

A review of genes that act downstream of the DAF-16 FOXO transcription factor to influence the life span of *C. elegans*

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Summary

In the work we have described here (Murphy et al. 2003), an unbiased microarray analysis was used to identify transcriptional targets of the DAF-2/DAF-16 pathway, and the identified genes were tested for their individual contributions to longevity. Because we applied an unbiased method of gene expression profiling of a combination of multiple mutant alleles as well as a time course of RNAi treatment, resulting in data from over 70 arrays, we were able to significantly reduce the number of false positives. We found not only the very few previously identified DAF-16 targets but also many novel targets, and the previously described DBE and a new motif were overrepresented in the promoters of the genes. The diversity of the core set of downstream targets suggests that the coordination of expression through DAF-16 is critical in the regulation of longevity.

DAF-16, a FOXO-family transcription factor, influences the rate of aging of *C. elegans* in response to insulin/IGF-1 signaling. Using DNA microarray analysis, we found that DAF-16 affects the expression of a set of genes during early adulthood, the time at which this pathway is known to influence aging, and we have shown that many of these genes influence the aging process (Murphy et al. 2003). We also identified a DNA motif, in addition to the canonical DAF-16 motif, that is overrepresented in the promoters of the DAF-16-regulated genes. The insulin/IGF-1 pathway functions cell non-autonomously to regulate life span, and our findings suggest that it signals other cells, at least in part, by feedback regulation of two insulin/IGF-1 homologs.

Our findings suggest that the insulin/IGF-1 pathway ultimately exerts its effect on life span by up-regulating a wide variety of genes, including cellular stress response and anti-microbial genes, fat and steroid hormone synthesis genes, and many genes of unknown function, and by down-regulating specific life-shortening genes. Because the genes seem to act in a cumulative manner to affect life span, this study demonstrates the power of functional microarray analysis for dissecting complex regulatory systems.

C. elegans has proven to be an excellent model system for the study of aging. In addition to its general utility in genetic approaches (Brenner 1974), the worm also displays distinct phenotypes of aging during its short life span, allowing researchers to study long- and short-lived mutants (Johnson 1990; Kenyon et al. 1993; Lin

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et al. 1997; Lakowski and Hekimi 1996; Ewbank et al. 1997; Dillin et al. 2002a) as well as treatments that affect longevity (Lakowski and Hekimi 1998; Melov 2002). Two recent studies (Herndon et al. 2002; Garigan et al. 2002) carefully described the stochastic progression of aging in worms, which is marked by general tissue deterioration and reduction of motility. Age-related changes include sarcopenia, distortion of the cuticle, collapse and bacterial packing of the pharynx, distortion of gonadal nuclei, and the accumulation of fat in droplets in the head (Herndon et al. 2002; Garigan et al. 2002). Many of these phenotypes are reminiscent of human aging, and the fact that the genetic pathways known to affect life span in worms are highly conserved (Kenyon 2001; Guarente and Kenyon 2000) suggests that what we glean through studies of *C. elegans* aging will shed light on the mechanisms of aging regulation in humans.

Among the genetic mechanisms known to affect aging in *C. elegans*, the DAF-2/Insulin-IGF-like receptor (IIR) pathway (Kenyon et al. 1993) has perhaps the most dramatic effects and is one of the best studied. *daf-2* mutants are not only long-lived, with a life span of two to three times that of wild type worms, but the mutants are also extremely healthy and active much later than wild type (Kenyon et al. 1993). Many of the components of this signaling pathway have been cloned and characterized and include a PI3-kinase (*age-1*; Morris et al. 1996), a PTEN phosphatase (*daf-18*; Ogg and Ruvkun 1998), and additional kinases (*akt-1*, *akt-2*, *pdk-1*, and *sgk-1*; Paradis et al. 1999; Paradis and Ruvkun 1998; Hertwick et al. 2004). The activation of DAF-2/IIR activates this kinase cascade, culminating in the phosphorylation and nuclear exclusion of the DAF-16/FOXO transcription factor (Lin et al. 1997; Ogg et al. 1997; Lee et al. 2001). The activity of DAF-16/FOXO is required for all of the known phenotypes of *daf-2* mutants, including its extended longevity (Kenyon et al. 1993). However, the downstream targets of this transcription factor were largely unknown before the work we will describe here. Additionally, the only mechanism that had been hypothesized to function downstream of DAF-16/FOXO to extend life span involved the mediation of reactive oxygen species (Honda and Honda 1999). This paper will describe the work that we have done to discover these genes and test their roles in longevity. (For additional details and supplementary data, please see Murphy et al. 2003.)

