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Genes that may modulate longevity in *C. elegans* in both dauer larvae and long-lived *daf-2* adults

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Summary

We used Serial Analysis of Gene Expression (SAGE) to compare the global transcription profiles of long-lived mutant *daf-2* adults and dauer larvae, aiming to identify aging-related genes based on similarity of expression patterns. Genes that are expressed similarly in both long-lived types potentially define a common life-extending program. Comparison of eight SAGE libraries yielded a set of 120 genes, the expression of which was significantly different in long-lived worms versus normal adults. The gene annotations indicate a strong link between oxidative stress and life span, further supporting the hypothesis that metabolic activity is a major determinant in longevity. The SAGE data show changes in mRNA levels for electron transport chain components, elevated expression of glyoxylate shunt enzymes and significantly reduced expression for components of the TCA cycle in longer-lived nematodes. We propose a model for enhanced longevity through a cytochrome c oxidase-mediated reduction in reactive oxygen species commonly held to be a major contributor to aging.

Keywords

aging; *C. elegans*; gene expression; longevity; reactive oxygen species

Introduction

Aging is a process of gradual deterioration characterized by a declining ability to respond to stress, an increasing homeostatic imbalance and an increasing incidence of pathology. There are multiple models of aging described in the literature (reviewed in Finch and Ruvkun, 2001; Weinert and Timiras, 2003), and likely no single cause can be attributed, such as change in expression of a single gene or decline in function of a specific biological process. Rather, it is a complex, multi-factorial process that takes place on molecular, cellular and systemic levels.

C. elegans is a well-established model for the study of aging (Finch and Ruvkun, 2001; Jazwinski, 2000; Johnson, 2003; Luo, 2004). The life span of this soil-dwelling nematode can

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be extended by at least six different mechanisms, including reduced Insulin/IGF-1 signaling, germline ablation, food-sensing amphid ablation, caloric restriction, mitochondrial deficiency and a decrease in temperature. To date, a number of gene mutations that extend lifespan have been described, and a network of genetic interactions has been derived (Jia et al., 2004; Tissenbaum and Ruvkun, 1998).

Mutations in the insulin/IGF-1 receptor gene *daf-2* (Kimura et al., 1997) double adult life span via up-regulation of the *daf-16* FOXO transcription factor (Lin et al., 1997; Ogg et al., 1997), which is conserved between nematodes and mammals. Partial loss of function in *daf-2* results in a temperature-sensitive phenotype. Mutant larvae grown at 25° C constitutively enter dauer stage, whereas adults cultivated at 25° C have extended longevity (Gems et al., 1998). Insulin/IGF-1 signaling has also been implicated in the aging of both flies and mice, which implies a conserved mechanism (Tatar et al., 2003).

The discovery that *C. elegans* insulin/IGF-1 signaling plays a role in aging resulted from studying the formation of the natural long-lived stage - the dauer larva (Klass and Hirsh, 1976). In wild-type worms, the formation of dauer larvae is induced by food restriction and exposure to a dauer-inducing pheromone (Golden and Riddle, 1984), the concentration of which is proportional to population density. Dauer larvae do not feed, and typically live at least four to eight times the normal 2-week life span of *C. elegans*. At the dauer stage, we expect changes in the gene expression profile associated with a life-extending mechanism.

In this study, we have attempted to gain insight into specific changes in gene expression in both wild-type dauer larvae and *daf-2* mutant adults compared to normal adults. Whereas McElwee et al. (2004) used cDNA microarrays for a similar comparison, we used Serial Analysis of Gene Expression, or SAGE (Velculescu et al., 1995) to identify potential novel regulators of life span, and to characterize the expression profiles of different gene families that were previously shown to be associated with aging (Lund et al., 2002; Murphy et al., 2003). SAGE data for *daf-2* and wild-type adults were compared in a previous study (Halaschek-Wiener et al., 2005), which showed general inhibition of metabolism in the middle-aged *daf-2* adults.

We identified 120 genes, the expression of which in worms with extended life spans (either in dauer or *daf-2* adults) was significantly higher or lower than in adults that display normal longevity. Such genes may be involved in a life span-controlling mechanism common to dauer larvae and *daf-2* adults. The annotation of putative longevity regulators on our list shows that there is a strong link between oxidative stress and a long-lived phenotype, adding further to previous work inferring that a major determinant in longevity is metabolic activity (Balaban et al., 2005; Brand, 2000). In addition, our data show changes in transcript level for certain components of the electron transport chain (ETC), which is probably the main source of damaging reactive oxygen species in mitochondria. Finally, the glyoxylate shunt appears to be up-regulated in longer-lived nematodes, perhaps complementing the tricarboxylic acid (TCA) cycle in producing the substrate for mitochondria-driven generation of ATP. This may also lower the amount of reactive oxygen species (ROS) in the cell.

Materials and Methods

Strains and times used for RNA preparation

We used the following strains of *C. elegans* in this study: *fer-15* (*b26ts*), a sterile mutant of the *C. elegans* N2 (Roberts and Ward, 1982) N2 dauer larvae, long-lived *fer-15*; *daf-2*(*m41*) mutants (Gems et al., 1998) and wild-type N2 adults. The *fer-15* mutation was introduced to eliminate embryonically expressed transcripts from the expression profile of adults. Adults with normal life span carrying the *fer-15* mutation were harvested on the first and sixth days

of adulthood. Long-lived *fer-15*; *daf-2* adults were harvested on the first, sixth and 10th day of adulthood. All RNA preparations were made from worms grown in liquid culture, with the exception of the N2 preparation, which was from worms grown on plates and harvested on the first day of adulthood. The young (2-day) and old (14-day) dauer libraries were generated from mRNA by the procedures described earlier for the young dauer library. Sequence reads were processed, and their quality was assessed by use of *Phred* (Ewing and Green, 1998; Ewing et al., 1998). The sequences of linkers and duplicate ditags were removed, and only tags of equal or more than 95% accuracy (derived from *Phred* quality scores) were kept for further analysis. SAGE libraries were constructed from polyA⁺ RNA as previously described (Halaschek-Wiener et al., 2005).

Databases and tools used for gene annotation

For the analysis of expression data for genes related to functional categories we used the Gene Ontology (GO) database, release Sep. 2004. We also used databases Kyoto Encyclopedia of Genes and Genomes (KEGG), release 31.0, July 5, 2004 and KOGs (clusters of euKaryotic Orthologous Groups), release Apr. 2003. SAGE expression information for corresponding genes was stored locally in a MySQL database and accessed either with Perl scripts using a DBI module, or alternatively, Multisage tool (McKay et al., 2007). We also used online tools, such as Genome Browser and Batch Genes, on the Wormbase web site. We used two releases of Wormbase (www.wormbase.org), WS110 and WS160. Tag-to-gene mapping was done using release WS110.

