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Transcriptional Profile of Aging in *C. elegans*

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Summary

Background: Numerous gerontogene mutants leading to dramatic life extensions have been identified in the nematode *Caenorhabditis elegans* over the last 20 years. Analysis of these mutants has provided a basis for understanding the mechanisms driving the aging process(es). Several distinct mechanisms including an altered rate of aging, increased resistance to stress, decreased metabolic rate, or alterations in a program causing organismic aging and death have been proposed to underlie these mutants.

Results: Whole-genome analysis of gene expression during chronological aging of the worm provides a rich database of age-specific changes in gene expression and represents one way to distinguish among these models. Using a rigorous statistical model with multiple replicates, we find that a relatively small number of genes (only 164) show statistically significant changes in transcript levels as aging occurs (<1% of the genome). Expression of heat shock proteins decreases, while expression of certain transposases increases in older worms, and these findings are consistent with a higher mortality risk due to a failure in homeostenosis and destabilization of the genome in older animals. Finally, a specific subset of genes is coordinately altered both during chronological aging and in the transition from the reproductive form to the dauer, demonstrating a mechanistic overlap in aging between these two processes.

Conclusions: We have performed a whole-genome analysis of changes in gene expression during aging in *C. elegans* that provides a molecular description of *C. elegans* senescence.

Introduction

Aging is among the most universal of biological processes and perhaps also among the most mysterious [1]. The modern era of genetic research into aging began with the identification of long-lived variants in tractable model systems including the nematode [2, 3], fruit fly [4–6], and yeast [7]. For example, genetic experiments in *Caenorhabditis elegans* have shown that genes that are part of an insulin-related signaling pathway are involved in specifying longevity [8].

The aging phenotype has been characterized at every level of organization, from the organismic to the cellular and the molecular [1]. Numerous age-related changes are apparent at the organismic level, but we are only now starting to understand age-related changes at the molecular level. In *C. elegans*, proteomic analyses with two-dimensional analyses of proteins labeled at different stages over the course of the normal life span have suggested that there are few changes in relative protein expression as chronological age increases [9]. Similarly, few genes were found to be differentially expressed over the life of the worm [10], and there was little change in the abundance of total RNA or rRNA, although moderate change in the abundance of polyA$^+$ with increasing chronological age was observed in hermaphrodites [11].

While age-related changes in gene expression have been found in studies of small numbers of genes [10], it has taken the development of microarray technology to make a genome-wide survey possible. DNA microarray expression studies in flies, mice, and monkeys have identified genes that change over the course of the life span [12–17]. The completion of the genome sequence of *C. elegans* has allowed for the production of full-genome oligonucleotide chips and DNA microarrays [18, 19], and one preliminary study compared aging-related expression differences by using a single sample from old worms [19].

We have used DNA microarrays to profile changes in gene expression during aging in the nematode *C. elegans*, from young adults to animals at and beyond the median life expectancy. Combining results from replicate samples has allowed us to apply rigid statistical criteria to the analysis of these data. These data present a robust molecular profile of normal aging and allow us to define complex changes associated with aging at the molecular level.

Results and Discussion

RNA was isolated from age-synchronous cultures of worms at six different times during their life span, starting at the first day of adult life (3 days from fertilization) to an age at which 90% of the population was dead (16–19 days of age) (see the Experimental Procedures) [20]. To produce populations of synchronously aged worms, we used temperature-sensitive sterile mutations that inactivate sperm and hence block reproduction but do not affect longevity [20–22]. We wished to identify genes whose expression levels change as a function of chronological age, and we were concerned that there may be differences between strains. Thus, we used three different sterile strains that have previously been used in aging studies (*fer-15*, *spe-9;fer-15*, and *spe-9; emb-27*) and have been shown to age at a similar rate based on time of fertility, movement, and life span [21]. To assess which gene expression changes are statisti-

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Table 1. Time Course of *C. elegans* from 3 to 19 Days of Age

Day	3	4	6–7	9–11	12–14	16–19
fer-15	2	0	1	1	1	0
spe-9;fer-15	3	3	2	5	2	3
spe-9;emb-27	1	0	1	0	1	
Total = 26	6	3	4	6	4	
	Young adult		Oocyte production ends			<25% survival

The number of arrays and the strain of worm included in each time point are shown. Notable characteristics of the population are indicated.

cally significant, we pooled samples of different strains and isolated a total of 3–6 samples for each time point (Table 1).