What are the transcriptional targets of DAF-16/FOXO?

Previous work to identify the targets

Genetic screens to find genes downstream of DAF-16/FOXO were largely unsuccessful (Lin et al. 2001; Lee et al. 2002), identifying only genes that act in parallel to DAF-16, such as mitochondrial genes (Lee et al. 2002). One interpretation of these results is that, rather than acting through a single linear pathway, many genes may act in concert downstream of DAF-16.

A few DAF-16/FOXO target genes have been identified through candidate gene approaches. Because *daf-2* worms are not only long-lived but also exhibit resistance to oxidative stress, heat, and heavy metals, genes that might confer such resistance are good candidates for regulation by the DAF-2/DAF-16 pathway. Su-

peroxide dismutase (*sod-3*; Honda and Honda 1999), a metallothionein (*mtl-1*; Barsyte et al. 2001), and a small heat-shock protein (*hsp-16* Walker et al. 2001) were all shown to be upregulated in the long-lived *daf-2* or *age-1* mutants. The absence of these genes had not yet been shown to suppress *daf-2* life span, however, suggesting that additional targets remained.

Our approach

Another method of identifying putative targets of a transcription factor is to find genes that contain the factor's binding site in the gene's promoter region. The DAF-16 binding element (DBE) TTGTTTAC had been identified by Furuyama et al. (2000) *in vitro*, so initially we looked for genes that contained the DBE within the first 1 kb upstream of the start site. We found that nearly one of every six promoters in the *C. elegans* genome contained a DBE. This number of genes seemed impractically high. [It should be noted, however, that in a simultaneous study, Lee et al. (2003) compared a list of DBE-containing *C. elegans* genes to a list of *Drosophila* orthologs that also contained this promoter element to obtain a smaller list of candidate DAF-16 targets to test.]

Because the principal role of DAF-16 is to control transcription, we decided to use expression analysis to identify the DAF-16/FOXO targets. We would like to point out some important points regarding our experimental approach that helped us to obtain a relatively small but significant group of DAF-2/DAF-16 targets:

- 1) unbiased selection of genes: we built full-genome *C. elegans* PCR microarrays for this analysis rather than examining a pre-defined set of genes;
- 2) predictive viewpoint: we looked at gene expression differences in early adulthood, before any aging or death has occurred, to identify differences that should **predict** the life span of the animal, rather than differences that are due to late aging;
- 3) biological redundancy and timecourse experiments: we used replicates of three alleles of *daf-2* (*e1368*, *e1370*, and *mu150*), *age-1* (*hx546*), and a DAF-16 overexpression strain compared with their respective *daf-16* double mutants or wild type background to identify DAF-2/DAF-16 differentially expressed genes that are shared between all of the long-lived strains. Ten of these experiments were included in the analysis.

We also took advantage of the fact that in *C. elegans*, RNA interference is very simple; worms may be fed bacteria containing the double-stranded RNA of a gene of interest, and the gene's transcript will be knocked down. RNAi of *daf-2* recapitulates the long-lived phenotype of the *daf-2* mutant (Dillin et al. 2002b). We carried out two 10-point time courses of *daf-2* RNAi, *daf-2* + *daf-16* RNAi, and control vector treatment. Sixty arrays were included in this analysis. From these two experiments, we were able to obtain information about both the DAF-2/DAF-16 pathway and normal early aging. Time course analysis helps eliminate a problem that is common to many microarray experiments that rely on a single time point, that is, generation of false positives through the misinterpretation of slight

differences in developmental timing. In general, by studying multiple alleles and RNAi treatments that share the trait we are interested in (i.e., long life span as a result of altered insulin signaling), as well as by biologically reversing the treatment (*daf-16* RNAi) and by using a time course, we were able to significantly reduce the number of false positives in our data set;

