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The search for DAF-16/FOXO transcriptional targets: Approaches and discoveries

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Abstract

The insulin/IGF-1 receptor (IIR)/FOXO pathway is remarkably conserved in worms, flies, and mammals, and downregulation of signaling in this pathway has been shown to extend lifespan in all of these animals. FOXO-mediated transcription is required for the long lifespan of IIR mutants; thus, there is great interest in identifying FOXO target genes, as they may carry out the biochemical activities that extend longevity. A number of approaches have been used to identify the transcriptional targets of FOXO. Thus far, the best data available on the components downstream of this pathway are from experiments involving the *Caenorhabditis elegans* FOXO transcription factor, DAF-16; some of these targets have been tested for their contributions to longevity, dauer formation, and fat storage. Here, I examine and compare the approaches used to identify DAF-16/FOXO targets, review the genes regulated by DAF-16, and discuss the processes that may be at work to extend lifespan in IIR mutants. Rather than upregulating every possible beneficial gene, DAF-16 appears to selectively upregulate genes that contribute to specific protective mechanisms, while simultaneously downregulating potentially deleterious genes. In addition to genes that carry out expected roles in stress protection, many previously unknown targets have been identified in these studies, suggesting that some mechanisms of lifespan extension still await discovery. These mechanisms may act cooperatively or cumulatively to increase longevity, and are likely to be at least partially conserved in higher organisms.

Keywords: *daf-2*; *daf-16*; FOXO; *C. elegans*; Longevity; Aging; Dauer; Fat

1. Introduction

The *Caenorhabditis elegans* FOXO transcription factor, DAF-16, is controlled by the activity of the DAF-2 insulin receptor, repressing DAF-16 activity through phosphorylation and cytoplasmic retention (Lin et al., 2001; Henderson and Johnson, 2001). In the absence of DAF-2/insulin receptor signaling, DAF-16/FOXO moves into the nucleus and regulates transcription of its targets. The identification of these target genes has been the focus of much attention because they may mediate the various phenotypes of *daf-2* mutants, including longevity determination (Kenyon et al., 1993), fat metabolism (Lee et al., 2003; Ashrafi et al., 2003), and formation of the diapause state known as dauer (Riddle and Albert, 1997). The genes making up the DAF-16-regulated transcriptome are likely to carry out the biochemical activities necessary for these phenotypes. The roles that DAF-16/FOXO performs in *C. elegans* may be broadly conserved, as its homologs in flies (dFOXO) and mammalian cells (FOXO3a, FOXO1) are also critical for lifespan (Hwangbo et al., 2004) and cell survival (Brunet et al., 2004). Elimination of the insulin receptor specifically from adipose tissue (the Fat Insulin Receptor KnockOut, or FIRKO, mouse) results in increased lifespan (Bluher et al., 2003), highlighting the importance of the longevity-determining genes regulated by FOXOs in mammals. The identification of FOXO target genes in all of these organisms will help unravel the mechanisms underlying longevity and cell survival. Once the roles of individual genes are elucidated, some may serve as targets for clinical intervention, both for normal aging and for age-related disease.

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2. Approaches used to identify DAF-16 targets

Of all the FOXO transcription factors, most is known about the targets of the *C. elegans* homolog, DAF-16. Identifying DAF-16 targets through classical genetics is challenging: an EMS screen for suppressors of the dauer-constitutive phenotype of *daf-2* in the presence of a *daf-16* transgene yielded many alleles of *daf-18* (the PTEN phosphatase upstream of *daf-16*) rather than genes downstream of *daf-16* (Lin et al., 2001). This is perhaps less surprising in light of later evidence that the functions of multiple genes may collectively contribute to *daf-2* phenotypes (Murphy et al., 2003).

Candidate gene tests have focused on a small number of genes implicated in processes regulated by DAF-16, such as resistance to such stresses as heat shock, oxidative stress, and heavy metal toxicity. The expression levels of superoxide dismutase (*sod-3*) (Honda and Honda, 1999), heat shock proteins (*hsp-16*) (Walker et al., 2001), and metallothionein (*mtl-1*) (Barsyte et al., 2001) were each found to be highly regulated by *daf-2* and *daf-16*. More recently, the *C. elegans* homolog of Raptor (a regulator of the nutrient-sensing TOR pathway), *daf-15*, was found to be negatively regulated by DAF-16 (Jia et al., 2004). Like *daf-2* mutants, *daf-15*/raptor loss of function results in lifespan extension, constitutive dauer formation, and fat accumulation (Jia et al., 2004). Additionally, DAF-16 regulates transcription of the cyclin-dependent kinase inhibitor *cki-1* during starvation-induced L1 arrest (Baugh and Sternberg, 2006). Thus, DAF-16-regulated transcription is an integration point for nutrient sensing, development, and insulin receptor signaling.

Ideally, one would be able to identify the complete suite of DAF-16 transcriptional targets, and unbiased methods can help overcome the limitations of the candidate gene approach. In a precursor to whole-genome expression array approaches, Yu and Larsen used differential display RT-PCR to find transcripts that differ in abundance between *daf-2* (*m41*) mutants and wild type worms, and identified a set of nine *dao* (Dauer or Aging adult Overexpression) genes (Yu and Larsen, 2001). *dao* genes have been identified in other *daf-2*/*daf-16* studies (Murphy et al., 2003; McElwee et al., 2003), and *dao-3* is required for *daf-2*'s long lifespan (Murphy et al., 2003).