PolyA$^+$ RNA isolated from the staged samples was used to synthesize Cy3-labeled cDNA from the experimental samples, and a single preparation of mixed-stage hermaphrodite polyA$^+$ RNA was used to synthesize a Cy5-labeled cDNA reference probe that was used for all experiments (see the Experimental Procedures). Each of the staged samples was hybridized along with the same reference probe to *C. elegans* DNA microarrays; this method allows the comparison of relative levels of gene expression among worm populations of different ages. The DNA microarrays contain PCR fragments of genomic DNA corresponding to 17,871 of the 19,626 currently predicted genes in *C. elegans* (91% of open reading frames) arrayed on a glass slide [18]. The DNA microarrays were scanned, and the ratio of the RNA levels for the experimental to reference signal intensities were determined. The $\log_2$(expression ratio) was calculated for every gene, and then the average of the $\log_2$(expression ratio) from all of the repeats at each time point was determined. These calculations resulted in a gene expression time course for nearly every gene over the life span. The expression profiles reflect changes in expression as a function of chronological age over several strains (see the Experimental Procedures). The entire data set is available at http://cmgm.stanford.edu/~kimlab/aging/, which provides copies of the Supplementary Figures and Tables and programs that can be used to view the individual genes that are shown in the figures (see Table S1 at http://cmgm.stanford.edu/~kimlab/aging/).

Identification of Aging-Regulated Genes

We used one-way analysis of variance (ANOVA) to identify 201 genes that show expression changes over the aging time course ($p < 0.001$; see Table S1 at http://cmgm.stanford.edu/~kimlab/aging/); this number is much greater than the 18 genes that are expected to occur due to random chance alone. We further divided the set of 201 genes into two groups: maturity genes that change between day 3 and day 4 but that remain relatively constant thereafter, and aging genes that show changes following day 4 (see the Experimental Procedures). The maturity class consists of 34 genes, 33 of which fall in abundance between days 3 and 4, and one that rises (Figure S1).

Small differences between laboratory wild-type strains of N2 have been observed [23]. We wish to characterize only those genes that show expression changes with age that are independent of background. The expression ratio and experimental variability for these genes would be the same across different strains, and they would be identified in our experiment because mixing strains should not diminish the statistical power of the analysis. For genes that are age-regulated differently in different strains, our experimental design would have less statistical power than a design using a single strain, and these genes may not be identified by the ANOVA analysis used here.

We also considered whether our experimental design might result in selecting genes whose expression changes were due to differences in strain rather than age. First, we used two-way ANOVA to individually analyze each of the 167 genes showing significant changes in the time course, and we found that the variance in gene expression due to age was greater than that due to strain in all but three cases (Table S1); these three genes were not analyzed further. Second, we plotted the data points used to generate the averages for each age in the time course for the remaining 164 genes and found no genes in which the change in the time course was clearly due to strain rather than age differences (see Figure S2 at http://cmgm.stanford.edu/~kimlab/aging/). Third, for each of the microarray hybridizations, we calculated which other hybridization correlated with it the most and found that a microarray hybridization from the same age range had the highest correlation in 21 out of 26 cases (see the Experimental Procedures). In summary, chronological age plays a stronger role than strain differences for a set of 164 genes that exhibit changes in expression as worms age (after day 4). Figure 1 shows this set of genes clustered hierarchically based on similarities in gene expression along the aging time course. Of the 164 aging-regulated genes, 72 encode proteins similar to proteins from other organisms (Table S2).

