Co-Regulation of the DAF-16 Target Gene, cyp-35B1/dod-13, by HSF-1 in C. elegans Dauer Larvae and daf-2 Insulin Pathway Mutants

Wendy B. Iser1, Mark A. Wilson1, William H. Wood III2, Kevin Becker2, Catherine A. Wolkow1*

1 Invertebrate Molecular Genetics Unit, Laboratory of Neurosciences, Research Resources Branch, NIA Intramural Research Program, NIH Biomedical Research Center, Baltimore, Maryland, United States of America
2 Gene Expression and Genomics Unit, Research Resources Branch, NIA Intramural Research Program, NIH Biomedical Research Center, Baltimore, Maryland, United States of America

Abstract

Insulin/IGF-I-like signaling (IIS) has both cell autonomous and non-autonomous functions. In some cases, targets through which IIS regulates cell-autonomous functions, such as cell growth and metabolism, have been identified. In contrast, targets for many non-autonomous IIS functions, such as C. elegans dauer morphogenesis, remain elusive. Here, we report the use of genomic and genetic approaches to identify potential non-autonomous targets of C. elegans IIS. First, we used transcriptional microarrays to identify target genes regulated non-autonomously by IIS in the intestine or in neurons. C. elegans IIS controls expression of a number of stress response genes, which were differentially regulated by tissue-restricted IIS. In particular, expression of sod-3, a MnSOD enzyme, was not regulated by tissue-restricted IIS on the microarrays, while expression of hsp-16 genes was rescued back to wildtype by tissue restricted IIS. One IIS target regulated non-autonomously by age-1 was cyp-35B1/dod-13, encoding a cytochrome P450. Genetic analysis of the cyp-35B1 promoter showed both DAF-16 and HSF-1 are direct regulators. Based on these findings, we propose that hsf-1 may participate in the pathways mediating non-autonomous activities of age-1 in C. elegans.

Citation: Iser WB, Wilson MA, Wood WH III, Becker K, Wolkow CA (2011) Co-Regulation of the DAF-16 Target Gene, cyp-35B1/dod-13, by HSF-1 in C. elegans Dauer Larvae and daf-2 Insulin Pathway Mutants. PLoS ONE 6(3): e17369. doi:10.1371/journal.pone.0017369

Editor: Matt Kaebelser, University of Washington, United States of America

Received January 13, 2011; Accepted February 1, 2011; Published March 9, 2011

This is an open-access article distributed under the terms of the Creative Commons Public Domain declaration which stipulates that, once placed in the public domain, this work may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose.

Funding: This work was carried out with funding from the NIA Intramural Research Program (AG000320) and from an Ellison Medical Foundation New Scholar in Aging award (www.ellisonfoundation.org). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: wolkowca@mail.nih.gov

Introduction

Insulin/IGF-I-like signaling (IIS) is a highly conserved pathway for promoting growth under replete conditions. Growth control is cell-autonomously regulated by IIS to determine cell and organ size in the affected tissue [1,2]. In addition to this well-conserved cell-autonomous function, IIS can also have non-autonomous effects on other parts of the body. These actions of IIS have been uncovered in C. elegans, Drosophila and mice, through studies of mosaic animals with IIS restricted to specific tissues. In worms and flies, tissue-restricted IIS non-autonomously regulates development and adult longevity [3,4,5,6,7,8]. In mice, brain-specific insulin receptor deletion is associated with obesity and low fertility, likely reflecting hormonal disruptions [9]. Tissue-restricted IIS could confer these non-autonomous effects either specifically, through endocrine outputs, or non-specifically, through pleiotropic phenotypes resulting from tissue dysfunction due to inadequate growth. Thus, these findings raise new challenges for identifying the downstream pathways mediating non-autonomous effects of IIS [10].

This question can be investigated in C. elegans, for which the major IIS pathway components have been identified. These include daf-2, encoding the sole C. elegans insulin/IGF-I receptor-like protein, and age-1, encoding a p110 PI3K catalytic subunit that is the primary DAF-2/IR effector [11,12,13,14]. The major downstream target of daf-2 and age-1 is daf-16, which encodes a FOXO transcription factor antagonized by DAF-2 signaling [15,16]. In C. elegans, the daf-2 pathway acts at both the cellular and organism level. At the cellular level, the daf-2 pathway cell-autonomously regulates sod-3 expression [6,17,18]. A second cell-autonomous output of the daf-2 pathway is the regulation of FIRE response sensitivity in intestinal cells [8]. Two types of behavioral plasticity are also regulated cell-autonomously by daf-2 [19]. The non-autonomous outputs of daf-2 regulate organismal phenotypes. The daf-2 pathway promotes reproductive development and prevents dauer larval arrest under replete conditions [14,20,21,22]. In adult animals, the daf-2 pathway promotes wildtype longevity and normal stress resistance [11,23,24,25,26,27]. Both dauer arrest and adult longevity are controlled non-autonomously by daf-2 and age-1 activity from several cell types [3,7,8].