Genome-wide approaches were made feasible by the availability of the complete *C. elegans* genome sequence (*C. elegans* Genome Sequence Consortium, 1998). One important step was the identification of the FOXO binding sequence, as promoters containing these regions are likely to be direct FOXO targets. Furuyama, et al. used an iterative *in vitro* method (four rounds of oligonucleotide selection and amplification) to find a core sequence that binds DAF-16, identifying TTGTTTAC as the consensus DAF-16 binding element (DBE) (Furuyama et al., 2000). This sequence differs slightly from the previously defined mammalian FOXO insulin-response element (IRE) (O'Brien et al., 1990), TT(g/a)TTTT(c/g), and *in vitro*, DAF-16 binds to the DBE more strongly than to the IRE (Furuyama et al., 2000). With a luciferase reporter assay in mammalian cells, they demonstrated that the DBE is functional for FOXO/FKHR activation *in vivo*; additionally, they found that the *C. elegans sod-3* gene (Honda and Honda, 1999) contains at least one copy of the DBE in its promoter (Furuyama et al., 2000). (The promoters of *mtl-1*, *hsp-12.6* (Murphy et al., 2003) and *daf-15* (Jia et al., 2004) contain DBE-like sequences, suggesting that these candidate genes are also direct DAF-16 targets.)

Lee et al. (2003) took advantage of this knowledge to tease out putative DAF-16 targets bioinformatically: they identified genes with the *in vitro*-defined DBE in the first 1 kb of their promoters in *C. elegans* (947 genes) and *Drosophila* (1760 genes), compared these with a list of 3283 worm/fly orthologs, and identified 17 candidates to study in detail. They found that six of the 17 genes are differentially regulated by *daf-2* and *daf-16*, and using double-stranded RNA interference (RNAi) to test biological function, they showed that four of the 17 (*rbp-2*, *hpd-1*, *mrp-5*, and *pnk-1*) affect lifespan. *mrp-5* and *hpd-1* also affect dauer formation, while *pnk-1* RNAi reduces fat storage (Lee et al., 2003) (Table 1). Notably, the activities of the genes affect the phenotypes in the predicted direction: *daf-2*-upregulated genes (Class 1 genes) would be predicted to promote dauer formation, fat storage, and lifespan, while *daf-2*-downregulated (Class 2) genes might suppress these phenotypes. Similarly, Ookuma et al. (2003) searched for genes with DBE-containing promoters, restricted the list to 159 genes with mammalian homologs, and identified *scl-1*, an SCP-like extracellular protein, as a *daf-2*-upregulated DAF-16 target. In RNAi experiments, *scl-1* was shown to be required for longevity, fat storage, and heat and UV stress resistance (Ookuma et al., 2003). Several members of the SCP family, including *scl-1* and *tpx-1*, were also identified as strongly regulated DAF-16 targets in microarray studies (Murphy et al., 2003; McElwee et al., 2003).

Because DAF-16 is a transcription factor, expression microarrays are an ideal approach for identifying its downstream targets, and have been used in several studies of the *daf-2*/*daf-16* pathway (Murphy et al., 2003; McElwee et al., 2003, 2004; Golden and Melov, 2004). McElwee et al. used four microarrays to compare *daf-2*(*e1370*) and *daf-16*(*m27*); *daf-2*(*e1370*) (McElwee et al., 2003), and in a later study, used five replicates of *daf-2*(*e1370*) and *daf-2*(*m577*) vs. *daf-16*(*mgDf50*); *daf-2*(*e1370* or *m577*) (McElwee et al., 2004) to identify differentially regulated genes in the two mutants on the first day of adulthood. Surprisingly, the two studies revealed only partially overlapping sets of genes: 15% (504) of the 3354 genes were identified as differentially expressed between the long- and short-lived mutants in both studies (McElwee et al., 2004). In the initial study, the authors chose 35 genes to study further by RNA interference, initially testing dauer formation; four of these genes were further tested and were found to affect either lifespan or development. RNAi of ZC334.2/*ins-30* and ZK430.3/*sod-5* increased lifespan, while ZK384.3/

aspartyl protease and C08A9.1/*sod-3* RNAi shortened lifespan (McElwee et al., 2003). Curiously, the expression of ZC334.2 and ZK430.3, which are upregulated in *daf-2* mutants (suggesting that their upregulation promotes longevity), did not correlate with their effects on lifespan by RNAi reduction of function (Table 1).

Another expression analysis study (Murphy et al., 2003) used an experimental design that sought to reduce false positives while identifying *daf-2*/*daf-16*-dependent genes by combining the analysis of multiple mutant strains with *daf-2* and *daf-16*-RNAi treatments, which recapitulates *daf-2* and *daf-16* mutant lifespans (Dillin et al., 2002). First, gene expression in *age-1* (the PI-3-Kinase downstream of the insulin receptor (Johnson, 1990; Morris et al., 1996)), three different alleles of *daf-2*(*e1370*, *e1368*, and *mu150*), and *daf-16* overexpression strains were compared with wild type and *daf-16*; *daf-2* double mutants to identify differentially expressed genes that are shared across these genotypes on the first day of adulthood (13 arrays). SAM (Significance Analysis of Microarrays (Tusher et al., 2001)) was used to identify genes that are consistently differentially expressed in these mutant comparisons. Additionally, two 10-point timecourses (0–48 h and 0–8 days) of *daf-2*, *daf-16*, and control RNAi-treatments of isogenic adult worms were used to distinguish age-dependent changes from true *daf-2*/*daf-16*-dependent changes (60 arrays). When hierarchically clustered (Eisen et al., 1998), the set of genes that are regulated by both *daf-2* and *daf-16* clusters separately from genes that change with age in all RNAi treatments (including control RNAi), such as collagens.

Reasoning that true DAF-16 targets should change across all of the *daf-2*/*daf-16* conditions, the data from this set of 73 microarrays was incorporated into a global analysis and rank ordered by three criteria: (1) presence in the *daf-2*/*daf-16*-dependent cluster, (2) magnitude of expression change, and (3) the significance of expression change, as defined by SAM analysis. The top 58 candidates were tested for lifespan phenotypes. From this unbiased selection, the majority of these high-scoring genes (50 of 58, or 86%) were found to have significant effects on longevity ($p \leqslant 0.05$, 10–20% change). Strikingly, 49 of these 50 genes affected longevity in the direction predicted by their expression patterns in *daf-2* worms. A number of these top hits were also identified in an independent RNAi screen for fat content (Ashrafi et al., 2003), including two cytochrome P450s, the *pep-2*/*opt-2* oligopeptide transporter, *dod-20* (*downstream of DAF-16-20*), and *fat-7* (Table 1).