Changes in Expression of Regulatory Genes Specifying Longevity

The age-dependent genes include a *C. elegans* insulin homolog, *ins-2*. *ins-2* expression is highest in young adults at day 4 and then decreases starting at day 7 until the end of life (Figure 2A). Mutations in an insulin-like signaling pathway can affect the *C. elegans* life span; for example, loss-of-function mutations in an insulin-like receptor gene (*daf-2*) or a PI3-kinase gene (*age-1*) result in increased longevity [8]. The observation that *ins-2* expression is age dependent prompted us to examine other genes encoding insulin-like proteins to see if they might also vary as a function of age, but at a level

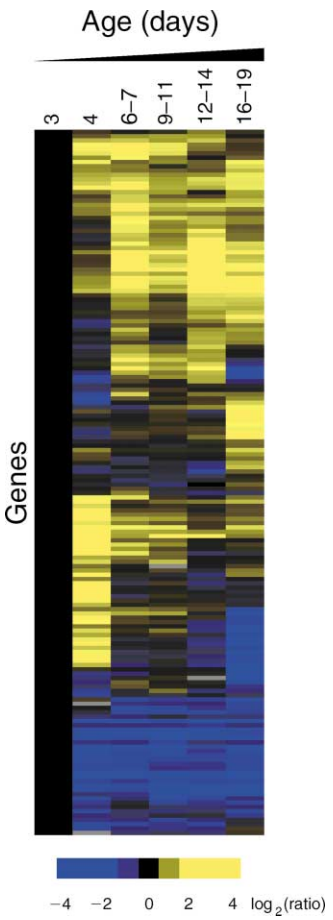

Figure 1. Expression Profile of 164 Aging-Regulated Genes

Each column shows the average of the $\log_2$(experiment/reference) ratio, normalized to expression at day 3. The scale shows the level of expression. Genes were hierarchically clustered based on Pearson correlation coefficients. Full data showing genes and expression values for all of the figures can be found at http://cmgm.stanford.edu/~kimlab/aging/.

below the stringent threshold used in our microarray analysis. We analyzed the expression of all 12 insulin-related genes present on the DNA microarrays and found 3 other insulin-related genes that changed expression during aging ($p < 0.05$). *ins-17* and *ins-18* expression levels are 3-fold higher at day 16–19 relative to day 3, and this is similar to the expression level of *ins-2*. In contrast, *ins-7* expression decreases 3.3-fold throughout life (Figure 2A). Genes in the insulin-like signaling pathway itself (such as *daf-2* and *age-1*) do not show concerted change in expression during aging (data not shown).

Expression of *sir-2.1* changes during aging ($p < 0.007$); it shows a mild increase during days 6–11, followed by a decrease at the end of life (Figure 2B). *sir-2.1* represses the activity of the insulin-like signaling pathway, and extra copies of *sir-2.1* prolong life [24]. Sir2 also regulates longevity in yeast by a mechanism involving chromatin silencing [25, 26]. *F46G10.3* encodes another *SIR2* homolog, and its expression decreases about 7-fold in old worms ($p < 0.006$). Two other