The downstream effectors for daf-2 non-autonomous regulation of dauer arrest and adult longevity are not known. daf-16, the major cell-autonomous daf-2 target, regulates longevity primarily from intestinal cells [6]. A working model proposes that daf-2 activity can regulate daf-16 through both the cell-autonomous pathway, via age-1 and akt-1, and non-autonomously, through unidentified pathways [8]. The daf-2 pathway’s non-cell autonomous actions may reflect crosstalk with other signaling pathways that convergently regulate dauer arrest and adult longevity. One candidate is the heat-shock transcription factor, encoded by the hsf-1 gene, which regulates lifespan, proteotoxicity and dauer arrest in collaboration with daf-16 [16,27,28,29]. HSFs are highly conserved and direct the expression of heat-shock proteins in
response to thermal stress. In C. elegans, hsf-1 also promotes the expression of other, non-hsp, targets in daf-2 mutants through both daf-16-dependent and independent mechanisms [20].

To identify factors mediating the non-cell autonomous effects of the daf-2 pathway upon dauer arrest, we searched for transcriptional targets regulated non-autonomously by age-1 and then analyzed factors directing their regulation in response to the daf-2/age-1 pathway. Using microarrays, we examined gene expression in animals with age-1 activity restricted to neurons or gut, and the results were compared with gene expression in wildtype animals and zygotically null age-1 mutants (m+)z. This approach identified a collection of transcripts that were potentially regulated by age-1 in a non-cell autonomous fashion. We characterized the cis- and trans-requirements for daf-2-dependent expression of on of these non-autonomous targets, cyp-35B1/dod-13. The findings suggest that hsf-1 may be a component of pathways mediating age-1 non-autonomous activities.

Results

Gene expression patterns in animals with tissue-restricted age-1 activity

In order to search for targets regulated non-autonomously by age-1, gene expression was analyzed in animals with tissue-restricted age-1 activity. This analysis compared gene expression in zygotically null age-1 mutants (age-1(mg44)/+z) with that in age-1(mg44) animals carrying transgenes directing neuronally-restricted (CY251) or intestinally-restricted (CY262) age-1 expression (Fig. 1A). Both neuronal and intestinal age-1 expression rescued constitutive dauer arrest of age-1(mg44) [8]. The extended lifespan of age-1(mg44) adults was also rescued by age-1 expression in either tissue, although CY262 more strongly rescued adult longevity than CY251, consistent with a critical role for intestinal daf-16 activity for extended lifespan [6].

Since age-1 and daf-2 mutants share many phenotypes, we expected that the age-1 and daf-2 transcriptomes would be similar. Therefore, we compared our results for age-1(mg44) and those of a previous study of gene expression in daf-2 pathway mutants [30]. Of 113 daf-2 targets which were also significantly changed in our experiment, 73% were changed concordantly in age-1(mg44) adults (fold-change p<0.05, t-test) (Table S1). Considering the differences in reference pools and growth conditions, these results indicate high concordance of the age-1 and daf-2 transcriptomes, consistent with the fact that age-1 and daf-2 have similar mutant phenotypes [14]. These findings support the role of AGE-1/PI3K as the major effector for DAF-2 signaling.

The goal of this analysis was to identify age-1 target genes that could be regulated non-autonomously by the age-1 pathway.
reasoned that non-autonomous targets would be rescued to wildtype levels in the strains CY262 and CY251, which express age-1 only in the intestine or neurons, respectively. Using these criteria, we examined the effect of tissue-restricted age-1 activity on the 82 transcripts whose expression was regulated concordantly in age-1 and daf-2 mutants. We found that 30% (25 targets) were rescued in both CY262 and CY251, one target was rescued only in CY251, three targets were rescued in only CY262 and 65% (53 targets) were not rescued in either strain or had inconclusive data (Table S1). The targets whose expression was rescued in both CY251 and CY262 are potential non-autonomous age-1 targets (Fig. 1B, Table S1). These included genes involved in a variety of processes, such as antimicrobial defense (clec-13, lvs-7), reproduction (zi-2, -4 and -5), catalysis (f09f7.7 dioxygenase) and metabolism (acr-2). Little expression data was available for these targets, although two are reportedly intestinal (lvs-7, bpl-4) and four have complex expression patterns (zi-2, f09f7.7, acr-2 and f552h3.5).

We then conducted this comparison for 791 transcripts that were significantly and reproducibly overrepresented in age-1(ng44) adults versus wildtype adults (at least 2-fold overrepresented, p<0.05), without regard to their inclusion in the daf-2 transcriptome (Table S2). We set two criteria for rescue of age-1 targets in these strains. First, age-1 targets were defined as being more than 2-fold overexpressed in comparison with wildtype levels with a p-value<0.05 (t-test vs wildtype). For rescue in CY262 or CY251, expression was both significantly different from that in wildtype animals (p<0.05) and significantly different from that in age-1(ng44) animals (p<0.05). Within the group of 791 age-1-upregulated transcripts, 127 (16%) were rescued in both CY262 and CY251, and were potentially regulated non-autonomously by age-1. This group was composed of genes involved in a variety of biological processes, including defense or signaling (7 glutathione S-transferases, 5 cytochrome P450s, 6 lectins, 3 alcohol dehydrogenases, 4 glucuronosyltransferase and 3 nuclear hormone receptors). We expect that some non-autonomous age-1 targets should be expressed outside of the age-1-expressing tissues in CY262 and CY251. Therefore, we surveyed the available expression data for these genes. Of the 127 potentially non-autonomous age-1 targets, expression data was available for 18 (Wormbase, release 190). Eleven genes were reportedly expressed in the intestine, with 5 exclusively intestinal, although the significance of intestinal expression is unclear since this is a common site for promiscuous transgene expression in C. elegans [8]. The targets that were not exclusively intestinal were expressed in a variety of tissues, including neurons (7), hypodermis (8), the gonad (2), epidermal seam cells (3), muscle (5) and the pharynx (5). This finding is consistent with the idea that age-1 non-autonomous outputs might target many of the body’s tissues.