The promoters of genes identified through cluster analysis can also be mined for regulatory information. Using unbiased overrepresentation motif analyses, rather than simply searching for a known site, is extremely useful both for the discovery of new motifs and for testing the hypothesis that a presumed transcription factor binding site is actually present in regulated genes' promoters. Two different algorithms (Bussemaker et al., 2000; van Helden et al., 1998) were used to examine the 1 kb region upstream of genes identified in the *daf-2*/*daf-16* cluster analysis (Murphy et al., 2003). This analysis revealed that (1) the DBE is indeed overrepresented in these genes' promoters, but exhibits some variation from the *in vitro*-identified canonical DBE sequence at particular nucleotides *in vivo* (Tg/aTTTAC), (2) the DBE is present in both up- and downregulated genes' promoters, suggesting a direct role for DAF-16 in transcriptional repression, and (3) a second motif, the DAF-16 Associated Element (DAE), CTTATCA, is highly overrepresented in the promoters of DAF-16-regulated genes. Because this motif is very different from the DBE, it is unlikely to be bound directly by DAF-16 and suggests the role of an additional factor (Murphy et al., 2003). Therefore, previous searches for DAF-16 targets using the canonical DBE (Lee et al., 2003; McElwee et al., 2003) may have missed some true targets, and the presence of the DBE in the whole promoterome should be re-assessed (Kenyon and Murphy, 2006): 13% of all *C. elegans* promoters contain the DBE within 1 kb upstream (this rises to 45% over 5 kb), and 22% contain either the DBE or the DAE in 1 kb of the promoter (and climbs to 78% if 5 kb upstream is included) (Kenyon and Murphy, 2006). Thus, it is remarkable that while the top 58 candidates were selected exclusively by expression criteria, the DAF-16 binding elements were highly overrepresented in the promoters of this set of genes relative to the rest of the promoters in the genome (57 of 58, or 98%). This suggests that many of these targets, including downregulated genes, are directly regulated by DAF-16 (Murphy et al., 2003; Kenyon and Murphy, 2006).

Serial analysis of gene expression (SAGE), which uses sequence tags for an entire sample to identify transcripts, has also been used to compare the *daf-2* and wild type transcriptomes (Halaschek-Wiener et al., 2005). Halaschek-Wiener et al. built two SAGE libraries from adult control worms (day 1 and day 6), and three libraries from *daf-2*(*m41*) worms (day 1, day 6, and day 10). They compared these SAGE libraries to find transcripts that were upregulated in long-lived worms (*daf-2*) and in worms in early stages of aging (wild type day 6 and *daf-2* day 10). The rest of their analyses focused on genes with known or inferred functions (406 genes). They used a statistical analysis of Gene Ontology classes to identify major biological pathways represented by the transcripts. The major classes included metabolism and oxidative stress (Halaschek-Wiener et al., 2005). While there were no functional assays of the candidates, the genes and biological categories overlap significantly with those identified through microarray studies (Murphy et al., 2003; McElwee et al., 2003).

Expression microarray and SAGE analyses identify transcripts that are differentially expressed between two samples, but cannot distinguish direct from indirect targets. To solve this problem, direct binding techniques are necessary. Chromatin immunoprecipitation (chIP) methods use antibodies to pull down proteins along with crosslinked DNA, and have the potential to identify sequences directly bound by transcription factors. Oh et al. (2005) immuno-

Table 1
Unbiased/whole-genome methods used to identify DAF-16-regulated genes

Reference	Technique	Conditions	# Targets ID'd	Method used to select genes for phenotypic analysis	Targets with phenotypes		% Functional hits, correlation w/expression in *daf-2* mutants
Yu and Larsen (2001)	Differential display RT-PCR	Wild type (N2) vs. *daf-2(m41)*	9 (*dao-3*[b])	None tested			
Lee et al. (2003)	Bioinformatic search for orthologs in flies & worms w/canonical DBE	*In silico*; *C. elegans* and Drosophila genomes; TTGTTTAC in 1 kb upstream of ATG; list of 3283 worm/fly orthologs	17	Tested all 17 genes for lifespan (LS), dauer (D), and fat storage (F)	*rbp-2* *hpd-1* *mrp-5* *pnk-1*	LS LS, D LS, D LS, F	4 of 17 (23%) genes identified have at least one phenotype 4 of 4 (100%) match direction of expession in *daf-2*
Ookuma et al. (2003)	Bioinformatic search for genes with DBE & mammalian homologs	*In silico*; *C. elegans* genome; genes w/mammalian homologs	159	Tested one gene, *scl-1*, for lifespan, fat storage, stress resistance (SR)	*scl-1*[b]	LS, F, SR	1 of 159 (0.6%) matches *daf-2* expression direction
McElwee et al. (2003)	Expression microarray	4 Arrays of *daf-2(e1370)* vs. *daf-16(m27)*; *daf-2(e1370)*; synchronized adults	1646	Tested 4 genes for lifespan, dauer formation after 35 chosen by >1.5x expression change	*ins-30*[a] *sod-5*[a] ZK384.3[b] *sod-3*[b]	LS LS LS LS,devel.	4 of 1646 (0.2%) total genes with phenotypes, or 4 of 35 (11.4%) chosen by dauer pre-screening 2 of 4 (50%) match *daf-2* expression direction
Murphy et al. (2003)	Expression microarray	73 Arrays; All synchronized adults: 1. Mutants •3 × *daf-2(e1370)* vs. *daf-16(mu86)*; *daf-2(e1370)* •*fer-15*; *daf-2(e1368)*; *fem-1* vs. *fer-15*; *fem-1* •*daf-2(mu150)* vs. wild type (N2) •4 × *fer-15*; *daf-2(mu150)*; *fem-1* vs. *fer-15*; *fem-1* •*daf-2(e1370)*; *daf-16*::*gfp in daf-16(mu86)*; *daf-2(e1370)* vs.*daf-16(mu86)*; *daf-2(e1370)* •*age-1(h × 546)* vs. wild type (N2)	~500 Ranked by priority score (see next column)	Prioritized genes into 6 groups by combination of (a) Fold expression change, (b) consistency (*p*-value), (c) presence in *daf-2*/*daf-16*-dependent cluster; Tested 58 genes in the top priority group for lifespan effects (all statistics available in Murphy et al., 2003)	1. Class I (upreg'd by DAF-16 in *daf-2* mutants): *ctl-2* *dod-1* *hsp-16.1* *lys-7* *dod-2* *hsp-12.6* *mtl-1* *gei-7* *dod-3* *dod-4* *dod-5* *fat-7* *dod-6* *hsp-16.49* *bir-2* *hsp-16.11* *bir-2*	 LS LS, F[d] LS LS LS LS LS LS LS LS LS LS, F[d] LS LS LS LS LS	50 of 58 (86%) top priority genes have lifespan effects ($p \leqslant 0.0001$–0.05); 49 of 50 (98%) with lifespan effects match *daf-2* expression direction