Statistical filtering of expression data

Eight SAGE libraries were used to extract the genes with expression pattern changes correlated with the long-lived phenotype. Prior to the analysis we normalized the tags in all libraries to 100,000 counts, and then picked the genes with at least three standard deviations between expression values in the two longevity classes (*daf-2*/dauer versus normal life span). Genes expressed only in the *daf-2*/dauer or only in the 'normal life span' subset of libraries had low levels of expression (less than 10 genes had an average tag count of 15 or more in both sets). We selected 10 genes with the highest cumulative tag count from each of these two subsets, considering them as putative aging-related genes.

We employed the Audic-Claverie test (Audic and Claverie, 1997) for direct comparisons of tag frequencies between libraries. This test was used to identify genes with significantly altered expression levels by estimation of p-value (p in the text) for the comparison. We also used the coefficient of variation (CV), calculated for individual genes, to evaluate the variation of the SAGE signals between libraries. In combination with a standard deviation threshold, the quality of filtering was further increased using the CV. Each gene had its expression level examined in pair-wise comparisons (values from long-lived vs. normal worms) using the Audic-Claverie test, and selecting for genes with higher CV resulted in a smaller set of genes with an improved ratio for the number of statistically significant changes. The threshold used (CV = 85%), which removed about 50% of the genes from the list after initial filtering (using calculated standard deviation), increased the ratio of significant changes 2-fold for comparisons, in which significance was tested with the Audic-Claverie test using $p = 0.05$ (data not shown). Genes that were expressed only in one of two subsets generated from long-lived worms were excluded from this stage of filtering. The described filtering criteria allowed extraction of a manageable list of 120 genes, which was functionally annotated. This final list represents the candidate genes involved in aging-related processes.

Semi-quantitative RT-PCR and northern blot analyses

For semi-quantitative RT-PCR analysis we used total RNA from wild-type N2, *fer-15*, two independent preparations from *fer-15*; *daf-2* and two independent preparations from dauer

larvae. Total worm RNA was extracted using Trizol[™] (Invitrogen) according to the manufacturer's recommendations. Three RNA preparations were the same ones used for generation of the SAGE libraries (*fer-15*; *daf-2* days 1 and 6 of adulthood and *fer-15* day 1 of adulthood).

Reverse transcription followed by PCR was performed to determine transcript levels from different samples. The linear range of PCR amplification was determined by running PCR reactions for 20–30 cycles. Amplification of *rpl-21* transcript, which encodes ribosomal protein L21, was used for normalization of the template amount in PCR reactions. The following gene-specific primers were used to amplify transcripts of interest: MTCE.31F: gttatatagattgattcatagg, MTCE.31R: ctatgattgctccacaaatc, F26E4.9F: atggtcaacttgctaagacg, F26E4.9R: gatggattctgggtcgtcggc, MTCE.23F: ctgagccggaatgtaagatc, MTCE.23R: gaattccgtgaaatcccgtag, Y37D8A.14F: tcgttgaccgcagctgtaac, Y37D8A.14R: gcacgaagaattctggctct. The results of semi-quantitative PCR were analyzed using the Histogram tool in Photoshop as described previously (Lehr et al., 1997). Intensities of the bands were analyzed using images from two separate experiments. Values were adjusted for background signal and normalized against the loading control (intensities of PCR products with *rpl-21* as a template).

For northern blot analysis, total RNA was isolated from *fer-15* and *fer-15*; *daf-2* worms grown in liquid culture on day 6 of adulthood. Total RNA (20 µg/lane) was analyzed by Northern blot hybridization with ³²P-labeled cDNA probes (Sambrook and Russel, 2001). Concentration was determined by UV absorbance, and confirmed by observing the intensity of ribosomal RNA bands on an agarose gel. Probes were prepared using PCR-amplified fragments of worm cytochrome c oxidase subunits II (MTCE.31), III (MTCE.23), Va (Y37D8A.14) and Vb (F26E4.9). Gel-purified double-stranded cDNA probes were labeled with [α -³²P] dCTP (3000 Ci/mmol⁻¹, Amersham) by random primer extension using Klenow polymerase. After hybridization, blots were exposed with Kodak BioMax XAR film. Northern blots were analyzed using Photoshop, as done with RT-PCR. The values obtained were adjusted by subtracting background signal.

Experimental Results

In total, we compared eight libraries: three *fer-15*; *daf-2* libraries generated at 1, 6 and 10 days of adulthood, two dauer libraries and three libraries generated from adults with wild-type life span. One of the libraries made from RNA extracted from two-day-old dauer larvae has been previously described (Holt and Riddle, 2003; Jones et al., 2001). The second dauer library was prepared from 2-week old dauer larvae. The control libraries (from normal adults) were represented by one library of wild-type N2 nematodes generated on day 1 of adulthood (prior to egg laying), and two libraries of *fer-15*; *daf-2*(+) mutants, made from day-1 and day-6 adults. The *fer-15* mutation results in infertility (no sperm production) and allows the analysis of the exclusively adult transcriptome with no contribution from developing embryos (Friedman and Johnson, 1988).

For mapping tags to transcripts, we used the virtual transcriptome of *C. elegans* (McKay et al., 2003; Pleasance et al., 2003). Genome matches (tags that map to the genomic DNA sequences, but not to any known transcript) were excluded from initial analysis, as were tags that matched multiple genes. The excluded dataset also contained 1294 tags that could not be aligned with genomic DNA. Such tags may result from novel alternatively spliced transcripts (manuscript in preparation).

Of 39,461 unique tag sequences, we unambiguously assigned 20,551 to 8,124 genes, for an average of 2.5 unique tags/gene. The assignment of multiple tags is due to alternative splicing

or polyadenylation of mRNA transcripts, which appears to be more widespread than previously documented in *C. elegans* (McKay et al., 2003). We aimed to narrow down this gene list to ones that had similar expression profiles in both *daf-2* adults and dauer larvae, but were expressed differently between the long-lived groups (*daf-2* and dauer), and adults with normal life spans (*fer-15* and N2). Using the statistical filtering described in *Experimental procedures*, we derived a list of the most prominent candidates, for which transcript abundance was most affected in long-lived animals.