Using these rescue criteria, we also found that 12 targets were preferentially rescued in CY251 animals, while 37 were preferentially rescued in CY262 animals. The targets rescued preferentially in CY251 included one metalloprotease (nas-9), one NADH oxidase (f1749.5) and one lectin (clec-4). Expression data was only available for nas-9, which is expressed in the hypodermis. The targets preferentially rescued in CY262 functioned in a variety of processes including stress resistance (3 glutathione S-transferases, 3 glucuronosyltransferases) and metabolism (1 fatty acid desaturase and 2 lipases). Expression data is available for 10 of the CY262-rescued targets. Eight of these targets are expressed intestinally, with 5 expressed exclusively in the intestine (fat-5 fatty acid desaturase, f99c3.1 phospholipase, F34F3.3 lipase, F42A10.6 unknown function and lce-6 lectin). We note that the proportion of intestinal genes was the highest among the CY262-rescued targets (8/10, 80%) and was lower in the group of targets rescued in both CY251 and CY262 animals (5/11, 45%). We propose that the targets preferentially rescued in CY262 animals represent age-1 targets that are autonomously regulated by intestinal age-1 activity.

**age-1 non-autonomously regulates a subset of stress response genes**

Adult longevity and stress resistance in daf-2 and age-1 mutants results, at least in part, from transcriptional upregulation of stress-response genes [27,31,32]. Therefore, we examined our microarray data to determine whether the stress-resistance genes regulated by the daf-2 and age-1 pathway were autonomously or non-autonomously regulated (Table S3). Interestingly, sod-3 expression was not rescued in CY262 or CY251 animals, suggesting this target is regulated cell-autonomously and is not under endocrine control. The heat shock genes targeted by heat shock factor, HSF-1, are major contributors to daf-2 longevity [27,28,29,33]. Our array results revealed that overexpression of hsp-16 genes and hsp-17 was rescued in CY262 and CY251, suggesting that small heat-shock proteins may be non-autonomous age-1 targets. We note that, although the hsp-16 genes were robustly upregulated in one age-1(ng44) sample, the were less robustly upregulated in an ng44 replicate sample, so that the average change was not statistically significant. Nevertheless, these findings indicate that a subset of the stress response genes induced in age-1 animals are non-autonomously regulated by age-1.

**Expression of an endocrine age-1 target, cyp-35B1, is daf-16 and hsf-1-dependent**

One of the putative endocrine targets of age-1 that we identified was cyp-35B1, which had previously been described as a daf-2 pathway target named dod-13 [30]. The cyp-35B1 transcript was 17.8-fold overexpressed in age-1(ng44) hermaphrodites compared to wildtype and was rescued in both CY262 and CY251 (1.64-fold and 2.73-fold overexpressed vs wildtype, respectively). The cyp-35B1 gene encodes a cytochrome P450 enzyme implicated in xenobiotic detoxification [34]. The cyp-35B1 mRNA declines with age in wildtype hermaphrodites and cyp-35B1 is a target of the elt-3 GATA transcription factor that initiates and maintains intestinal cell fates in C. elegans [34].

Our microarrays showed that the age-1-dependent endocrine pathway could rescue hsp-16 overexpression in age-1 mutants. This observation led us to hypothesize that the hsf-1 heat-shock transcription factor which regulates hsp-16 expression and can collaborate with daf-16, might be important for expression of other endocrine age-1 targets [28,29]. We therefore examined whether hsf-1 RNAi was also required for cyp-35B1 expression in response to lowered age-1 activity. Using semi-quantitative RT-PCR, we examined transcript levels for cyp-35B1 and sod-3 in daf-2(e1370) adult hermaphrodites treated with daf-16 or hsf-1 RNAi or an empty vector RNAi control (Fig. 2A). As expected, sod-3 overexpression in daf-2(e1370) adults was suppressed by daf-16 RNAi, consistent with previous evidence showing that sod-3 is a direct DAF-16 target [17]. Treatment with daf-16 RNAi also reduced cyp-35B1 mRNA in daf-2(e1370), consistent with the identification of cyp-35B1 as a target of the daf-2 and age-1 pathway ([30]; this work). In contrast, only cyp-35B1 mRNA was significantly reduced in daf-2(e1370) animals treated with hsf-1 RNAi, while sod-3 levels were unaffected. This result demonstrates that hsf-1 activity is required for induction of cyp-35B1, but not sod-3, in the absence of daf-2 and age-1 activity.