(*continued on next page*)

Table 1 (*continued*)

Reference	Technique	Conditions	# Targets ID'd	Method used to select genes for phenotypic analysis	Targets with phenotypes		% Functional hits, correlation w/expression in *daf-2* mutants
Murphy et al. (2003)		•3 × *fer-15 age-1*(*h* × 546); *fem-1* vs. *fer-15*; *fem-1*			T20G5.8	LS	
					spp-1	LS	
					dod-7	LS	
					dod-8	LS	
					ctl-1	LS	
		2. RNAi timecourses			*dod-9*	LS	
		•60 microarrays of RNAi-treatment (*daf-2*, *daf-16*+*daf-2*, and *control RNAi*):			*sip-1*	LS	
					dod-10	LS	
					gcp-1	LS	
		•3 × 10 timepoints, 0-48 h			*dod-11*[c]		
		•3 × 10 timepoints, 0–8 days			*ges-1*[c]		
					dod-12[c]		
					asp-3[c]		
					hsp-16.2	LS	
					dod-13	LS, F[d]	
					mdl-1	LS	
					sod-3	LS	
					gpd-2[c]		
					dao-3	LS	
					ins-18 [e]	LS,D	
					dod-14	LS	
					dod-15[c]	LS	
					dod-16[a]	LS	
					2. Class 2 (downreg'd by DAF-16 in *daf-2* mutants):	LS	
					nuc-1	LS	
					dod-18	LS	
					dod-19	LS	
					gcy-6	LS	
					dod-20	LS, F[d]	
					dod-21	LS	
					vit-5	LS	
					mtl-2	LS	
					dod-22	LS	
					dod-23	LS	
					lbp-7	LS	

					pep-2	LS, F[d]	
					ins-7	LS, D	
					pes-2[c]		
					old-1	LS	
					dod-24	LS	
					gcy-18	LS	
					vit-2	LS	
McElwee et al. (2004)	Expression microarray	5 replicates of *daf-2*(*m577*) and *daf-2*(*e1370*) vs. *daf-16*(*mgDf50*); *daf-2*(*e1370* or *m577*); Synchronized adults	1890	None tested			
Halaschek-Wiener et al. (2005)	Serial Analysis of Gene Expression (SAGE)	Synchronized adults: •*daf-2*(*m41*), day 1, day 6, and day 10 •Wild type (N2) day 1, day 6	406	None tested			
Oh et al. (2005)	Chromatin IP, subcloning, and sequencing (320 clones)	Mixed (not synchronized) cultures of *daf-2*(*e1370*)	130	Tested 33 genes containing a DBE or DAE within 5 kb promoter region for dauer formation, lifespan, and fat storage; Lifespan statistics not reported	C01A2.2[a]	F	18 of 130 (14%) total genes identified have at least one phenotype, or 18 of 33 (54.5%) genes tested; 3 out of 18 (17%) genes with phenotypes (*glh-1*, K06A9.1, and F42G10.1) match *daf-2* expression direction.
					C01B7.1[ab]	LS, D	
					aqp-2[a]	D	
					C36A4.9[a]	F, D	
					srh-99[a]	F, D	
					D1054.6[a]	F	
					F07F6.1[a]	F	
					lin-2[a]	LS	
					egl-10[a]	LS	
					F42G10.1	LS	
					unc-84[a]	F	
					zfp-1[a]	LS, F, D	
					K06A9.1	F	
					ldb-1[a]	LS	
					sca-1[a]	LS, D	
					glh-1	F, D	
					Y38E10A.4[a]	D	
					Y48A6B.6[a]	D	

D, F, LS, SR: Authors report dauer formation (D), fat storage (F), lifespan (LS), or stress response (SR) phenotype.

[a] Does not match direction of expression change and/or other *daf-2*-associated phenotypes.

[b] Also identified in Murphy et al., 2003.

[c] Small lifespan effect.

[d] Also found in screen for fat content (Ashrafi et al., 2003).

precipitated DAF-16 and DNA from mixed-stage cultures, amplified and subcloned the DNA, and sequenced 320 clones, of which 140 were bacterial and 130 were *C. elegans* genes. Surprisingly, only one of the 130 genes had been identified in previous DAF-16 studies. Eighty-eight of these genes contain either the DBE or the DAE (DAF-16 Associated Element) (Murphy et al., 2003) in the 5 kb region upstream, and were thus considered putative DAF-16 targets. (It should be noted, however, that this fraction (67%) is lower than the average occurrence in the promoterome (78%), that the DAE is unlikely to be directly bound by DAF-16 and the fraction of these genes containing just the DBE in 5 kb upstream (33.8%) is lower than in the general promoterome (45.1% (Kenyon and Murphy, 2006)). Thirty-three genes were studied further by RNAi treatment and phenotypic analysis (Table 1). Eighteen of these genes affected fat accumulation, dauer formation, or lifespan in some manner, and six affected more than one phenotype. Oddly, of the 18 genes with phenotypes, only three of the genes (*glh-1*/germline helicase, K06A9.1/mucin, and F42G10.1/neprilysin) have activities that correlate with their regulation by DAF-16. That is, *glh-1*, K06A9.1, and F42G10.1 expression is reduced in *daf-16* mutants relative to wild type, and RNAi of these genes decreases fat storage, dauer formation, or lifespan (Oh et al., 2005), as one would expect. RNAi of the rest of the tested genes show conflicting relationships between expression and dauer formation, lifespan, and fat storage.

