahringer Library (Source BioScience LifeSciences) and spread onto standard culture plates containing ampicillin and tetracycline. L4440 vector alone was used as a negative control. Individual colonies were grown in HT115 overnight culture with 50ug/mL of ampicillin.

Lifespan Assays

Lifespan assays were performed at 20°C. For *glp-1* animals, NGM + 5 mM IPTG + 25 mg/ml carbenicillin plates were seeded with HT115 containing empty vector or the corresponding RNAi clone from overnight culture. dsRNA production was induced overnight by IPTG. Synchronized Day 2 adult animals were added and assayed for viability every 1-2 days. The same procedure was followed for *daf-2* mutant animals, however, the NGM plates also included 25 lg/mL (100 lM) 5-fluoro-2′-deoxyuri- dine (FUDR; Sigma-Aldrich, St. Louis, MO, USA). n = 105 for *Y39G8B.7* and vector; n = 60 for *daf-16*.

Dauer Assays

Dauer assays were performed at 25°C as previously described (Hu, Xu, & Ruvkun, 2006). NGM + 5 mM IPTG + 25 mg/ml carbenicillin plates were seeded with 250uL of overnight culture containing HT115 transfected with empty vector or the designated RNAi clone. A synchronized 3-5 hour egglay was performed at 20°C, then the animals were removed. Animals grew at 25°C on the RNAi, then were assayed for the dauer phenotype at ~48-60 hours.

Results

***Y39G8B.7* expression levels are elevated in *daf-2* mutants and diminished by subsequent *daf-16* mutation**

Both reduced IIS and germline ablation extend lifespan in a DAF-16/FoxO dependent manner in *C.elegans* (Hsin & Kenyon, 1999; Kenyon, 1993; Murakami & Johnson, 1996). We utilized two mutant alleles of *daf-2/InsR* (*e1368* and *e1370*) and the germline ablation mutant, *glp-1* in order to identify *daf-16/FoxO* target genes that may be involved in multiple contexts of lifespan extension. As the RNA-seq results in Table 1 show, *Y39G8B.7* expression levels were upregulated in both long-lived *daf-2(e1368* and *e1370*) backgrounds and downregulated upon addition of either *daf-16(mg54* or *mu86)* mutation to these and the *glp-1* background. These expression patterns are consistent with a *daf-16* target gene with a potential role in lifespan extension. In order to validate *Y39G8B.7* as a *daf-16* target gene, I performed quantitative real-time PCR (qPCR) in the same genetic backgrounds and compared the expression changes in the same manner as in Table 1.

Compared to its expression levels in N2, *Y39G8B.7* was upregulated in both mutant *daf-2* backgrounds. The magnitude of increase in *daf-2(e1368)* was marginally significant ($p=0.1$), while the increase in *daf-2(e1370)* was statistically significant ($p=0.03$) (Fig. 2, Table 2). These increases in *Y39G8B.7* expression were drastically reduced by the subsequent deletion of *daf-16/FoxO* by either mutant allele (Fig. 2). Again, the magnitude of reduction was marginally significant for both *daf-16* alleles in *daf-2(e1368)* and statistically significant for both alleles in *daf-2(e1370)*. The expression changes for all seven genetic backgrounds are similar to those observed by RNA-seq. Furthermore, they mirror the expression trends of a known *daf-16/FoxO* target gene that plays a role in longevity, *sod-3* (Fig. 3). Therefore, the expression data by qPCR

further validates *Y39G8B.7* as a *daf-16/FoxO* target gene with a potential role in lifespan extension.