We next examined the tissue distribution of cyp-35B1 by constructing a transcriptional reporter expressing GFP from a cyp-
35B1 promoter. A previously-described cyp-35B1:gfp reporter was expressed in an elicited-dependent manner, suggesting the possibility of intestinal expression [34]. We identified the cyp-35B1 promoter as a 660-bp sequence between the cyp-35B1 initiating methionine and the next upstream gene, cyp-35A3 (Fig. 2B). During reproductive development and in wildtype adults, cyp-35B1::GFP was either not detectable or expressed at very low levels in posterior hypodermal cells (Fig. 2B, w). In dauers and daf-2(e1370) adults, cyp-35B1::GFP was upregulated and expressed solely in the intestine (Fig. 2B, w). Intestinal cyp-35B1::GFP levels in daf-2(e1370) adults were substantially reduced by daf-16 RNAi (Fig. 2B, w). Treatment with hsf-1 RNAi also reduced levels of cyp-35B1::GFP in daf-2(e1370) adults, although not to the same extent as for daf-16 RNAi (Fig. 2B, w). These findings together demonstrate that cyp-35B1 expression under conditions of low daf-2 pathway activity is regulated by coordinate action of daf-16 and hsf-1. In some lines, we observed increased or broader intestinal expression of cyp-35B1::GFP in hsf-1 RNAi-treated animals. We attribute these changes to misregulation of the GFP reporter transgene, as we never observed increased levels of the endogenous cyp-35B1 mRNA under hsf-1 RNAI conditions.

Direct regulation of intestinal cyp-35B1 expression by DAF-16 and HSF-1

Based on our results, we hypothesized that cyp-35B1 may be a direct or indirect target of DAF-16 and/or HSF-1. To test these possibilities, we studied the cyp-35B1 promoter by deletion using gfp reporters. Deletion of 150-bp from the 5' end of the 0.6-kb cyp-35B1 promoter abolished intestinal GFP in dauers, without significantly affecting the weak hindgut expression observed in developing larvae (Fig. 3). Inspection of the deleted region identified two sequences (TTAAAGA & AAAACA) resembling the previously-identified DAF-16 binding element (DBE, GTAAAC/TA) [17]. This suggested that cyp-35B1 might be a direct DAF-16 target. Smaller deletions were made from this 150-bp region removing one or both of the DBEs. Deletion of sequences containing one DBE reduced, but did not eliminate, cyp-35B1::GFP in dauers (pWB1076), while deletion of both DBEs eliminated dauer expression (pWB1077). The DBE-containing region was not sufficient for intestinal cyp-35B1::GFP expression in dauers, however. In addition, maximal cyp-35B1::GFP expression required an adjacent 100-bp sequence which lacked any DBE-like sequences (pWB1072).

To test whether DAF-16 and/or HSF-1 directly bind to the promoter regions shown to be required for cyp-35B1::GFP expression in dauers, we utilized the yeast 1-hybrid assay for DNA-protein interactions [35,36,37,38]. We first constructed yeast expression plasmids for the two prey we wished to test, DAF-16 and HSF-1, utilizing a copper-inducible yeast expression system to drive expression of full-length daf-16 or hsf-1 cDNAs [39,40]. Next, we constructed bait plasmids by inserting the 150-bp dauer CRM or a dispensable downstream region in front of a minimal promoter driving ß-galactosidase [40]. Four bait plasmids were tested, containing either the dauer CRM or the downstream region in the sense or antisense orientation with respect to the β-galactosidase reporter (pWB1084/085 and pWB1082/083, respectively) (Fig. 4A). Reporter expression was activated by HSF-1 in yeast cells containing pWB1084, which contains the dauer CRM in the sense orientation with respect to the β-galactosidase reporter. In the opposite orientation (pWB1085), β-galactosidase expression was enhanced in cell expressing DAF-16. In contrast, we did not detect any stimulation of β-galactosidase expression by the DAF-16 or HSF-1 prey in cells containing baits with promoter fragments that were dispensable for dauer cyp-35B1::gfp expression (pWB1082 or 083).

To further delineate the binding regions for DAF-16 and HSF-1 binding within the promoter fragments in pWB1084/085, we subdivided this 160-bp fragment into three overlapping fragments of 60–65-bp each. In yeast expressing DAF-16, β-galactosidase expression was stimulated in the presence of the pWB1127 bait plasmid, which contains the cyp-35B1 promoter fragment with the 2nd DBE (Fig. 4C, D). The adjacent promoter fragment, carried in pWB1129, stimulated beta-galactosidase reporter expression in yeast expressing HSF-1. HSF transcription factors bind as trimers to the heat shock element (HSE) 5'-AGAANNTTCTAGAAN-3', consisting of three inverted repeats of the 5'-AGAAN-3' monomeric sequence. Inspection of the cyp-35B1 promoter element contained in pWB1129 identified a sequence on the bottom strand which resembles this consensus (5'-AGAGAACCAGCCTGGAGA-3'), but may be an imperfect inverted pentamer repeat of the 5'-AGAAN-3' sequence (Fig. 4F). The presence of an HSE-like sequence in pWB1129 is consistent with the observation of HSF-1 binding in the 1-hybrid assay.