In summary, at least five different approaches (differential display PCR, bioinformatic searches, expression microarrays, SAGE, and chIP) have been used to identify DAF-16 targets thus far (Table 1). Many putative targets have been identified, and some have been verified phenotypically, but additional functional studies will be necessary to clarify the roles of these targets in *daf-2* animals.

3. Why might the DAF-16 target sets vary?

It is fair to ask at this point how much overlap exists between the target sets produced by these studies, and if there are differences, what are the sources of this variation? In fact, many of the significantly changed genes have been identified in multiple studies (Murphy et al., 2003; McElwee et al., 2003, 2004; Halaschek-Wiener et al., 2005), so it is likely that most of the approaches have resulted in valuable data. However, both biological factors and technical methodologies may lead to variations in the sets of identified genes. Candidate and non-genome-wide approaches (e.g., differential-display PCR, chIP with sequencing) cannot, of course, be comprehensive, so these techniques will only yield small lists of select DAF-16-regulated genes; most of these techniques have now been supplanted by genome-wide approaches (expression arrays, SAGE, chIP/chip). The targets identified by some of these more limited approaches would be expected to be a subset of the previously identified sets; for example, the chIP-identified set (Oh et al., 2005) should, in theory, be a subset of the expression set (Murphy et al., 2003; McElwee et al., 2003, 2004; Halaschek-Wiener et al., 2005), which may include both indirect and direct DAF-16 targets, so it is surprising that none of these chIP targets have been found by other genome-wide approaches. This could be due simply to the non-comprehensive nature of chIP/sequencing and the limited number of clones sequenced, or to the use of mixed-stage cultures, rather than the synchronized adult cultures used in other genome-wide studies (Murphy et al., 2003; McElwee et al., 2003, 2004; Halaschek-Wiener et al., 2005). Whole-genome target-binding approaches, such as chIP/chip (Iyer, 2001; Lieb et al., 2001) and STAGE (SACO) (Kim et al., 2005; Impey et al., 2004), use full-genome microarrays or sequencing of hundreds of thousands of tags thereby reducing the occurrence of false positives. The application of these whole-genome approaches to DAF-16/DNA binding should identify true direct targets of DAF-16.

Genome-wide techniques have limitations as well. For example, differences between target lists have been attributed to differences in microarray platform (McElwee et al., 2003, 2004), although these differences should, in theory, be restricted to low-abundance transcripts and can be eliminated by more stringent false positive elimination (Murphy et al., 2003). Additionally, non-tiling microarrays require each open reading frame to have been predicted and deposited on the array prior to the experiment, precluding the identification of any transcripts corresponding to unpredicted ORFs. The development of whole-genome tiling arrays should resolve these issues. In principle, SAGE is not limited by *a priori* gene predictions, and in fact, the results from many expression analyses and SAGE have largely overlapped (Murphy et al., 2003; McElwee et al., 2003, 2004; Halaschek-Wiener et al., 2005).

More fundamentally, the biology of *daf-2* mutants and the aging transcriptome must be taken into account when evaluating the results of each study. *daf-2* mutants develop slowly, and experiments that use a single timepoint for comparison are especially vulnerable to the mis-identification of developmental or aging targets as *daf-2*/*daf-16*-dependent targets. Some of these issues can be addressed through the incorporation of additional timepoints (Golden and Melov, 2004; Halaschek-Wiener et al., 2005) or through detailed timecourses that profile control, long-lived, and short-lived animals. Another factor is the synchrony of the animals; most, but not all, of the studies described here used synchronized populations to identify *daf-2*/*daf-16* targets. The genes identified from an unsynchronized, mixed-stage population may indeed be DAF-16 targets, but should not necessarily be expected to overlap with the target sets identified from synchronized adult populations.

In addition to the technical aspects of the different approaches, the selection criteria used to define the target gene set varies. The use of Gene Ontology (GO) terms has been quite useful in creating hypotheses of the underly-

ing biological mechanisms at work (McElwee et al., 2004; Halaschek-Wiener et al., 2005; McCarroll et al., 2004). One danger in relying too heavily on GO terms, however, is the temptation to ignore or eliminate genes of unknown function from analysis, restricting interpretation of function to previously described mechanisms. This issue is amplified when candidates are not tested for phenotypes, because genes of minor functional importance may be given undue weight. Both problems reduce the advantage that unbiased genome-wide approaches offer over candidate gene approaches.

For a set of transcriptional targets to be useful, the biologist would like not only a list of genes, but also a metric of the confidence that the particular gene is actually regulated by the pathway. It is important to consider the notion of false positives in any discussion of genome-wide approaches; longer lists will provide more overlap between sets, but will also increase the number of false positives. In practice, reducing false positive rates involves not only performing a sufficient number of replicates to achieve statistical significance, but also doing multiple types of experiments to be sure that the observed differences in expression are due to biological differences, rather than to real but biologically uninteresting sources of variation. In addition to time-courses, the analysis of multiple alleles and genes in the same pathway, the use of RNAi to mimic phenotypes, and alternative methods of identification can help reduce the number of false positives included in the target gene list (Lee et al., 2003; Murphy et al., 2003). Although the resulting list may be shorter, its value to the biologist is higher. Other metrics, such as the presence of the DBE in the promoters of selected genes, have been used as validation (McElwee et al., 2003, 2004; Halaschek-Wiener et al., 2005; Oh et al., 2005), but all bioinformatic approaches should consider the rate of enrichment relative to the random appearance in the whole genome or promoterome (Kenyon and Murphy, 2006).