- 4) two independent analyses: we used two completely different methods to analyze our data. First, we submitted the 60 RNAi time course arrays and five of the mutant arrays to Cluster (Eisen et al. 1998) as a single data set; after hierarchical clustering, we were able to easily identify two groups of genes: Class 1 genes were upregulated under *daf-2(-)* conditions and downregulated under *daf-16(-)* conditions, whereas Class 2 genes met the opposite criteria. Because of the large number of arrays that we used for the experiment, genes that changed randomly were extremely obvious in the Cluster dendrogram, and these false positives did not cluster with our genes of interest. In fact, the Class 1 and 2 clusters contained fewer than 200 genes total. Additionally, we performed Significance Analysis of Microarrays (SAM) on the 10 mutant arrays and were able to find the set of genes that were consistently up- or downregulated. Most of the genes in the SAM list had already been identified through the Cluster analysis, but we did find some genes that had small but significant (consistent) expression changes in the mutants;
- 5) prioritization: by examining the data from the Cluster and SAM analyses, we were able to prioritize each gene according to a) its significance score from the mutant SAM analysis, b) its presence in the Class 1 or Class 2 cluster, c) its expression pattern under different conditions, and d) the consistency of the gene's expression within the cluster. The 250 genes were then placed into categories of 1 (best) through 6 (marginal);
- 6) testing the candidate target genes: we used RNAi to test each gene's contribution to the life span extension seen in *daf-2* mutants, starting with Priority Group 1 and working our way downwards. We tested 58 of the highest priority genes multiple times for their roles in longevity.

By taking these factors into account during the experimental set-up, rather than simply doing a small number of arrays of one *daf-2* allele at a single point, we were able to eliminate false positives and focus quickly on a small set of genes. Additionally, our system allows us to easily test the candidate genes for their functional significance.

Our results

Microarray analysis

Using Cluster to group genes with similar expression profiles, we were able to identify two major clusters of DAF-2/DAF-16-regulated genes (Fig. 1). The Class 1 genes are upregulated in the long-lived *daf-2* mutants and under *daf-2(RNAi)* conditions while downregulated under *daf-16(RNAi)* conditions, and the Class 2 genes show the converse transcriptional profile. The previously identified targets