Correlation of the SAGE data with differences in longevity

Tag-to-gene mapping showed that 3097 individual genes were shared between *daf-2* and dauer subsets, which comprises 43% of the genes mapped for dauer libraries and 68% of the genes mapped for *daf-2* libraries. It is important to note that 2733 of these 3097 genes were also found in normal libraries. Those genes were subjected to statistical filtering in order to extract those with significantly different transcript abundance.

Fig. 1 illustrates the relationship between three classes of transcripts – (1) 364 transcripts detected in both *daf-2* adults and dauer larvae but not in worms with normal life spans, (2) 932 transcripts detected only in adults with wild-type longevity, and (3) 2733 transcripts detected in all three subsets of SAGE libraries. Among these three classes, we identified 120 genes, the expression of which is most strongly changed in long-lived animals (Suppl. Table 1). Sixty-one of the 120 genes have a similar expression pattern in both types of long-lived worms, and these are considered to be the strongest candidates for longevity controllers.

Our list of 120 also included genes with transcript levels higher or lower relative to worms with wild-type longevity in just one of the long-lived phenotypes. Therefore, the 120 genes may be grouped into four sets, which include: up- or down-regulated genes in both long-lived forms, and up-regulated genes in either *daf-2* or in dauer larvae. Suppl. Table 1 also includes SAGE data for the L3 stage (McKay et al., 2003), to identify genes having a similar level of expression in young dauer larvae and in the growing L3 larva. We made this comparison to determine which genes are expressed in a ‘L3-like’ manner to distinguish them from genes for which expression changes were more specific to the dauer stage. Forty of the 120 genes had similar expression in the young dauer and L3 libraries. These genes are bold faced in Suppl. Table 1, and are included for completeness. However, none of them are material to our conclusions.

Cytochrome c oxidase subunits- I, II, III and V (designated as MTCE.26, MTCE.31, MTCE.23 and F26E4.9, respectively) were found among the genes with increased transcripts in both dauer larvae and *daf-2* adults. Elevated transcript abundance for cytochrome c oxidase subunits in dauer larvae was demonstrated earlier (Jones et al., 2001), but our observation also suggests a role for these genes in adult longevity. The average increase in expression for these genes was 2 to 6-fold ($p < 0.001$). To our knowledge, this study is the first time up-regulation of these genes has been demonstrated for both dauer larvae and *daf-2* adults (Table 1).

Autophagy has been highlighted as an important aging-related process in *C. elegans* (Melendez et al., 2003). Perhaps because of low tag counts, we did not find autophagy-related genes among those showing statistically significant expression changes in long-lived nematodes. However, we found significant changes in transcripts for two genes related to apoptosis. In *daf-2* adults, tags corresponding to the cystatin-like protease inhibitor *cli-2* (K08B4.6) were five times more abundant ($p < 0.001$) than in adults with a normal life span (Suppl. Table 1). Based on the RNAi phenocopy (Kamath et al., 2003; Sonnichsen et al., 2005), cystatin is thought to be at least superficially dispensable for viability and gross morphology. However, the elevated level of transcripts for this gene in *daf-2* adults provokes a question about the possible importance of this gene for longevity. Another gene, T28F4.5 (encoding death-associated protein DAP-1),

also had greater abundance (about a 2-fold difference, $p < 0.01$) in both dauer larvae and *daf-2* adults.

Genes previously associated with longevity are among the 46 genes that showed an increased transcript level in dauer larvae and adults. The *asc-1* gene (Y53F4B.15), a homolog of human activating signal cointegrator-1 (Jung et al., 2002), showed an average 28-fold increase in tags ($p < 0.001$). The non-coding transcribed telomeric sequence (*tts-1*) transcript (F09E10.11, reported in Jones et al., 2001) was also found at higher levels (average 57 fold increase) in both *daf-2* adults and dauer larvae ($p < 0.001$). However, the expression level of *tts-1* decreased with time in dauer larvae (from 1622 in young dauer to 611 normalized tag counts in 2-week old dauer larvae).

Tags for metallothionein *mtl-1* (K11G9.6) (Freedman et al., 1993), which participates in metal detoxification and stress adaptation, were also higher in long-lived animals (more than a 10-fold increase on average, $p < 0.001$), however relatively low in the old dauer library (3 normalized tag counts). This is consistent with earlier observations (McElwee et al., 2003; Murphy et al., 2003) and corroborates the strong association of this gene with long life.

Among the genes down-regulated in long-lived animals, we found collagens *col-77*, *col-112*, *rol-1* and collagen-like *dpy-5* (M195.1, Y73B6BL.34, Y57A10A.11 and F27C1.8, respectively). These genes had a strong SAGE signal in adults with wild-type lifespan (up to 60 normalized tags), but were practically undetectable in long-lived worms (Table 2). Conversely, we found tags for F41E7.5, which encodes a collagen-like protein, to be much more abundant in long-lived worms (more than nine folds on average). Tags for *col-106* (Y77E11A.15) were approximately five times higher (when compared to worms with normal life spans) in the *daf-2* dataset (Suppl. Table 1). These observations raise the possibility that changes related to the structural organization of connective tissue may be beneficial for longevity.

Analysis of SAGE data for genes related to energy generation and stress response

Fig. 2 shows the cumulative tag counts (sums for all genes in a group) for genes related to cellular metabolism and stress resistance. Oxidative phosphorylation is the energy-producing pathway utilized by the cell for aerobic synthesis of ATP. ROS, the by-products of oxidative phosphorylation, are considered to be the primary source of damage, which leads to a gradual degradation of cellular components (recently reviewed in Barja, 2004). A list of 55 *C. elegans* genes associated with oxidative phosphorylation was generated using Wormbase online tools and annotations taken from the Gene Ontology (GO) database (The Gene Ontology Consortium, 2000). Tags for cytochrome c oxidase subunits I, II, III and V all had significantly increased abundance in worms with longer life spans. However, transcripts for ATP synthase subunits (we examined a list of 31 genes), and other genes related to oxidative phosphorylation did not change significantly.

We also observed higher transcript levels for mitochondria-encoded cytochrome c oxidase subunits with semi-quantitative RT-PCR and northern blots (Fig. 5). Although we did not observe the same magnitude of changes in abundance of tested transcripts as seen in the SAGE data, the changes were reliably detected by both methods. MTCE.23 and MTCE.31 primers detected more template in *daf-2* adults and dauer larvae than in wild-type young adults, but there was no obvious change in the levels for two nuclear transcripts, F26E4.9 and Y37D8A.14 (Fig. 5A). On northern blots, *daf-2*(-) produced a stronger signal for mitochondria-encoded MTCE.31 than did the *daf-2*(+) strain (Fig. 5B). Quantification of the Northern blot results correlated with SAGE and RT-PCR data, except for MTCE.23, which had a low (difficult to quantify) signal in both *daf-2*(-) and *daf-2*(+) samples. These observations provide consistent evidence for differential expression of cytochrome c oxidase (ETC complex IV) subunits,

correlated with longevity. In long-lived adults there was a substantial increase in transcript levels for the core mitochondria-encoded subunits.