Of course, the real test of biological significance is function. Many of the studies mentioned here have taken the necessary next step of testing at least some of their candidates for roles in *daf-2*/*daf-16*-associated phenotypes, such as dauer formation, fat storage, and longevity (Lee et al., 2003; Murphy et al., 2003; Jia et al., 2004; McElwee et al., 2003; Ookuma et al., 2003; Oh et al., 2005). The development of RNAi techniques, especially by feeding in *C. elegans*, has greatly improved the potential throughput of phenotypic analysis of large numbers of candidate genes from genome-wide approaches. The availability of *C. elegans* deletion strains is also growing (*C. elegans* Gene Knockout Consortium), and in a few cases they have confirmed initial RNAi results or resolved off-target effect questions (Petriv and Rachubinski, 2004). Deletion strains may be the "gold standard" for phenotypic analysis, especially for neuronally expressed genes, since neurons are refractory to RNAi. However, RNAi can also be used to knock down gene function solely in adulthood, avoiding deleterious developmental effects, and may often be a better tool for lifespan studies (Dillin et al., 2002). In any case, RNAi clones for most of the *C. elegans* genome have been available for several years now (Fraser et al., 2000), and the ease of feeding RNAi means that even long lists of candidate genes can be followed up by phenotypic analysis. Functional testing of candidates will not only help eliminate false positives, but can also determine the degree to which each DAF-16 target gene functions in the determination of a particular *daf-2* phenotype (Murphy et al., 2003). Usually, the positive or negative contribution of a gene's function to a phenotype, such as longevity or fat storage, correlates with its *daf-2*/*daf-16*-dependent expression (Lee et al., 2003; Murphy et al., 2003; Jia et al., 2004; Ookuma et al., 2003). Such information for all putative DAF-16 target genes will aid in building models of pathway function.

In addition to the genes found through differential *daf-2*/*daf-16* approaches, it is likely that some of the genes identified in whole-genome RNAi longevity screens will be found to be DAF-16 targets (Hansen et al., 2005; Hamilton et al., 2005). These connections are not yet obvious for several reasons: (1) the RNAi longevity screens have screened for maximum, rather than mean, lifespan changes, and many of the DAF-16 targets seem to act cumulatively to affect lifespan (Murphy et al., 2003); (2) these screens have looked for lifespan increases, which would identify only Class 2 (DAF-16-downregulated) genes, rather than the more familiar Class 1 (DAF-16-upregulated) genes (Murphy et al., 2003); and (3) epistasis tests have identified genes acting upstream of known longevity pathways (Hansen et al., 2005; Hamilton et al., 2005), but would not have been able to identify genes downstream of DAF-16. In fact, 5 of the 19 DAF-16-downregulated (Class 2) genes from microarray analysis (Murphy et al., 2003) were among the 89 genes identified in a recent whole-genome RNAi longevity screen (Hamilton et al., 2005). A *daf-2* suppressor RNAi screen would be interesting, and could serve as a nice verification of the roles of putative *daf-16*-activated DAF-16 targets.

Eventually, expression experiments, direct binding approaches, and candidate phenotype testing will help to identify a complete, single set of true DAF-16 targets. In addition to the current set of *daf-2*-dependent *daf-16* targets, future data should include genes that act downstream of DAF-16 through interactions with other genes, including *jnk-1* (Oh et al., 2005), *bar-1* (Essers et al., 2005), *smk-1* (Wolff et al., 2006), *sir-2* (Tissenbaum and Guarente, 2001) and *cst-1* (Lehtinen et al., 2006). The definition of a complete DAF-16 target set and the contributions to *daf-2* pathway activity, therefore, is an ongoing project.

4. DAF-16 target genes: clues to underlying biological mechanisms

Now that several approaches have identified DAF-16 targets, we can begin to think about how they contribute to *daf-16*-mediated phenotypes. Many of the DAF-16

targets revealed by genome-wide approaches were expected, as they had already been studied in candidate approaches. Additionally, many are shared with the dauer transcriptome (McElwee et al., 2004; Halaschek-Wiener et al., 2005; Wang and Kim, 2003); this is not surprising, given that *daf-2* mutants form dauers constitutively at high temperatures, and dauers are long-lived (reviewed elsewhere in this volume). A large number of these genes may play a role in protection from stresses. For example, genes involved in oxidative stress response, such as superoxide dismutases, catalases, and glutathione S-transferases, were found to be upregulated in *daf-2* mutants by candidate as well as genome-wide approaches (Murphy et al., 2003; McElwee et al., 2003; Halaschek-Wiener et al., 2005; Oh et al., 2005). Heat shock proteins, in particular *hsp-16*, *hsp-12.6*, and *sip-1* as well as other anti-toxicity genes (the metallothionein *mtl-1* and xenobiotic metabolism genes) are upregulated in both *daf-2* and dauer worms (Halaschek-Wiener et al., 2005; Murphy et al., 2003; Barsyte et al., 2001; McElwee et al., 2004; Hsu et al., 2003). Genes involved in pathogen resistance, such as *lys-7*, *spp-1*, and thaumatins and hypertonic stress resistance (trehalose 6-phosphate synthases *tps-1* and *tps-2*) are also upregulated, extending the theme of protection from many different kinds of stresses (Murphy et al., 2003; Mallo et al., 2002; Lamitina and Strange, 2005). Interestingly, rather than upregulating whole classes of genes, as is observed upon stress treatments (heat, heavy metal toxicity, etc.), DAF-16 appears to select specific members of a variety of classes to achieve increased stress protection.