Discussion

The goal of this study was to identify potential endocrine targets of age-1 activity that could be regulated non-autonomously from
the nervous system and/or the intestine. Expression of wildtype age-1 within neurons or intestinal cells rescues dauer arrest and lifespan phenotypes of age-1(mg44) animals [7,8]. This evidence led to a working model whereby age-1 activity within signaling tissues regulates an endocrine output that, in turn, can direct dauer morphogenesis and aging in target tissues [8]. The major genetic target of age-1 is daf-16, encoding a FOXO transcription factor [15]. All available evidence indicates that the major mechanism for AGE-1/PI3K regulation of DAF-16 is cell-autonomous, via phosphorylation by AKT kinases regulated by AGE-1/PI3K phospholipid products [41,42,43]. Thus, the finding that daf-16 acts cell-autonomously in the intestine to promote longevity was an apparent contradiction to earlier mosaic and transgenic analyses showing that the upstream regulators, daf-2 and age-1, regulate these processes non-autonomously [6]. To resolve this conflict, we proposed that age-1 can regulate daf-16 activity in target tissues through convergent cell-autonomous and non-autonomous mechanisms [8].

To investigate possible effectors through which age-1 might non-cell autonomously regulate daf-16, we used transcriptional microarrays to identify age-1 target genes that could be non-autonomously regulated by age-1. This search identified 127

![Figure 3](https://www.plosone.org/figure3.jpg)

**Figure 3. Identification of cis-regulatory modules (CRM) for dauer cyp-35B1:gfp expression by deletion analysis.** Deletions in the cyp-35B1 promoter were constructed to identify cis-regulatory modules necessary for directing dauer-specific expression in the intestine. (A) Diagram of cyp-35B1 promoter constructs; promoters are black bars, brown hatches designate potential DAF-16 binding elements (DBE, [17]), pink boxes designate cyp-35B1 exon 1 sequence fused to GFP (green bar). (B) Representative fluorescence images of cyp-35B1:GFP expression in dauer larvae carrying transgenes described in panel (A); bar, 100 μm. (C) GFP expression in dauer fore-, mid- and hindgut from indicated transgenes; black bars indicate % of dauers with medium or bright GFP fluorescence. GFP expression was scored in 20–59 dauers for each construct.

doi:10.1371/journal.pone.0017369.g003
Figure 4. Yeast 1-hybrid analysis to detect DAF-16 and HSF-1 binding to cyp-35B1 dauer CRM. Yeast 1-hybrid assays were performed to detect DAF-16 and HSF-1 binding to cyp-35B1 dauer CRM (A, B) and subfragments (C, D). DAF-12 binding was also examined for the cyp-35B1 dauer CRM (A, B), but produced negative results. (E) As a positive control, HSF-1 could bind to the heat-shock element (HSE)-containing region in the hsp-16 promoter in pWBI113. In DAF-16- or HSF-1-expressing cells, transcription factor binding to the promoter fragments was measured as beta-galactosidase reporter activity in yeast cell extracts, normalized for cell density. Charts show average beta-galactosidase activity, relative to vector (pRS424) controls. Number of trials: pWBI082, pWBI083, 4 trials; pWBI084, 9 trials; pWBI085, 8 trials; pWBI124, 6 trials; pWBI127, 5 trials; pWBI129, 6
potential non-autonomous age-1 target genes. We found that one of these, cyp-35B1/dod-13, which is expressed in the intestine of dauer larvae and daf-2(e1370) adults, could be directly regulated by both HSF-1 and DAF-16. Furthermore, other HSF-1 target genes, the hsp-16 genes, were non-autonomously regulated by age-1. Expression of a subset of daf-2 target genes is known to be hsf-1-dependent, although HSF-1 has not been shown to directly regulate these targets [28].

These observations provide circumstantial evidence placing hsf-1 in the pathway for daf-2 and age-1 non-autonomy. We propose that DAF-2/IR and AGE-1/P3K signaling in non-intestinal cells regulates an endocrine output that affects HSF-1 activity in intestinal cells (Fig. 5). We hypothesize that HSF-1 and DAF-16 can interact within intestinal cells to optimize expression of HSF-1 and DAF-16 target genes that may extend lifespan. An alternative model is that HSF-1 and DAF-16 function independently to promote expression of longevity target genes in intestinal cells. Distinguishing between these possibilities will likely require characterization of other non-autonomous age-1 targets and clearer descriptions of the interactions between hsf-1 and daf-16 in the regulation of C. elegans dauer arrest and longevity.

Materials and Methods

Strains and growth

C. elegans strains were maintained at 15°C on NG agar medium with nystatin and streptomycin with OP50 bacteria as a food source [44]. The following strains were used: Bristol N2 (wildtype); SP75 (sqt-1(sc13) age-1(mg44)/mnC1); CY251 (sqt-1(sc13) age-1(mg44); bvIs2); CY262 (sqt-1(sc13) age-1(mg44); bvIs1); CY312 (daf-16(mgDf50); daf-2(e1370)); DR10 (daf-1(m40)); bvIs1 and bvIs2 were previously described [8].