No significant differences were seen in nucleus-encoded transcripts for F26E4.9 and Y37D8A.14 between *daf-2* and normal young adults or dauer larvae, although quantification of the RT-PCR results suggested a modest increase of the F26E4.9-specific product in the case of long-lived worms (Fig. 5A). Northern blots revealed no obvious change in the two nuclear transcripts between *daf-2*(+) and *daf-2*(-).

We also considered the hypothesis that enhanced longevity is the result of switching to anaerobic fermentation for energy production (Holt and Riddle, 2003). According to this hypothesis, shifting energy metabolism to an anaerobic mode would allow the cells to produce ATP through a less harmful, ROS-free process. In *daf-2* adults, there was no significant increase in transcripts for alcohol dehydrogenase (K12G11.3) until day 10, when the corresponding tags increased from 3 to 22 ($p < 0.001$). The latter value is comparable to the 27 tags in 2-day-old dauer larvae. Tags for fumarate reductase (F48E8.3) were more abundant in the dauer (22 and 16 tags) than in normal adults (1–3 tags), but in *daf-2* adults, tags for this gene were at wild-type levels. Tags for fumarate reductase were higher in both dauer libraries, whereas tags for alcohol dehydrogenase (an alternative electron sink), were increased only in young dauer larvae. These observations suggest that an anaerobic metabolic shift is not needed for the long-lived phenotype of *daf-2* adults.

The TCA cycle provides intermediate compounds, such as reduced coenzymes NADH and FADH₂, to donate protons and electrons to the ETC. The ETC then generates additional molecules of ATP through oxidative phosphorylation. SAGE tags for 10 of the 19 genes encoding the components of the TCA cycle are less abundant in worms with extended life spans (in the range of 2 to 5-fold, $p < 0.05$).

The glyoxylate shunt is more active in dauer larvae than other stages (O’Riordan and Burnell, 1990). The shunt produces succinate and glyoxylate by splitting isocitrate, a TCA cycle intermediate. Succinate is channeled back into the TCA cycle where it eventually forms oxaloacetate, whereas glyoxylate condenses with acetyl-CoA (usually derived from the oxidation of fatty acids) to form malate, another TCA cycle intermediate. The glyoxylate shunt also enables the cell to use oxidation of fatty acids for gluconeogenesis, which becomes possible as both oxaloacetate and malate can be converted into phosphoenolpyruvate in mitochondria.

The *gei-7* isocitrate lyase/malate synthase (C05E4.9) has already been associated with long-lived phenotypes in microarray studies (Murphy et al., 2003). The *gei-7* transcripts were significantly elevated ($p < 0.001$) in the day-10 *daf-2*(-) library with 65 tags versus 7 tags in day-6 *daf-2*(+) library. Therefore, the glyoxylate shunt is a candidate process involved in the extension of longevity in nematodes. It uses isocitrate, the production of which depends on the first two TCA enzymes, citrate synthase and aconitase. Although the transcription level for some of the TCA cycle components is lower in long-lived worms, the SAGE data for citrate synthase T20G5.2 and aconitase F54H12.1 indicate that isocitrate may still be produced and available for the glyoxylate shunt. Among all of the TCA cycle enzymes, these two have the highest cumulative SAGE tag counts in long-lived worms. At day 10, *daf-2* adults have an even higher level of citrate synthase mRNA (49 vs. 12 tags in the day-6 *daf-2*(+) library) (also see Suppl. Table 3). Our SAGE data also indicated an increase in transcripts for the key gluconeogenesis enzyme *pyc-1* (D2023.2), although the tag counts for this transcript were very low (less than 10). There was no indication of an increase in transcripts of the other genes in this pathway (Suppl. Table 3).

Fatty acid oxidation is another way to provide NADH for the mitochondrial electron transport chain and the proton-pump machinery, but we were unable to detect any changes in the expression profile for these enzymes. We generated a list of genes related to fatty acid oxidation using the Gene Ontology database, and SAGE data were available for 28 genes from this list. Only 12 genes from this pathway had ten or more SAGE tags (sum of tags in all eight libraries), and no significant changes in transcripts for those genes were observed.

Using terms from the GO database, we retrieved a list of 66 *C. elegans* genes related to DNA repair, 53 genes with chaperone activity (a class that includes numerous heat shock proteins) and 35 genes involved in responses to oxidative stress. The latter genes include those for nine enzymes that neutralize reactive oxygen species. Their transcript levels in long-lived worms was not significantly elevated (except for the day-2 dauer library, where transcripts for five of the nine genes increased significantly, $p < 0.01$), but actually decreased with adult aging (Suppl. Table 3).

Similarly, transcripts for genes involved in DNA repair were not elevated in long-lived animals, having a low abundance of SAGE tags. The day-6 *daf-2* library showed a relatively high level of transcripts for the group, but the signal was mostly contributed by a single gene - the excision DNA repair protein R10E4.5, the level of which was on average 15 fold higher ($p < 0.001$) in long-lived adults relative to the wild-type (Table 1).

The group of 43 genes predicted to have chaperone activity revealed an expression pattern different from that of the genes related to oxidative stress response and DNA repair. Expression of chaperones was higher in both *daf-2* adults and dauer larvae relative to normal adults. Changes in transcript levels for 36 of these were statistically significant with $p < 0.05$, implying a coordinated response for this group of genes. Among the most abundant transcripts (100 – 800 tags in at least one of the libraries), we found the *daf-21/hsp-90* (C47E8.5), *hsp-16.2* and *hsp-16.41* (Y46H3A.3 and Y46H3A.2). These three genes encode highly conserved molecular chaperones previously implicated in longevity (Murphy et al., 2003; Walker and Lithgow, 2003).