daf-2 and dauer worms increase fat storage and shift their metabolism, enabling the use of stored fat for energy (Riddle and Albert, 1997; Wang and Kim, 2003; Rea and Johnson, 2003), likely explaining the fact that a number of the DAF-16 regulated targets are metabolic. Lipid and carbohydrate metabolism are highly regulated by the *daf-2*/*daf-16* pathway, through genes involved in fat storage, ß-oxidation of fatty acids, coenzyme A synthesis, glyoxylate cycle regulation, and gluconeogenesis. The *daf-2*/*daf-16* pathway regulates the expression of fatty-acid desaturase *fat*-genes, lipid-binding protein *lbp-7*, acyl CoA dehydrogenase, pantothenate kinase *pnk-1*, the glyoxylate cycle regulator isocitrate lyase /malate synthase *gei-7*, and the rate-limiting gluconeogenesis enzyme PhosphoEnolPyruvate CarboxyKinase (PEPCK) (Murphy et al., 2003; Lee et al., 2003; Oh et al., 2005; Halaschek-Wiener et al., 2005; Wang and Kim, 2003). Intestinal genes, such as alcohol dehydrogenase *dod-11* (Murphy et al., 2003; Halaschek-Wiener et al., 2005), gut esterase *ges-1*, gut cysteine protease *gcp-1*/*cpr-1*, aspartyl protease *asp-3* (Murphy et al., 2003), and cholesterol-based bile acid and steroid hormone synthesis and transport genes (transthyretins, cytP450s, estradiol dehydrogenases (Murphy et al., 2003)) are also regulated by the *daf-2*/*daf-16* pathway. The *tps* (trehalose 6-phosphate synthase) genes might do double-duty in increasing lifespan of *daf-2* worms: trehalose may function metabolically as a sugar (Kormish and McGhee, 2005) in addition to its protein-protective role (Lamitina and Strange, 2005). Many of these genes were found by multiple approaches, and have been verified by phenotypic analysis or identified in phenotypic screens (Lee et al., 2003; Murphy et al., 2003; Oh et al., 2005).

Not all of the genes regulated by *daf-2* and *daf-16* are of the expected stress protection and metabolism classes, however. First, many genes appear to be negatively regulated by DAF-16 (Lee et al., 2003; Murphy et al., 2003; Oh et al., 2005), and many of these genes' promoters contain the DBE, suggesting a direct role for DAF-16 in transcriptional repression. This idea is consistent with the fact that mammalian FOXO repression of cyclin genes is a mechanism for suppression of tumor cell growth (Ramaswamy et al., 2002). The DAF-16 repressed set includes genes involved in neuronal signaling (*gcy* guanylate cyclases), apolipoprotein binding (*vit* genes), RNA-binding (Pumilio *puf* genes, ribosomal *rpl* and *rps* genes, DNA replication (*mcm* genes, *pcn-1*) and protein degradation (*skr* genes (Murphy et al., 2003; Halaschek-Wiener et al., 2005)). Additionally, DAF-16 represses the insulin-like peptide *ins-7*, providing a positive feedback mechanism of insulin pathway regulation (Murphy et al., 2003). Intriguingly, many genes of unknown function are also regulated by *daf-2* and *daf-16* (Lee et al., 2003; Murphy et al., 2003) (Fig. 1). Although often ignored during discussions of mechanism and not chosen for candidate studies, many of these unknown genes have significant effects on longevity. Thus, the studies of DAF-16 targets have uncovered both the identities of many of the targets, as well as a potential new function for DAF-16 in transcriptional repression. New mechanisms of longevity regulation are likely to be revealed through study of previously uncharacterized DAF-16 target genes.

It should be noted that a large fraction of the genes regulated by *daf-2* and *daf-16* are known to be expressed intestinally and/or map to the Kim laboratory's Intestinal "Topomountain" (Rea and Johnson, 2003; C.T. Murphy, unpublished data; Murphy et al., 2003; McElwee et al., 2004; Halaschek-Wiener et al., 2005; Kim et al., 2001). The observation that neuronally expressed DAF-2 shortens the lifespan of *daf-2* mutants (Wolkow et al., 2000) suggested that neurons are the critical lifespan-determining tissue, and that whole-animal transcriptional analyses may be poor representations of the longevity transcriptome. However, Libina et al. showed that intestinally expressed DAF-16 can extend the lifespan of *daf-16* worms by ~60% (Libina et al., 2003). Additionally, *daf-2* and *daf-16* are expressed in many tissues (Ogg et al., 1997) and it is possible that many of the same targets are engaged in most cells. Thus, given the bulk of the intestine and its large role in lifespan determination, it is possible that many of the relevant longevity genes have already been