Microarray analysis

Gene expression in CY262 and CY251 was compared to non-transgenic age-1(mg44) n.t.-adults of similar age, and all comparisons were in reference to wildtype young adults. Synchronized populations of animals at the late-L4/young-adult stage were obtained from embryos isolated by bleach treating gravid hermaphrodites. Embryos were hatched overnight in S medium without food, causing developmental arrest at the first larval (L1) stage. Arrested L1s were plated with food, grown for 72 hours at 20°C and washed in M9. Total RNA was isolated with Absolutely RNA miniprep kit (Stratagene, La Jolla, CA). cDNA was labeled with the Quick Amp 2 Color Labeling Kit (Agilent Technologies, Santa Clara, CA) and hybridized to Agilent 2×22 k oligo microarrays containing probes for nearly the complete C. elegans genome. Slides were scanned using the Agilent Microarray Scanner (G2565B). Three biological replicates were examined per strain. After hybridization, probes with raw fluorescence signals >100 in wildtype samples were selected for further analysis as being reliable signals. Expression ratios for each gene were calculated in all 3 strains with respect to wildtype controls at similar, or slightly later developmental stage. Expression ratios were calculated as z-scores for a statistical evaluation of relative expression, and as fold-changes for standardized displays of relative expression [45]. To determine fold change, raw fluorescence values were first normalized by dividing the fluorescence intensity for a given sample by the average intensity of all samples for the array. Fold change ratios were then determined by dividing mean normalized value for all replicates of test versus control conditions. Statistical significance was judged using t-test to compare signal intensity measurements among biological replicates. Two replicates were performed for age-1(mg44) and 3 for CY251, CY262 and N2. MIAME-compliant raw microarray data
is available from the NCBI Gene Expression Omnibus (Accession #GSE102000).

cyp-35B1: gfp reporter

The cyp-35B1 promoter was considered to be the sequences between the predicted translational start to the immediate upstream gene and was PCR-amplified from *C. elegans* genomic DNA using primers with unique restriction sites. Primer sequences were 5'-CACAAGGAGGACATTCGCC-3' and 5'-GGAAGAAGAAACAGGCTCCTGGTGCCG-3'. PCR products were purified, digested and ligated into pre-digested pPD95.75, which contains GFP and the uve-34 3' UTR (Addgene, Cambridge, MA). Constructs were confirmed by DNA sequencing. Transgenic animals were created by microinjection technique (100 ng/μl plasmid DNA with 50 ng/μl of the co-injection marker gcy-2:gfp). Stable transmitting lines were selected in the 2nd generation and analyzed.

For cyp-35B1 promoter deletions, 150-bp and 300-bp deletions were made in the cyp-35B1 promoter in pWB1066, to create pWB1066 and pWB1067, respectively, by PCR using upstream primers that hybridized to internal sites within the cloned promoter. The PCR primers also contained unique BamHI and KpnI restriction sites, which were used to insert the PCR fragments into pPD95.75. To further define the region responsible for dauer expression, smaller deletions were made from the region deleted in pWB1066, using the same PCR strategy, to make pWB1074 (59-bp deletion), pWB1075 (109-bp deletion), pWB1076 (159-bp deletion) and pWB1077 (209-bp deletion). Next, a 161-bp fragment containing both putative DAF-16 binding sites was PCR amplified with primers containing unique BamHI and NheI restriction sites and the digested product was ligated to a basal promoter in pWB1067, consisting of 398-bp upstream from the amplified promoter fragments with added NotI restriction sites. These PCR products were inserted in the place of the DAF-12 responsive element in pYSYR0002 and the resulting plasmids were sequence confirmed. Yeast were transformed with both a bait and prey plasmid, or negative controls, using the Yeastmaker Yeast Transformer Kit (Clontech, USA) with selection on SC medium lack tryptophan and uracil. For β-galactosidase assays, independent yeast colonies were inoculated into 3-mL of selective medium and grown overnight at 30°C. Prey protein expression was induced by inoculation with 100 μM CuSO4 for 4–5 hours at 30°C. β-galactosidase activity was assayed from pelleted cells using the Yeast β-Galactosidase Assay Kit (Thermo Scientific, USA). The parent vector for pYSYE0002 is pRS424, which was used as a negative control for bait auto-induction.

Supporting Information

**Table S1** mRNA expression levels of *daf-2* pathway class 1 and 2 target genes in *age-1* mutants with tissue-restricted *age-1* expression. Expression data for class 1 and 2 *daf-2* pathway targets (Murphy et al. 2003) were obtained from microarray data for *age-1(mg44)*, CY262 and CY251 relative to wildtype. Relative expression levels are presented as fold-changed relative to wildtype. “Missing from array?”, genes which were absent from the microarrays used in this study; “Our results”, indicates whether previous target classifications were consistent in the current study; “Rescue category”, indicates whether expression was rescued to wildtype or near-wildtype levels in CY262 and/or CY251 for targets that were congruent between the prior and current study; “Not rescued” indicates targets that maintained the mutant level of expression in CY262 and CY251; “Both” indicates targets whose expression was rescued to wildtype or near-wildtype levels in both CY251 and CY262.