Discovery of new aging-related genes

In principle, SAGE can provide information on virtually any polyadenylated transcript in the cell that possesses an anchoring enzyme restriction site (Velculescu et al., 1995). This makes SAGE useful as a gene discovery tool (Saha et al., 2002). In our study, approximately 5% of the tags (1918) were present in more than one library, but were unambiguously mapped to genomic regions outside any known genes. This represents evidence for new genes at these locations. Hence, we filtered an initial set of 69 tags (with unambiguous genomic matches, and five or more tags in at least one of the eight analyzed libraries) using the same statistical approach as in the case of tags mapped to genes (see *Experimental procedures*). Twenty SAGE tags were selected, nine of which were significantly more abundant in libraries from both types of long-lived worms (Fig. 3). The additional eleven tags were elevated in *daf-2* adults, but not in dauer libraries. Our analysis implies the existence of novel transcripts detected by SAGE, some of which are associated with longevity.

We initially used the data from Wormbase release WS110 (<http://ws110.wormbase.org>) for the tag-to-gene mapping, as well as for annotation of identified genes. Eleven of the selected 20 differentially expressed tags (Fig. 3) map to genomic locations that have no detectable sequence homology with the current *C. briggsae* genomic DNA sequence assembly. The other nine tags overlapped with the regions, homologous to *C. briggsae* genome. When we analyzed the later release WS160 (<http://ws160.wormbase.org>), four of these nine tags were found to overlap with newly established curated coding genes (Y82E9BR.22, T07A5.5, C08A9.10 and W10C8.6).

Comparison of SAGE results with published microarray and RNAi data

Lund et al. (2002) derived a list of 164 aging-associated genes using microarray analysis at different times in adulthood, from young adults to adults at and beyond the median life expectancy. Less than half (69 genes total) of the reported genes were detected in any of our eight libraries (8429 genes), and only four of the 164 genes were found on our list of 120 putative longevity-related genes (Suppl. table 2A). We also compared our results with gene subsets derived from microarray experiments by Murphy et al. (2003), who identified 514 genes for which expression appears to correlate with longevity. We were able to find only 14 of the 514 genes on our filtered list, 13 putative life extenders and one life shortener, according to the author's classification (Suppl. Table 2B). Randomization tests showed that this number is significantly higher ($p < 0.01$) than the number expected due to chance alone. The majority of the putative life extenders (9 of 13) were found in the fraction of the genes up-regulated both in *daf-2* adults and dauer larvae.

We also examined the expression of two groups of genes identified through RNAi experiments as those affecting the level of body fat in *C. elegans* (Ashrafi et al., 2003). We were able to detect 70 of 112 genes stimulating deposition of body fat, and 131 of 305 genes for which expression decreased the level of fat. The tags for genes increasing body fat content were about two times higher in both dauer libraries than in the normal adult libraries (241–256 vs. 94–166 normalized counts). However, in *daf-2* adults, the level of expression for these genes was on average 1.5 fold lower. The SAGE data do not provide convincing evidence that fat metabolism is significantly changed in long-lived animals, owing to the low expression level for the majority of the genes in both groups (average tag count > 5 only in 18 of 201 genes). In addition, we examined the list of 89 genes for which inactivation with RNAi results in life span extension (Hamilton et al., 2005) as well as the genes derived by McElwee et al., 2004. Although the number of exact matches with our list of 120 genes was negligible, we found that some of the genes belonged to the same biological process (discussed below).

In order to compare our data with that of McElwee et al. (2004), we generated lists of genes for the three groups mentioned above by examining GO and Interpro annotations with Wormbase online tools. In all eight of our libraries, the SAGE signal was low for most of the genes (107 of 112 tested genes had, on average, less than 5 normalized tag counts). However, one of the short chain dehydrogenases (T04B2.1) on our list of the 120 most prominent putative aging regulators, while undetectable in *daf-2* adults, had significantly more tags in long-lived worms, with 107 normalized tags in young dauer vs. 3–12 tags in worms with wild-type life span ($p < 0.001$). However, we did not see coordinated elevation of tags in any of the three groups of genes.

Discussion

We have identified common trends in the gene expression of two types of long-lived *C. elegans*, *daf-2* adults and wild-type dauer larvae. The dauer stage represents the natural ability of *C. elegans* to survive for an extended period during adverse conditions. Mutations in certain genes that regulate dauer formation are known to result in changes of the adult life span (Finch and Ruvkun, 2001; Jia et al., 2004; Vanfleteren and De Vreese, 1995), including genes for insulin/IGF-1 signaling (Gems et al., 1998; Kenyon et al., 1993). We aimed to identify changes in transcript abundance common to both types of extended longevity, which may represent a shared mechanism for modulating life span. We found that the most significant detectable changes in gene expression were identified as components of energy production pathways.

A metabolic model of aging explains the extension of life span as the result of reduced damage inflicted by ROS (reviewed in Rea and Johnson, 2003). Holt & Riddle (2003) showed a significant up-regulation of fermentation-related genes and an associated decrease in

transcripts for the TCA pathway genes in dauer larvae, consistent with the decline of TCA activity during dauer development (Wadsworth and Riddle, 1989). However, there was no consistent up-regulation for fermentation-related genes in *daf-2* adults (Suppl. Table 3), indicating that the anaerobic shift observed in dauer larvae is not required for *daf-2* longevity. However, we observed a 4–5-fold decline in TCA pathway transcripts in both dauer larvae and *daf-2* adults.

Increased tags for some of the components of oxidative phosphorylation in long-lived worms, suggests that this pathway might be stimulated. Indeed, the respiration rate in some long-lived mutants of *C. elegans* is not reduced relative to wild-type animals (Braeckman et al., 2002), and the longevity-enhancing effect of caloric restriction has been associated with a higher than normal rate of oxygen consumption (Barros et al., 2004; Lin et al., 2004). As we observed a decrease in tags for TCA cycle enzymes, a question arose about possible alternative sources of the NADH required for the membrane-associated ATP-generating mitochondrial complex.

We observed increased abundance of tags for a gene from the glyoxylate shunt in *daf-2* adults. In dauer larvae, the tag counts for the same gene were not significantly different from that in *daf-2(+)* adults. This observation implies that usage of the glyoxylate shunt, as an alternative source of substrate for oxidative phosphorylation, may not be utilized to the same degree by dauer larvae, but is influenced by the insulin/IGF-1 signaling pathway. However, this aspect of nematode physiology may not pertain to the study of human longevity, since the components of glyoxylate shunt are found in plants, bacteria and relatively primitive metazoans, but not in mammals.