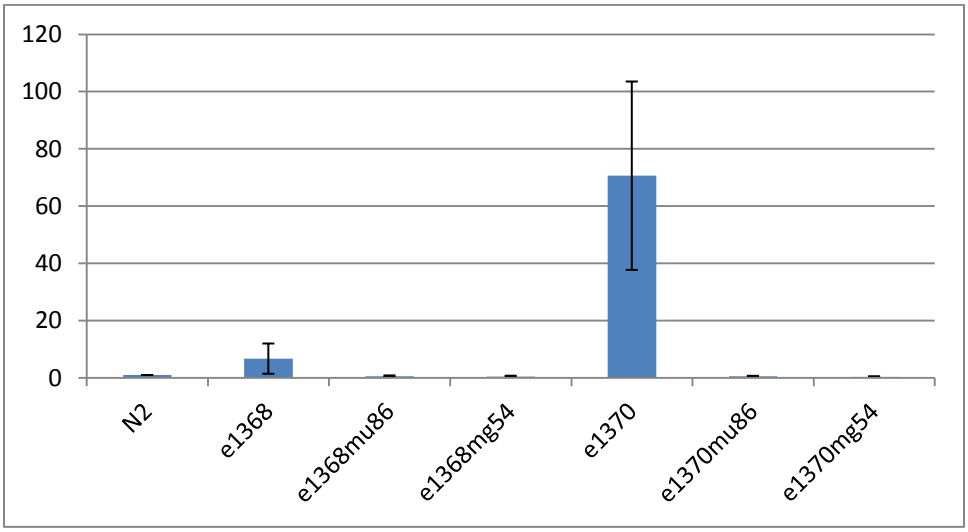

Figure 2: Relative expression of *Y39G8B.7* by qPCR. *e1368* and *e1370* are mutant alleles of *daf-2/InsR*. *mu86* and *mg54* are mutant alleles of *daf-16/FoxO*. The data shown is an average of three biological replicates; the error bars denote biological standard deviation.

Genetic Background Comparison	p value
WT vs *daf-2(e1370)*	0.03353438
daf-2(e1370) vs. *daf-16(mg54);daf-2(e1370)*	0.033172899
daf-2(e1370) vs. *daf-16(mu86);daf-2(e1370)*	0.032901018
WT vs daf-2(e1368)	0.100704638
daf-2(e1368) vs. daf-16(mg54);daf-2(e1368)	0.080152938
daf-2(e1368) vs. daf-16(mu86);daf-2(e1368)	0.089747302

Table 2: paired one-tail T-test analysis of genetic background comparisons.

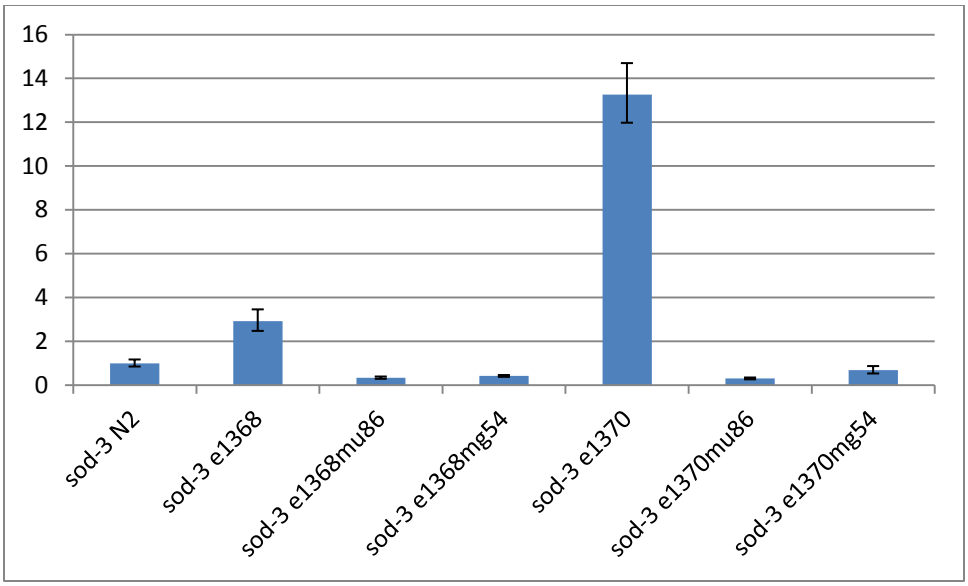

Figure 3: Relative expression of *sod-3*, a validated *daf-16/FoxO* target gene that plays a role in lifespan.

Functional Characterization

Effects of Y39G8B.7 RNAi on lifespan

As previously mentioned, both reduced IIS and germline ablation extend lifespan in a DAF-16/FoxO dependent manner in *C.elegans* (Hsin & Kenyon, 1999; Kenyon, 1993; Murakami & Johnson, 1996). Validated *daf-16/FoxO* target genes such as *sod-3* and *mtl-1* have also been shown to reduce *daf-2* mediated lifespan extension through RNAi (Murphy et al., 2003). *Y39G8B.7* RNAi was shown to reduce lifespan in a statistically significant fashion in our pilot lifespan assay (Fig. 1) and has been shown to be a *bona fide* target gene of *daf-16/FoxO*. Consequently, I evaluated the role of this gene in *daf-2* and *glp-1* mediated lifespan extension in a more rigorous fashion. As opposed to the 50 animals on vector and *Y39G8B.7* RNAi in the pilot lifespan, this assay had 105 animals on each and 50 on *daf-16* RNAi.