**Table S2** mRNA and anatomical expression data for genes upregulated by ≥2-fold in *age-1(mg44)* adults relative to wildtype. Expression data, as fold-change relative to wildtype and *age-1(mg44)*, is shown for each strain (*age-1(mg44)*, CY262 and CY251). “262 rescue”, “251 rescue” and “Category” indicate whether upregulation in *age-1(mg44)* was determined to be rescued in CY262 or CY251 and the corresponding rescue category (262, 251, Both, None). Anatomical expression data were obtained from the curated expression pattern annotations for each gene in WormBase (www.wormbase.org).

**Table S3** Expression of stress response genes in *age-1(mg44)*, CY262 and CY251. mRNA expression levels were obtained from microarray data for genes annotated as heat-shock protein, glutathione S-transferase, catalase, superoxide dismutase, lysozyme or metallothionein. Expression levels in *age-1(mg44)*, CY262 and CY251 are shown as fold-change relative to wildtype.

**Acknowledgments**

We are grateful to the following colleagues for providing reagents: Andrew Fire (Stanford University), Scott Alper (NIH/NIH), Patrick Hu (University of Michigan), Curtis Loer (USD) and Keith Yamamoto (UCSF). We thank Shailaja Kishan Rao for assistance with the microarray experiments. The Caenorhabditis Genetics Center and the Genome BC C. elegans Gene Expression Consortium provided several *C. elegans* strains [33,44]. We thank Mark Mattson for helpful suggestions and the members of the Wolkow lab for critical review of the manuscript.
Author Contributions
Conceived and designed the experiments: WBI MAW KB CAW. Performed the experiments: WBI MAW WHW CAW. Analyzed the data: WBI MAW CAW. Contributed reagents/materials/analysis tools: WBI MAW WHW KB CAW. Wrote the paper: CAW.