Two recent studies identify genes that affect life span in worms using whole genome-scale RNAi screens (Hamilton et al., 2005; Hansen et al., 2005). Lists of the aging-associated genes from these two studies, although quite different from each other and the 120 aging-associated ones that we found, share a few members that belong to the same biological pathways. Our results showed down-regulation of three TCA cycle enzymes, which supports the previously reported association of decreased TCA cycle activity with longevity (Hamilton et al., 2005). The latter RNAi screen also identified some structural proteins, i.e. collagen *col-93* (W05B2.5). We observed a lower abundance of tags corresponding to collagens in SAGE libraries from long-lived worms.

Mitochondria are considered an important site of aging-associated processes (Harman, 1972; Linnane et al., 1998; Singh, 2004). Genes related to the electron transport chain (ETC) and ATP synthase have been examined using RNAi (Dillin et al., 2002). For some of the genes, exposure of animals to RNAi after hatching resulted in extension of life spans, albeit accompanied by smaller body size, slower movement, lower rates of feeding and a reduction in ATP levels and oxygen consumption.

The fact that transcripts for electron transport machinery are expressed at higher levels in long-lived worms is consistent with the increased oxygen consumption in *daf-2* adults (Houthoofd et al., 2005). Expression levels of mitochondrial subunits of cytochrome c oxidase, comprising the catalytic core of complex IV (subunits I – III), are higher in long-lived *daf-2* adults and dauer than in wild-type adults. By contrast, the RNAi screens show that a knockdown in expression of the nucleus-encoded subunits of cytochrome c oxidase (F26E4.6, F26E4.9 Y37D8A.1 and W09C5.8) extends longevity, albeit with reduced growth and fecundity. Although a link between mutants with lowered metabolic rate and longevity has been established in *C. elegans* (Vanfleteren and De Vreese, 1995), it remains a possibility that the nematode may extend longevity not only by reducing oxidative phosphorylation, but by making it more efficient and reducing its production of ROS. A higher ratio of complex IV to complex III activity might accomplish this. Two sites of ROS generation in mitochondria have been

identified - ETC complexes III and I. Although the exact mechanism of ROS generation in complex I is not clear, it is known that ROS may emerge from interaction between intermediate electron-binding centers of complex III and stray oxygen (Balaban et al., 2005; Brand, 2000).

In the resource starved dauer stage, it may be highly adaptive to utilize the efficiencies in energy production realized through oxidative phosphorylation carried out by the ETC. Higher than normal expression of ETC complex IV may be beneficial for longevity for worms with a normal body size and rate of respiration. Assuming that increased SAGE tag abundance for complex IV subunits reflects a greater amount of functional protein in long-lived worms, we hypothesize that this may modulate ROS production, resulting in longer life span.

Changes in expression of some of the ETC components may reduce ROS production and extend life span. The insulin/IGF-1 pathway may be involved in transcriptional regulation of ETC complex IV components in mammals. In prostate cancer, which is associated with high levels of IGF-1 (Djavan et al., 2001; Jerome et al., 2003), differential expression of cytochrome c oxidase subunits was observed with the shift to nucleus-encoded components (Herrmann et al., 2003). This is consistent with the shift in the ratio of cytochrome c oxidase subunits in *daf-2* adults resulting from decreased insulin/IGF-1 signaling.

Fig. 4 shows the proposed model, explaining how modulation of cytochrome c oxidase (complex IV) expression may reduce the rate of ROS formation. For illustration, a partial outline of the ETC chain is used. With an increase in transcripts from ETC complex IV, oxygen metabolism may become more efficient, as more sites for oxygen turnover become available. This may reduce the chances of damaging ROS generation at the complex III site by lowering the local concentration of free oxygen. This model assumes that the number of functional cytochrome c oxidase molecules increases, although we did not see a significant increase in tags corresponding to the nucleus-encoded subunits of complex IV (except for F26E4.9 with up to 4-fold increase in long-lived worms, $p < 0.05$).

Taking our observations together, energy metabolism was significantly changed in long-lived worms. A general decrease in metabolic activity for *daf-2* adults was detected in a previous study (Halaschek-Wiener et al., 2005; McElwee et al., 2006), and here we describe the affected pathways, in common between *daf-2* adults and dauer larvae. There is an indication that transcripts for enzymes of the TCA cycle are lowered in both types of long-lived worms, although mechanisms that might compensate for reduced TCA cycle activity appear to involve different pathways, fermentation in dauer larvae versus the glyoxylate shunt in *daf-2* adults.

hsp-90 and *hsp-16* were among the most highly expressed genes, and both appeared on our filtered list of putative longevity-associated genes. The *hsp-16.2* tags were more than 10-fold higher in *daf-2* libraries (days 6 and 10, $p < 0.001$) than in normal adults and 6-fold higher than in dauer libraries. Also, the tag count for this gene increases with age in both dauer larvae and *daf-2* adults, whereas in normal adults the level of expression for *hsp-16.2* and *hsp-16.41* remains low. Our data confirm previous findings that show a link between aging and expression of *hsp16* genes. Increased expression of *hsp-16* is linked to *daf-16* activation (Walker and Lithgow, 2003). Stimulation of *hsp-16* expression may also result from reduced membrane potential of mitochondria (Kuzmin et al., 2004). In normal worms, *hsp-16* is expressed ubiquitously in somatic tissues and is likely to function as a passive ligand, temporarily preventing unfolded proteins from aggregating.

hsp-90 transcripts were significantly elevated in dauer larvae (Jones et al., 2001), but not in *daf-2* adults. As the exact function of *hsp-90* in the cell is unclear, it is difficult to conclude that increased expression of *hsp-90* is important in maintenance of the long-lived phenotype. Nevertheless, higher than normal levels of transcripts for the chaperone proteins may be

beneficial for longer life (reviewed in Kroll, 2004; Morrow and Tanguay, 2003; Soti and Csermely, 2003).

Comparison of our results with earlier microarray studies showed little overlap in candidate gene lists. McElwee et al. (2004) suggested that a group of stress response genes participating in detoxification might be a major factor in long life spans. The microarray data showed increased transcripts for three groups of genes: cytochrome P450s, short-chain dehydrogenase/reductases and UDP-glucuronosyltransferases. However, our SAGE data do not support this model. Nor was there substantial overlap with other genes previously identified as aging-related (Murphy et al., 2003), although a few genes (like *mtl-1*), clearly have a strong SAGE signal. McKay et al. (2007) cautioned that reliance on a single *C. elegans* global transcription profiling technique could give an incomplete or biased picture of the transcriptome. However, the set of genes that overlap between the SAGE and microarray experiments may be considered as especially strong candidates for aging-related function, as their selection was based on the analysis of data generated by two independent methods. It is also possible that the long-lived phenotype may be controlled by genes with low-abundance transcripts, making detection of the relevant expression changes difficult for both SAGE and microarrays.