While the assays are still ongoing, it is clear that the median percent survival and overall trends for *Y39G8B.7* RNAi are not the same as were observed in the pilot experiment. In the *glp-1* background, the median survival for *Y39G8B.7* was reached at 36 days, while the median for vector was 32. As determined by Chi-squared log-rank test (p=0.05), the *Y39G8B.7* RNAi and Vector curves were not significantly different (*glp-1*: p = 0.1726, *daf-2(e1368)*: p = 0.6177). The results were similar in the *daf-2(e1368)* background, where the median was 32 days for both vector and *Y39G8B.7* and the curves were not significantly different.

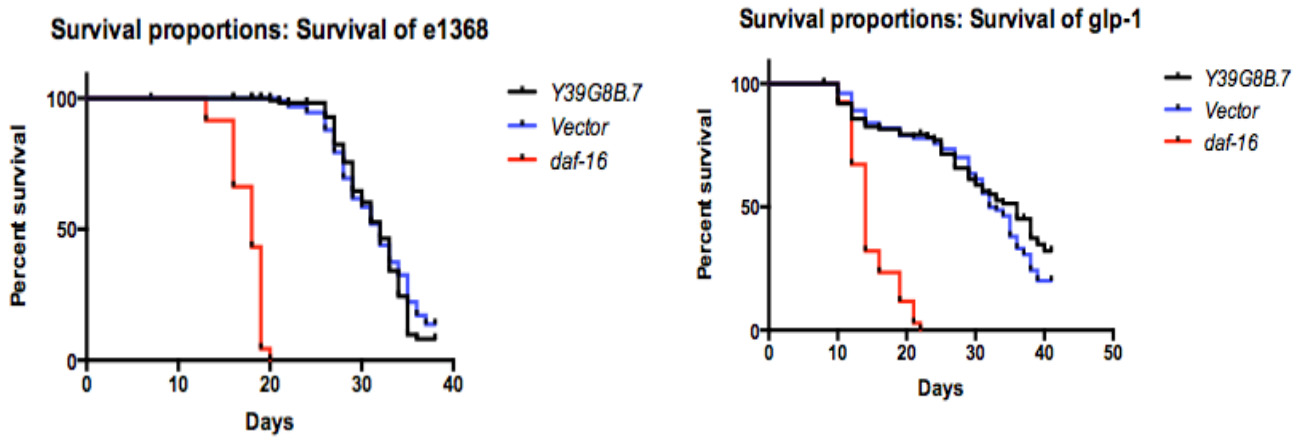

Figure 4: Effect of *Y39G8B.7 RNAi* on lifespan in both *glp-1* and *daf-2(e1368)* backgrounds. Both controls acted in a manner consistent with previous results. 105 animals were divided onto 7 plates for *Y39G8B.7* and vector, while 60 were spread across 4 plates for *daf-16* in both instances. Scoring of vector and *Y39G8B.7* will continue until completion.

Effects of Y39G8B.7 RNAi on dauer arrest

Dauer is a reversible developmental arrest stage that can be induced by stressful environmental conditions such as overcrowding, lack of food, or elevated temperature. The decision to enter dauer arrest occurs late in larval 1 (L1) stage and causes worms to undergo morphological remodeling into a wiry form with a resistant cuticle and a pharynx that does not pump. Additionally, the animals cease to exhibit food-seeking behavior and their metabolism is

drastically slowed (Hu, 2007). DAF-16/FoxO promotes dauer in *daf-2/InsR* mutants with reduced IIS (Gottlieb & Ruvkun, 1994; Vowels & Thomas, 1992). Therefore, I hypothesized that *Y39G8B.7* may influence this phenotype in its capacity as a *daf-16/FoxO* target gene.

Although no null mutant is available for *Y39G8B.7*, I was able to use *Y39G8B.7* RNAi to roughly assess its effect on dauer arrest in the *daf-2(e1368)* background of reduced IIS. Both the positive (*daf-16)* and negative (vector) control behaved as expected and *Y39G8B.7* RNAi did not appear to have a noticeable effect on dauer arrest in either direction (Fig. 4).