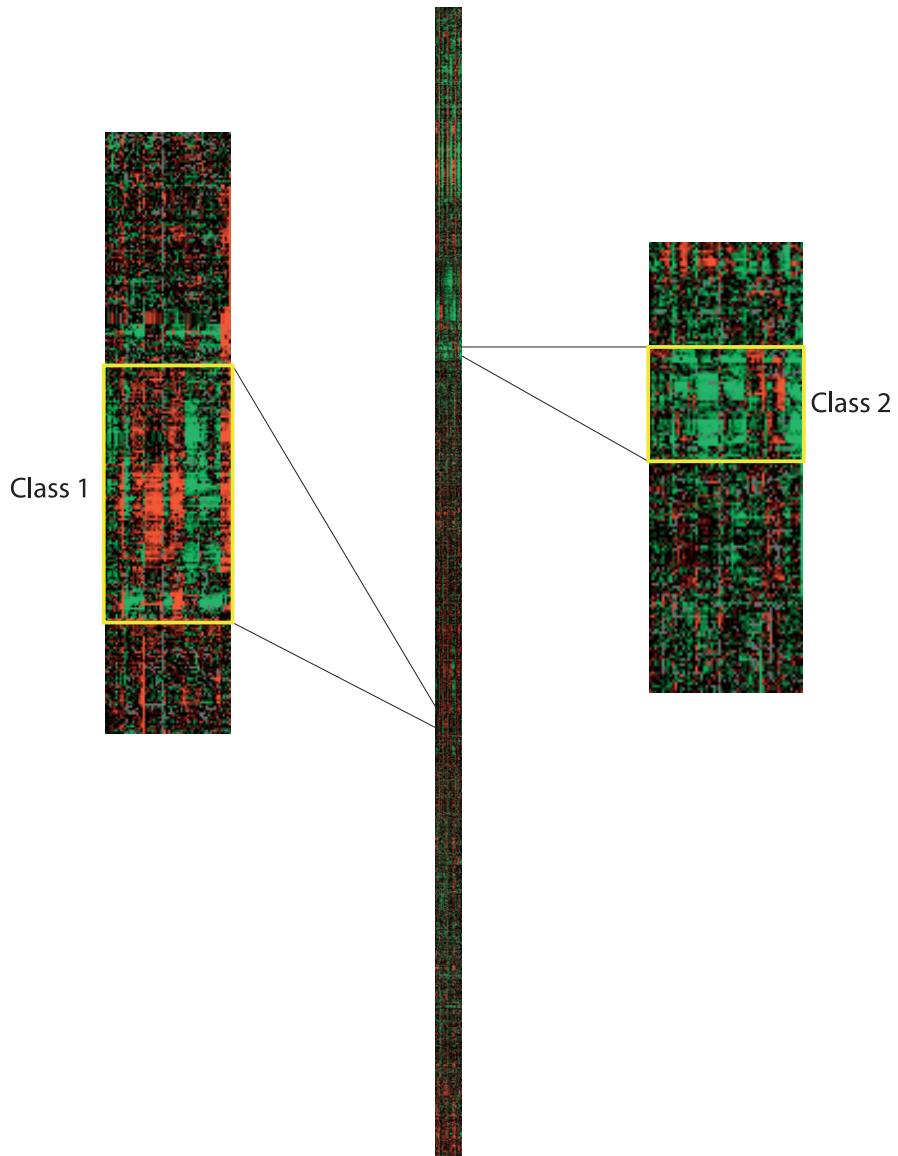


Fig. 1. Cluster of Class 1 and 2 genes.

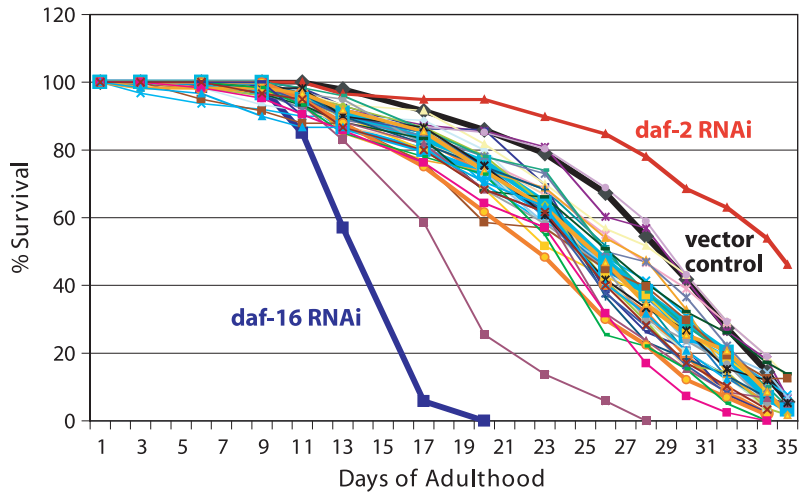


Fig. 2. Life span of *daf-2* worms on RNA interference of Class 1 genes. (Yves: is this sufficient explanation for all of the data on this graph?)