To date, the agreement between SAGE and microarray results has been generally low (Griffith et al., 2005; van Ruisen et al., 2005). Differences in expression data obtained by SAGE and microarray platforms are based upon difficulties in reliable probe design for microarrays, and upon problems associated with reliable assignment of SAGE tags to genes. Tags not mapped to a single gene (ambiguous mapping) are excluded from analysis, lowering the quality of the information obtained. Resolving these problems may improve the agreement between the two platforms, but the current users of these protocols should also rely on experimental evidence provided by alternative methods, as we do in this study.

For comparative analyses, both the Murphy et al. (2003) and McElwee et al. (2004) microarray studies pooled data from class 1 and class 2 *daf-2* alleles. We made our SAGE libraries with RNA from the reference class 1 allele *daf-2(m41)*, because it is much less pleiotropic than class 2 mutants, exhibiting only the *daf-2* longevity, stress resistance and fat accumulation traits (Gems et al., 1998). The *daf-2(e1370)* mutant exhibits additional traits, including low penetrance egg lethality, fertility and movement defects. We would expect these traits to be associated with changes in the gene expression profile that are unrelated to longevity. Hence, the use of pooled class 1 and class 2 data for the microarray studies could possibly account for differences in our results.

Unlike DNA microarrays, which scan populations of mRNAs with a fixed set of probes, SAGE is capable of detecting virtually any polyadenylated mRNA that contains a site for an anchoring enzyme. Theoretically, each SAGE tag with its unique sequence identifies one transcript, and using the information about tag counts we can estimate the level of expression for a particular mRNA. However, there is a sizable fraction of SAGE tags that cannot be matched with known genes, but can be aligned with a single genomic location. Such incidences present a chance to identify novel genes (Fig. 3). In fact, four gene models were established in later releases of Wormbase for locations associated with our candidate SAGE tags. Although all of these genes had regions of sequence homology with *C. briggsae*, an absence of homology does not necessarily mean the absence of a gene at a given location in *C. elegans*. Eight hundred of the predicted 19,500 *C. briggsae* genes (Stein et al., 2003) lack any obvious ortholog in *C. elegans*, whereas *C. elegans* has about 1300 genes with no identified ortholog in *C. briggsae*. Our selected candidate tags had between five and 459 normalized tag counts, and we believe that more of these proposed genes may be confirmed in future experiments.

The goal of our study was to highlight the most prominent gene expression changes in common for nematodes in the dauer and long-lived adult stages. There are distinct changes in expression for a few groups of genes common to both modes of longevity extension. Our findings corroborate the importance of metabolism in aging, and identify classes of genes that may be universally utilized for extending life span. In addition, our study demonstrates the application of SAGE for gene discovery, which allowed us to identify possible new longevity-associated genes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

SAGE tag-to-gene mapping. (A) Comparison of genes identified for three different subsets of SAGE libraries. Overlap between genes detected in *daf-2*, dauer and wild-type life span subsets is shown, with the number of genes identified as longevity-associated (either increased or decreased transcripts in long-lived worms) for each group of interest. (B) The proportion of SAGE tags unambiguously mapped to genes, and tags with an unambiguous match to genome sequence not at the site of an annotated gene. The remaining fractions include the tags mapped to more than one gene, and tags with no genomic match.

Fig. 2.

Transcript abundance for functional groups related to aging-associated biochemical processes. Level of expression in a group is calculated as cumulative tag count for genes in this group for each of the SAGE libraries. The stacked bars show the input from individual genes in a pathway (or functional family of genes).

Fig. 3.

Discovery of novel aging-associated genes. (A) Screenshots of diagrams generated with the GeneBrowser online tool (Wormbase, <http://www.wormbase.org/db/seq/gbrowse/wormbase>) using different versions (freezes) of Wormbase data. In this example, the newer release (WS160) has an additional gene model (T07A5.5), corresponding to SAGE tags differentially expressed in long-lived worms. (B) Novel tags with significantly different abundance in long-lived vs. normal worms. Normalized tag counts for each of eight libraries are shown. Asterisks mark tags aligned with sequence having homology with the *C. briggsae* genome. Gene names are given for the four new genes, to which tags were mapped using WS160 data.

Fig. 4. Possible mechanism for lowering ROS production (and extending life span) due to higher concentration of cytochrome c oxidase (complex IV of ETC). With the increased expression of complex IV the oxygen turnover intensifies, resulting in lower free oxygen concentration. That, in turn, reduces the concentration of oxygen at the active centers of complex III, one of the sites of ROS generation.

Fig. 5.

Changes of the transcript levels examined for the cytochrome c oxidase subunits using RNA samples from normal- and long-lived worms. (A) The results of semi-quantitative RT-PCR, conducted for two nucleus-encoded and two mitochondria-encoded cytochrome c oxidase subunits. Two independent RNA samples (1 and 2) were obtained from *fer-15; daf-2* adults and another two from N2 dauer larvae. These were compared with samples from two *daf-2(+)* strains, N2 and *fer-15* (day 1 of adulthood). Quantified results of two independent experiments were used to build the diagram with the standard deviation values for each mean on the right side of panel A (plotted in arbitrary units). (B) In Northern blot analysis we used RNA extracted from *fer-15; daf-2* and *fer-15* on day 6 of adulthood. PCR-amplified fragments of the corresponding genes were labeled with [α - 32 P] dCTP and used as probes. The band intensities

were used to build the diagram on the right side of panel B (plotted in arbitrary units) using the Histogram tool in Photoshop.

SAGE data are shown for genes with increased transcript abundance in *daf-2* adults and dauer larvae.