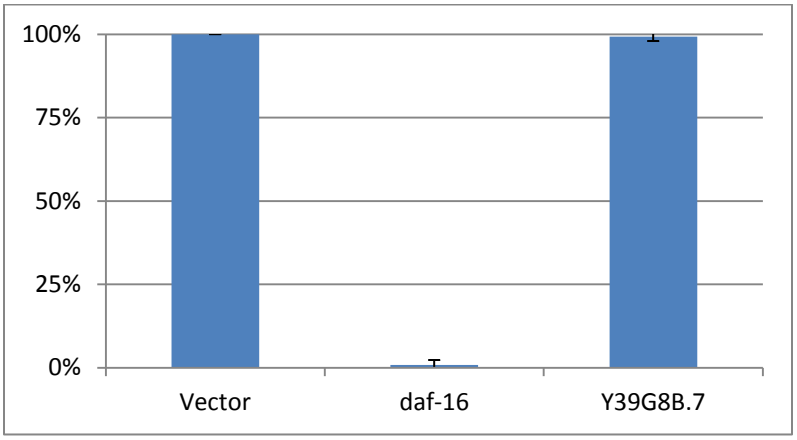

Figure 5: effect of *Y39G8B.7 RNAi* on dauer arrest in *daf-2(e1368)* background. Error bars denote standard deviation.

Discussion

FoxO transcription factors and the IIS pathway that regulates them are conserved in many species, where they promote metabolic homeostasis, stress resistance, and longevity (Accili, 2004; Arden, 2008). Despite a large body of research on FoxOs, relatively little is known about the group of FoxO target genes that play a functional role in the intriguing lifespan extension phenotype observed in worms and flies - a phenotype which is likely to be observed in

vertebrates as well. *C. elegans* is a useful model organism for approaching this problem, as it has only one ortholog of FoxO, DAF-16 and a short lifespan. Our lab's unique genetic filers that we employed allowed us to identify genes whose expression levels are altered in three different long-lived backgrounds as well as by specific deletion of the DAF-16/FoxO isoforms that play a role in longevity. As a member of this list of 44 putative *daf-16/FoxO* target genes and due to preliminary lifespan data, *Y39G8B.7* is a promising potential modulator of lifespan. While there does not appear to be any obvious conservation of the protein in vertebrates, this may be due to its small size (179aa) which can make it very difficult to identify homologs using BLAST.

qPCR validation of *Y39G8B.7* expression levels in several long-lived backgrounds confirmed this gene is indeed upregulated in these strains and a single additional mutation in *daf-16/FoxO* could significantly reduce this upregulation (Fig. 2). This information, in combination with our previous RNA-seq data, corroborate *Y39G8B.7's* status as a *daf-16/FoxO* target gene.

Unlike so called "gold standard" *daf-16/FoxO* target genes that are involved in lifespan, *Y39G8B.7* RNAi demonstrated very little phenotypic effect. Two of these "gold standard" genes, *sod-3* and *mtl-1* have been shown to suppress lifespan in the *daf-2/InsR* mutant background by RNAi alone (Murphy et al., 2003). The first pilot replicate of *Y39G8B.7* RNAi lifespan showed significant suppression (Fig. 1), but the more robust second assay did not show any effect (Fig. 4). This disparity may be due to differential knockdown by RNAi in the two experiments, or the emergence of the true phenotypic effect with a larger sample size. Incomplete RNAi knockdown is also a possible explanation for a lack of a differential dauer phenotype. This shortcoming can only be truly overcome using a full knockout, but a second dauer assay in which the parental generation is raised on RNAi as well is under way. This should serve to maximize RNAi exposure time and to eliminate the possibility of maternal rescue. It is assumed that the target

genes are acting in the intestines, where DAF-16/FoxO exerts its effects, but it is also possible that *Y39G8B.*7 functions in an RNAi-resistant tissue and this is affecting phenotypic outputs. There are numerous shortcomings and caveats of using RNAi knockdown, and obtaining a null mutant from the National Bioresource Project (http://www.shigen.nig.ac.jp/c.elegans/) would help to address these.

Other than the unavoidable possibility of incomplete knockdown by RNAi, there are two other reasons that no phenotypic effect was observed in lifespan or dauer assays. The first is that *Y39G8B.7* simply is not involved in lifespan control. This is a possibility that is hard to test without a full knockout, but one that was hopefully avoided through our vetting system of multiple genetic filters for *daf-16/FoxO* targets involved in longevity. The second, more likely possibility is that *Y39G8B.7* is involved in a more complex system that affects lifespan. In order to investigate the possibility of functional redundancy, I compared our list of 47 putative target genes with the list of *Y39G8B.7* paralogs without finding any matches.