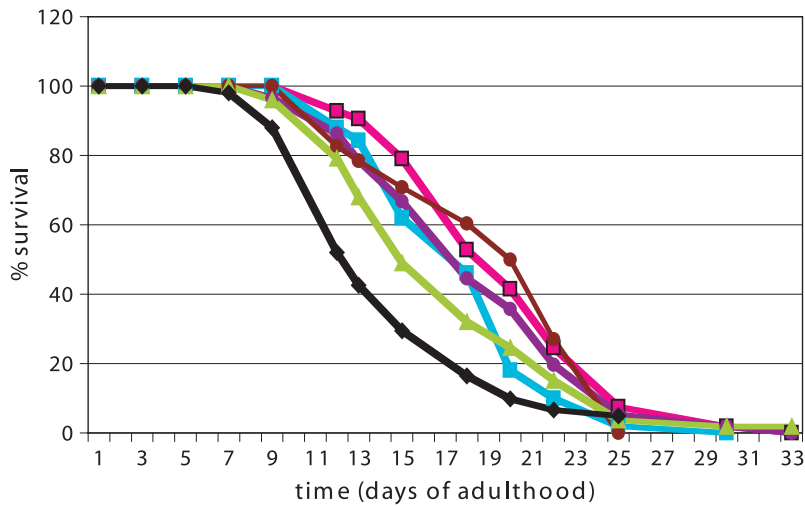


Fig. 3. Life span of wild type worms on RNA interference of Class 2 genes. (Yves: as with figure 2 legend, is tis sufficient? Should the capitalization of the wordng on the axes be made consistent on th 2 figre?)

sod-3, *mtl-1*, and *hsp-16.2* were present in our Class 1 cluster, verifying our approach. Class 1 includes not only the expected genes involved in oxidative and heat stress response but also genes that function in activities not previously associated with prevention of aging in worms: antibacterials, cytochrome P450s, steroid and lipid synthesis, and specific metabolism genes. One of the 38 insulin-like peptide genes, *ins-18*, showed a small but significant increase in expression across all of the *daf-2* mutants. Additionally, novel genes, which we are naming *dod* for “downstream of DAF-16,” were present in Class 1.

Class 2 includes genes involved in ubiquitin-mediated protein degradation (*skr*), peptide transport, peptidases, apolipoproteins (vitellogenins), some neuronal (*gcy-6*, *gcy-18*) and signaling (*nhr*) molecules, and many novel genes. Additionally, *ins-7*, another insulin-like peptide gene, is significantly downregulated in *daf-2* worms. It should be noted that this is the first evidence that DAF-16 may play a direct role in gene repression.

Independent of the DAF-2/DAF-16-regulated genes, collagens and histones each show age-dependent changes, with collagen expression decreasing over time and histone expression increasing. Other early adult age-dependent expression is also apparent in our data.

Promoter analysis

We examined the promoters of the Class 1 and Class 2 genes and found that the canonical DAF-16 binding element (DBE), with variation at the seventh nucleotide, was overrepresented, suggesting that many of the genes are indeed direct targets of DAF-16 transcription. Additionally, a novel element (the DAF-16-associated element, or DAE) with some similarity to the GATA transcriptional motif, was also overrepresented, suggesting the involvement of a co-factor or downstream transcription factor. Interestingly, both motifs are present in the promoters of both classes. Also, the presence of the DBE in the Class 2 genes suggests that DAF-16 is directly involved in these genes' repression.

Life span analysis: RNAi of individual candidate genes

We were interested in establishing the contribution of each gene to *daf-2*'s long life span. To do this, we first prioritized the genes by the magnitude and consistency of expression changes. We then used feeding RNAi of the top genes first in life span tests, working our way down the priority list. Knockdown of many of the top-priority Class 1 genes shortened the life span of *daf-2* mutants (Fig. 2), whereas reduction of the lower priority genes had little to no effect. With the exception of T10B9.1, a cytochrome P450, reduction of most of the genes had no effect on wild type life span; RNAi of T10B9.1 affected development and dauer exit as well as shortening *daf-2*'s life span greatly. Another exception was B0213.15, also a cytochrome P450, which did not affect life span of wild type but extended *daf-2*'s life span significantly; this gene was the single exception to the general

observation that RNAi of Class 1 genes either shortened or had no effect on *daf-2* life span. We also found that RNAi of many of the top-priority Class 2 genes extended the life span of wild type worms (Fig. 3), including the apolipoproteins, the *gcys*, several novel genes, and *ins-7*, which showed a very large increase in life span, reminiscent of *daf-2* RNAi treatment.