Gene description	daf-2(-)/fer-15(-)					Dauer			adult worms, normal life span			
	Gene id	Day1	Day6	Day10	young	2 week	day1	day1	day6	day1	day6	long/normal lifespan
										wt	fer-15(-)	
similarity to H.sapiens Interleukin-7 receptor	ZK355.3	107	571	95	13	2	0	0	1			
SPP-20 (Saposin-like type B, region 1)	K04A8.8	8	90	7	2	3	0	0	1			
similar to H.sapiens FLJ1043	F46A8.7	2	40	10	20	22	0	0	1			
CX module	Y60C6A.1	4	58	2	9	20	0	1	0			
lys-3 encodes one of a lysozyme genes	Y22F5A.6	3	4	1	52	14	0	0	2			
small protein with a CSL motif	K01H12.1	5	18	15	23	12	1	0	0			
similarity to Homo sapiens Dermcidin precursor	Y39H10A.1	4	16	2	19	24	0	0	0			
Similar to L.tarentolae NADH-ubiquinone oxidoreductase chain 5	C54F6.5	1	7	1	34	20	0	0	0			
Alpha-B-crystallin C. elegans HSP-12.3 protein	F38E11.1	9	2	22	21	1	0	0	2			
alcohol dehydrogenase	K12G11.3	3	0	22	27	3	1	0	0			
RNA gene of unknown function locus:ttts-1	F09E10.11	379	1207	1026	1622	611	1	2	48			57.0
similar to D.discoideum Ras interacting protein RIPA	Y39B6A.5	97	81	50	13	4	3	1	0			36.8
similar to S.cerevisiae DNA polymerase V	ZC395.5	91	138	102	18	17	1	4	1			36.6
RNA gene of unknown function locus:ttts-1	F09E10.11	14	28	5	20	106	0	2	1			34.6
C.elegans small nucleolar snRNA sn1502	F30H5.4	70	251	193	299	610	0	21	9			28.5
similar to M.martensii Neurotoxin BmK-VIII	F21C10.11	10	88	8	4	22	0	0	4			19.8
asc-1, homolog of human activating signal cointegrator-1	Y53F4B.15	131	405	86	34	22	0	12	15			15.1
hypothetical gene model	F45E4.5	39	82	25	36	118	2	4	7			13.8
similarity with Lectin C-type domain short and long forms	Y54G2A.6	108	61	24	107	6	0	12	3			12.2
C. elegans MTL-1 protein	K11G9.6	117	21	2	4	4	0	6	2			11.1
Trypsin Inhibitor like cysteine rich domain	C10G8.4	24	110	81	48	3	0	0	15			10.6
dehydrogenase	T04B2.1	19	97	19	33	53	6	4	3			10.2
RNA gene of unknown function	F55A4.9	33	7	1	25	75	0	7	2			9.4

Gene description	Gene id	daf-2(-)/fer-15(-)				Dauer		adult worms, normal life span			
		Day1	Day6	Day10	young	2 week	day1	day1	day6	long/normal lifespan	
		wt									fer-15(-)
similar to LPS-induced tumor necrosis factor alpha factor	Y22D7AL.15	146	45	39	0	27	8	7	3	8.6	
Putative cytochrome haem-binding periplasmic protein	C27B7.9	89	168	32	55	38	2	5	20	8.5	
hypothetical gene model	F15D3.7	169	551	220	30	57	4	15	57	8.1	
cytochrome_c_oxidase_subunit_III	MTCE.23	304	192	110	93	73	13	22	25	7.7	
hypothetical gene model	F15D4.3	5	5	13	52	77	2	5	5	7.6	
cytochrome_c_oxidase_subunit_II	MTCE.31	16	26	48	66	97	4	11	5	7.6	
cytochrome_c_oxidase_subunit_I	MTCE.26	36	124	10	9	56	1	1	17	7.4	
similar to TRAM, LAG1 and CLN8 homology	Y48G8AL.13	2307	2349	1086	1197	367	96	343	215	6.7	
collagen-like repeats	F41E7.5	3	166	238	7	36	15	17	10	6.4	
cytochrome C oxidase	F26E4.9	3	128	245	9	25	9	21	9	6.3	
microfilarial antigen like	ZC410.5	1166	1209	434	995	174	153	152	111	5.7	
similarity to Xenopus laevis Neuromedin B precursor	E04F6.8	34	57	15	13	10	3	5	6	5.5	
heat shock protein hsp16.4	Y46H3A.2	37	2	85	63	565	27	45	22	4.8	
heat shock protein hsp16.2	Y46H3A.3	874	1149	374	502	157	86	193	105	4.8	
similar to P.falciparum revertase II largest subunit	Y119D3B.21	10	23	7	63	14	2	2	11	4.7	
predicted glyceraldehyde 3-phosphate dehydrogenase	K10B3.7	8	20	13	75	54	4	11	14	3.5	
Histone H3	F45E1.6	25	17	11	52	11	10	3	7	3.5	
similar to Adenylosuccinate synthetase	C37H5.6	16	16	35	11	108	3	18	13	3.3	
Protein of unknown function (DUF1242)	K07F5.15	588	157	50	146	83	63	117	40	2.8	
methylmalonate-semialdehyde dehydrogenase	F13D12.4	37	9	83	32	13	13	18	8	2.7	
DAP-1 Death associated protein 1a	T28F4.5	17	11	72	63	22	15	14	19	2.3	

Table 2
SAGE data are shown for genes with decreased transcript abundance in *daf-2* adults and dauer larvae.

Gene description	Gene id	daf-2(-)/fer-15(-)				Dauer				adult worms, normal life span			
		Day1	Day 6	Day 10	young	2 week	day 1	day 1	day 6	normal/long lifespan			
								wt	fer-15(-)				
ATP citrate lyase	D1005.1	1	0	1	0	1	11		6	8			
cuticle collagen COL-77	M195.1	0	0	0	0	0	0		32	0			
Winged helix DNA-binding	R08E5.3	0	0	0	0	0	1		33	1			
cuticlin	F53F1.5	0	0	1	0	0	1		24	0			
Yeast RPD3 protein like	C53A5.3	0	0	1	0	1	13		3	13			
rol-1, cuticular collagen	Y57A10A.11	0	0	0	0	0	0		34	0			
C. elegans DPY-5 protein	F27C1.8	1	0	0	0	1	0		62	0			
hypothetical gene model	C02E7.6	2	0	0	0	1	1		76	0			
cuticle collagen COL-112	Y73B6BL.34	0	0	0	0	0	3		33	0			
hypothetical gene model	K03H9.1	0	0	2	0	1	1		36	1			
hypothetical gene model	W05F2.3	36	89	26	0	6	132		88	173			4.17
contains similarity to Xenopus tropicalis	Y18D10A.17	23	4	62	5	24	137		104	98			4.79
Klp-10 encodes a kinesin-like motor protein	C33H5.4	4	1	15	2	16	42		23	37			4.47
P97, ATPase associated with various cellular activities	C41C4.8	7	1	12	4	11	26		47	31			4.95
glucosamine-fructose-6-phosphate aminotransferase	F07A11.2	12	1	8	0	29	37		39	53			4.30
FKBP-type peptidyl-prolyl cis-trans isomerases	F31D4.3	4	3	20	2	28	47		81	62			5.56
Eukaryotic protein kinase domain bub-1	R06C7.8	5	4	6	2	7	25		38	42			7.29