References
1. Butler AA, Le Roth D (2001) Control of growth by the somatotropic axis: growth hormone and the insulin-like growth factors have related and independent roles. Annu Rev Physiol 63: 141–164.
2. Garofalo RS (2002) Genetic analysis of insulin signaling in Drosophila. Trends Endocrinol Metab 13: 156–162.
3. Apfeld J, Kenyon C (1998) Cell nonautonomy of C. elegans daf-2 function in the regulation of diapause and life span. Cell 95: 199–210.
4. Giaimakis ME, Gros M, Jungre MA, Hafen E, Leevers SJ, et al. (2004) Long-lived Drosophila with overexpressed dFOXO in adult fat body. Science 305: 361–364.
5. Hwangbo DS, Gershman B, Tu MP, Palmer M, Tatar M (2004) Drosophila dFOXO controls lifespan and regulates insulin signalling in brain and fat body. Nature 429: 560–566.
6. Libina N, Berman JR, Kenyon C (2003) Tissue-specific activities of C. elegans DAF-16 in the regulation of lifespan. Cell 115: 489–502.
7. Wolfow CA, Kimura K, Lee M, Ruvkun G (2000) Regulation of C. elegans life-span by insulinslike signaling in the nervous system. Science 290: 147–150.
8. Isler VB, Gami MS, Kenyon C (2007) Insulin signaling in C. elegans regulates both endocrine-like and cell-autonomous outputs. Dev Biol 303: 343–347.
9. Bruning JC, Gautham D, Burks DJ, Gillette J, Schubert M, et al. (2000) Role of brain insulin receptor in control of body weight and reproduction. Science 289: 2112–2125.
10. Broughton S, Partridge L (2009) Insulin/IGF-like signals in the central nervous system. Neuron 51: 613–625.
11. Larsen PL, Albert PS, Riddle DL (1995) Genes that regulate both development and longevity in C. elegans. Genetics 139: 1567–1583.
12. Kimura KD, Tissenbaum HA, Liu Y, Ruvkun G (1997) daf-2, an insulin receptor-like gene that regulates longevity and diapause in C. elegans. Science 277: 942–946.
13. Morris JZ, Tissenbaum HA, Ruvkun G (1996) A phosphatidylinositols-3-OH kinase family member regulating longevity and diapause in C. elegans. Nature 382: 536–539.
14. Gotlibb S, Ruvkun G (1994) daf-2, daf-16 and daf-23: Genetically interacting genes controlling dauer formation in C. elegans. Genetics 137: 107–120.
15. Ogg S, Paradies S, Gottlieb S, Patterson GI, Lee L, et al. (1997) The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature 391: 994–999.
16. Lin K, Dorman JB, Reedan A, Kenyon C (1997) daf-16: An HNF-3/forkhead family member that can function to double the life-span of C. elegans. Science 278: 1319–1322.
17. Furutama T, Nakazawa T, Nakano I, Mori N (2000) Identification of the differential distribution patterns of mRNAs and consensus binding sequences for DAF-16/PARP in C. elegans. Genes Dev 12: 2488–2498.
18. Tomiska M, Adachi T, Suzuki H, Komimoto H, Schafair WR, et al. (2006) The insulin/PI 3-kinase pathway regulates salt chemosensation learning in C. elegans. Neuron 51: 613–625.
19. Riddle DL, Swanson MM, Albert PS (1991) Interacting genes in nematode dauer larva formation. Nature 290: 668–671.
20. Thomas JH, Birshy DA, Vowels JJ (1993) Evidence for parallel processing of sensory information controlling dauer formation in C. elegans. Genetics 134: 1105–1117.
21. Vowels JJ, Thomas JH (1992) Genetic analysis of chemoosmosy control of dauer formation in C. elegans. Genetics 130: 105–123.
22. Vowels JJ, Thomas JH (1992) Genetic analysis of chemoosmosy control of dauer formation in C. elegans. Genetics 130: 105–123.
23. Gums D, Sutton AJ, Sundermeyer ML, Albert PS, King KV, et al. (1998) Two pleiotropic classes of daf-2 mutations affect larval arrest, adult behavior, reproduction and longevity in C. elegans. Genetics 130: 129–155.
24. Johnson T (1996) Increased life-span of age-1 mutants in C. elegans elav. lower Gompertz rate of aging. Science 249: 908–912.
25. Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R (1993) A C. elegans mutant that lives twice as long as wild type. Nature 366: 461–464.
26. Klass M (1983) A method for the isolation of longevity mutants in the nematode C. elegans and the initial results. Mech Ageing Dev 22: 279–296.
27. Walker GA, White TM, McColl G, Jenkins NL, Babich S, et al. (2001) Heat shock protein accumulation is upregulated in a long-lived mutant of C. elegans. J Gerontol A Biol Sci Med Sci 56: B201–207.
28. Hsu AV, Murphy C, Kenyon C (2003) Regulation of aging and age-related disease by DAF-16 and Heat-Shock Factor. Science 300: 1142–1143.
29. Morley JE, Morimoto RI (2004) Regulation of longevity in C. elegans by heat shock factor and molecular chaperones. Mol Biol Cell 15: 657–664.
30. Murphy C, McCarroll S, Bargmann C, Fraser A, Kamath RS, et al. (2003) Genes that act downstream of DAF-16 to influence the lifespan of C. elegans. Nature 424: 277–284.
31. Halashek-Wiener K, Khattra JS, McKay S, Pouyryev A, Stott JM, et al. (2005) Analysis of long-lived C. elegans daf-2 mutants using serial analysis of gene expression. Genome Res 15: 603–615.
32. Larsen PL (1993) Aging and resistance to oxidative damage in C. elegans. Prog Natl Acad Sci USA 90: 8903–8909.
33. Garigan D, Hsu AL, Fraser AG, Kamath RS, Ahringer J, et al. (2002) Genetic analysis of tissue aging in C. elegans: a role for heat-shock factor and bacterial proliferation. Genetics 161: 1101–1112.
34. Bodrichova YY, Wu K, Southworth LK, Jiang M, Tedesco P, et al. (2008) An elt-3/elt-5/sib-6 GATA transcription circuit guides aging in C. elegans. Cell 134: 291–303.
35. Li J, Herzkowitz I (1993) Isolation of ORC6, a component of the yeast origin recognition complex by a one-hybrid system. Science 262: 1870–1874.
36. Lehming N, Thanos D, Brickman JM, Ma J, Maniatis T, et al. (1994) An HMG-like protein that can switch a transcriptional activator to a repressor. Nature 371: 175–179.
37. Kim SH, Huang SB, Chung IK, Lee J (2003) Sequence-specific binding to telomeric DNA by C. elegans DAF-16/PARP and Heat-Shock Factor. Science 300: 1142–1145.
38. Schena M, Yamamoto KR (1993) Mammalian glucocorticoid receptor derivatives enhance transcription in yeast. Science 241: 963–967.
39. Schena M, Picard D, Yamamoto KR (1991) Vectors for constitutive and inducible gene expression in yeast. Methods Enzymol 194: 389–398.
40. Shostak Y, Van Gist MR, Antebi A, Yamamoto KR (2004) Identification of C. elegans DAF-16-binding sites, response elements, and target genes. Genes Dev 18: 2529–2544.
41. Lin K, Hian H, Libina N, Kenyon C (2001) Regulation of the C. elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. Nat Genet 28: 139–145.
42. Paradis S, Ruvkun G (1998) C. elegans lifespan reduction by DAF-16/PARP transcriptional activity from AGE-1 PI3 kinase to the DAF-16 transcription factor. Genes Dev 12: 2482–2490.
43. Lee RH, Hirsch J, Ruvkun G (2001) Regulation of C. elegans DAF-16 and its human ortholog FKHR1 by the daf-2 insulin-like signaling pathway. Curr Biol 11: 1930–1937.
44. Brenner S (1974) The genetics of C. elegans. Genetics 77: 71–94.
45. Cladde C, Vawter MP, Freed WJ, Becker KG (2003) Analysis of microarray data using Z score transformation. J Mol Diagn 5: 73–81.