Biological implications of our findings

Our results suggest that DAF-16 activity is important to the survival of *daf-2* mutants because it coordinates the expression of many different genes that may cumulatively contribute to longevity. The genes regulated by DAF-16 comprise a large number of functional classes, including stress response, antimicrobials, steroid synthesis, metabolism, lipid synthesis, apolipoproteins, protein and peptide degradation, and signaling. After identifying the genes downstream of DAF-16, we tested them individually for their life span effects, and we have shown here that many of the genes contribute individually to longevity. The wide variety of functions of life span-affecting genes emphasizes the importance of multiple systems in keeping the organism alive.

The presence of the DAF-16-binding element in the promoters of both Class 1 and Class 2 genes suggests that DAF-16 not only promotes but also represses expression of some genes. Additionally, the presence of the DAF-16-associated element (DAE) in these promoters suggests the activity of an additional co-factor or downstream signal.

The discovery of regulation of a pair of insulins, *ins-7* and *ins-18*, downstream of DAF-16 suggests the presence of a feed-forward mechanism that may coordinate the activity of the DAF-2 pathway in cells throughout the organism. This type of control could be especially important for longevity, since the survival of all the tissues is necessary for long life.

Future directions

We looked for the targets of the DAF-2/DAF-16 pathway in early adulthood because we were interested in genes that are predictive of life span. Future experiments will tell us whether, at different times in the life of *C. elegans*, the activities of these genes have any bearing on longevity. While we have briefly mentioned some genes associated with general early aging, in the future it will be interesting to know all of the genes that change with late aging (Lund et al. 2002), as well as how DAF-16 target genes change with age.

It is not yet known whether there is an additional systemic pathway downstream of DAF-16. We can surmise that those genes with DBEs are direct targets of DAF-16, but our experiment does not distinguish direct from indirect targets. Later experiments will address this through biochemical techniques, including ChIP/chip analysis.

While the neurons were previously thought to be the primary site of DAF-2-mediated longevity control (Wolkow et al. 2000; Apfeld and Kenyon 1998, 1999),

recent experiments with tissue-specific expression of DAF-16 suggest that the intestine may play a larger role in life span determination than previously appreciated (Libina et al. 2003). Our experiments were carried out on whole animals expressing DAF-16 in all tissues (or lacking DAF-16 in all tissues); it is possible that in the future we could dissect the pathway downstream of DAF-16 further by using strains expressing DAF-16 only in specific tissues.

Additionally, our microarray analyses were performed on populations of worms, to parallel our life span analyses, which are always done on populations as well. However, it would be interesting to combine the technique of single-worm microarray analysis³⁶ with the morphological analysis of aging (Herndon et al. 2002; Garigan et al. 2002). From this type of analysis we could obtain information about genes that change as an individual worm ages, and we could compare this information with that from our large-scale studies.

During our work, two other studies also looked for the downstream targets of DAF-16. As mentioned above, Lee et al. (2003) looked for genes that contained the in vitro-defined DBE and had orthologs with DBEs in flies. From this list they were able to find several targets that indeed were regulated in a DAF-2/DAF-16-dependent fashion and proved to have life span functions, as well. We have found an additional motif as well as a slight relaxation of the previously defined DBE in the promoters of our DAF-2/DAF-16 genes, and the genes with these motifs would not have been identified in the Lee et al. study. Thus, their approach, while avoiding many false positives and finding genuine targets that have life span effects, missed many other true DAF-16 targets.

A study by McElwee et al. (2003) also used microarrays to find targets of DAF-2/DAF-16. Their experiments were limited to four comparisons of one allele of *daf-2* with a *daf-16*; *daf-2* double mutant at a single time point, and they find thousands of genes varying between their samples. Due to this large number, they were unable to verify the life span contributions of the genes.

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