Y39G8B.7 contains two functional domains, Signal Peptide (SP), and ShK (metridin-like ShK toxin), that may play a role in its function. The presence of a SP domain means that Y39G8B.7 enters the secretory pathway and may be secreted, allowing for the possibility of cell-nonautonomous expression of the protein. The ShK toxin domain is found in metridin, a toxin produced from a type of sea anemone, where it serves as a potent inhibitor of potassium channels (Pennington et al., 1999). Some *C. elegans* proteins that contain the ShK toxin domain are candidate secreted antimicrobials that have been reported to be under the control of PMK-1, which encodes a mitogen-activated protein kinase (MAPK) that plays a critical role in the PMK-1 p38 MAPK pathway (Troemel et al., 2006). This pathway and the IIS pathway have been shown to control intestinal innate immunity (Garsin et al., n.d.; Dennis H Kim et al., 2002).

Y39G8B.7 fits the description of these ShK toxin-like secreted antimicrobials and is even co-expressed with three of the ShK toxin candidates identified by Troemel, et. al. - *F01D5.1, F01D5.2,* and *F01D5.5* (co-expression analysis by SPELL - (Hibbs et al., 2007)). However, *Y39G8B.7* does not fit the model proposed by Troemel, et. al. (Fig. 6). This model shows PMK-1 upregulating ShK toxin-like candidate antimicrobials, and DAF-16/FoxO inhibiting their expression. If *Y39G8B.7* is truly a secreted antimicrobial, either DAF-16/FoxO upregulates expression of some ShK toxin-like genes independently of PMK-1, or some ShK toxin-like genes are regulated by both PMK-1 and DAF-16/FoxO. Either way, this would elevate the role of DAF-16/FoxO in direct innate immunity and provide an indirect method by which *Y39G8B.7* could modulate lifespan through innate immunity.

Innate immunity's role in lifespan extension is not well understood. However, multiple DAF-16/FoxO target genes implicated in longevity have also been identified as secreted antimicrobials (Murphy et al., 2003; Troemel et al., 2006). Additionally, PMK-1 has been shown to contribute to lifespan extension in the *daf-2* background (Troemel et al., 2006). The potential role of innate immunity in longevity is furthered by the fact that aged animals have been shown to have extensive intestinal proliferation of *E. coli* and that nematodes grown on dead bacteria live longer than those grown on live bacteria (Garigan et al., 2002; Gems & Riddle, 1996). Consequently, innate immunity may be the complex system by which *Y39G8B.7*, which has been validated as a *bona fide daf-16/FoxO* target gene, exerts its effects on lifespan extension.

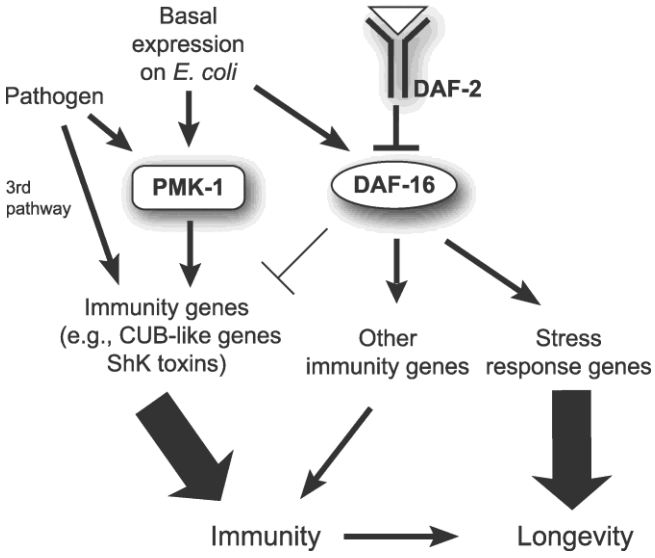

Figure 6: Model for Regulation of Immunity/Longevity by the PMK-1 p38 MAPK Pathway and the DAF-2, DAF-16 Insulin-Signaling Pathway in *C. elegans*(Troemel et al., 2006)

Acknowledgements

I would like to thank Patrick Hu for his phenomenal guidance on this project and thesis. In addition, I would like to thank Dr. Kumar for his contributions as my co-sponsor and Gyorgyi Csankovszki for agreeing to read my thesis. The other members of the Hu lab have been indispensible in preparing and assembling this project, and I would like to especially thank Albert Chen for his help.

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