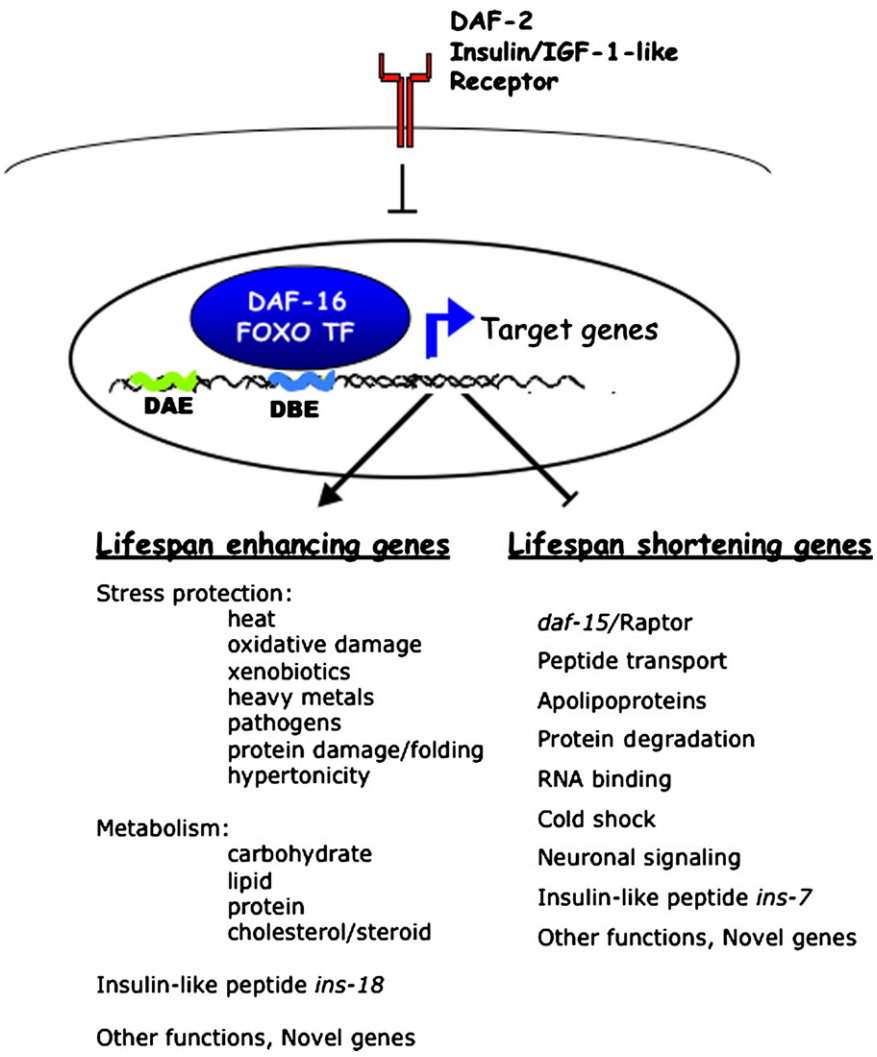

Fig. 1. DAF-16/FOXO regulates the transcriptional activation and repression of many genes; these genes participate in the promotion or prevention of longevity. DAF-16 targets are also involved in dauer formation/maintenance and fat storage.

identified, although further tissue-specific studies will test this hypothesis.[1]

Like DAF-16, *Drosophila* dFOXO activity regulates longevity (Hwangbo et al., 2004; Wessells et al., 2004), and the targets downstream of the mouse adipose insulin receptor must also control lifespan (Bluher et al., 2003). Although the full transcriptomes of *Drosophila* and mammalian FOXOs have not yet been described, among the known transcriptional targets of dFOXO are the translational inhibitor factor d4EBP, the insulin receptor dInR, IRS-2, (Puig and Tjian, 2005), and specific insulin-like peptides (dILP2) (Hwangbo et al., 2004). Thus, much like the regulation of the insulin pathway through insulin-like peptides in *C. elegans* (Murphy et al., 2003), feedback control of the insulin pathway is a major output of the *Drosophila* system (Puig and Tjian, 2005). Glucose homeostasis is an important regulatory target of the FOXOs, as PEPCK is regulated by the worm, fly and mammalian homologs (Murphy et al., 2003; Ramaswamy et al., 2002; Puig and Tjian, 2005). The various homologs of FOXO in mammals are likely to have specific targets, and tissue-specific forms may regulate subsets of these targets. Genes involved in cell death (BIM-1, bcl-6), metabolism (PEPCK, Glucose-6-phosphatase), DNA repair (GADD45, DDB1), cell differentiation (p21CIP1), and oxidative stress protection

[1] Although there is concern that genes that are regulated in a small number of cells might be missed in microarray analyses, in fact this may not be problematic. The gene may be lost from the analysis if it is expressed at low levels, and only exhibits small differences in different genotypes, in analyses that eliminate genes with low expression, but this is also true of genes expressed in many cells with small differences across conditions. Approaches such as SAM (Tusher, et al. 2001) are successful at identifying genes with small but consistent expression differences (e.g., *ins-18* in *daf-2* vs. *daf-16*; *daf-2* worms). Genes that are expressed in a small number of cells (e.g., *gcy-6*, which is only in ASEL in wild type animals) can be identified if they undergo large expression changes. Genes expressed in one set of cells in one condition, but in a different set of cells in another (so both the absolute levels and the ratio would remain largely unchanged) and genes expressed at low abundance levels remain difficult to identify, however.

(MnSOD, catalase) are all regulated by FOXO transcriptional activity (Ramaswamy et al., 2002; Puig and Tjian, 2005; Greer and Brunet, 2005; Puig et al., 2003; Puig and Tjian, 2006). Like *C. elegans* DAF-16's regulation of the cyclin-dependent kinase inhibitor *cki-1* (Baugh and Sternberg, 2006), mammalian FOXO regulates transcription of cell cycle arrest genes (p27KIP1, p21CIP1, and cyclin G2). While the most obvious shared genes are of the stress protection and metabolism classes, additional commonly regulated genes will become apparent through ortholog analyses (McCarroll et al., 2004), and will help tease out the general functions of FOXOs from those that are specific to particular tissues and organisms. It will also be interesting to find the FOXO targets in particular tissues, such as cardiac muscle and neurons (which are extremely metabolically active and are likely to require high levels of oxidative stress protection), and kidneys (which encounter high levels of toxins). Additionally, the FOXO transcriptional data should be compared with general aging transcriptional analyses to more fully understand which genes are likely to be critical in increasing longevity, and which may be used as age and age-related disease biomarkers (Murphy, 2006).

5. Conclusions

What has been learned by amassing this collection of genes regulated by *daf-2* and *daf-16*? While many of the targets confirm previous hypotheses (protection from a variety of stresses, overlap with dauer genes), many of the biological functions of FOXO/DAF-16 targets remain undiscovered. The vast majority of the genes have not yet been tested for their contributions to *daf-2* phenotypes; until those genes are analyzed, it seems premature to claim that the mechanisms by which insulin receptor mutants are long-lived are fully understood. However, it does seem clear that DAF-16 regulation is a critical integration point for signals, and its ability to select and regulate specific genes is remarkable. Interestingly, DAF-16 activation appears to selectively upregulate genes that contribute to specific protective mechanisms, which may act in a cooperative or cumulative manner to increase longevity; these mechanisms are likely to be conserved in higher organisms (Murphy, 2006). Another point is that many of the functions downstream of *daf-2*/*daf-16* seem to be cell autonomous, mediated by direct targets of DAF-16, rather than acting through a large network of transcription cascades, increasing the likelihood that the targets will be shared across phylogeny. By identifying DAF-16 target genes and establishing their individual roles in insulin receptor mutant phenotypes, we will be able to unravel these pleiotropies, which may allow the identification of specific intervention candidates. The identification of FOXO targets and the associated longevity-promoting mechanisms will augment our understanding of the biological mechanisms necessary to increase cell survival and longevity. This knowledge should enhance our ability to understand and treat age-related disease.

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