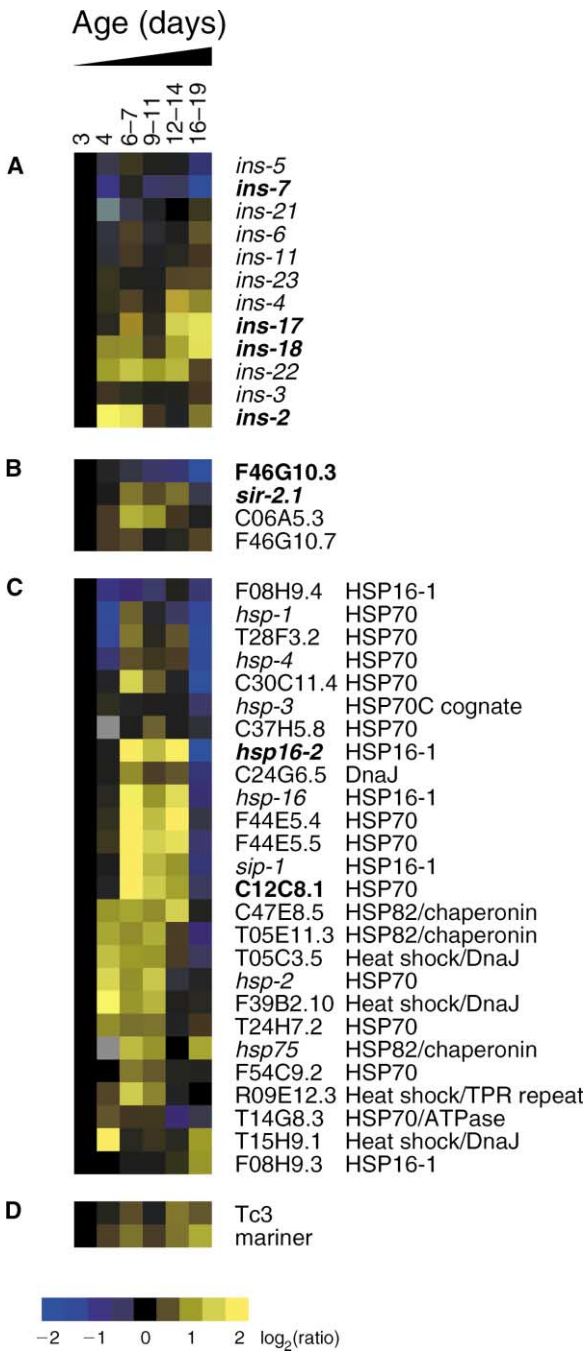

Figure 2. Aging Profiles for Specific Classes of Genes

The average of the $\log_2$(expression ratio) for each gene relative to day 3 is shown. Genes were hierarchically clustered based on Pearson correlation coefficients. The scale shows the level of expression.

(A) Insulin-related genes. Bold type indicates four insulin-like genes that change expression levels during aging ($p < 0.05$).

(B) Homologs of the yeast *SIR2* gene.

(C) Genes that encode heat shock proteins.

(D) Average expression of Tc3 and Mariner genes. For each transposon element copy, the average $\log_2$(expression ratio) was calculated from the microarray hybridization repeats, and then the average from all copies of each transposon was calculated (since each copy has nearly identical sequence). Data for each of the individual transposable elements are shown in Tables S3 and S4.

SIR2 homologs (*C06A5.3* and *F46G10.7*) do not show statistically significant changes. Dynamic changes in these four insulin-like genes and two *SIR2* homologs might play a role in specifying life span by interacting with the *daf-2/age-1* insulin-like signaling pathway and regulating downstream targets.

Changes in Expression of Stress-Resistance Genes

Aging can be thought of as the result of a lifetime of stress [27–30], and hence genes that are regulated by stress (such as heat shock genes) could show age-dependent expression changes. Previous experiments have reported both an increase and a decrease in heat shock gene expression with age, and these findings have led to different hypotheses about the potential role of heat shock genes during aging [12–15, 31, 32]. Part of the inconsistency arises because previous experiments used DNA microarrays representing only a fraction of the genome. Using our full-genome DNA microarrays, we found that two heat shock genes (*Y46H3A.D* and *C12C8.1*) show age-related changes in gene expression (Figure 2C). These two genes are members of the HSP16 and HSP70 gene families, respectively. The appearance of two heat shock genes in the set of aging genes is more than would be expected by random chance, given that there are 27 heat shock genes present on the DNA microarrays ($p < 0.006$). To evaluate expression of heat shock genes during aging more fully, we examined the entire set of 26 heat shock genes. We found that many showed a common expression profile; most genes rise in expression between day 3 and days 4–11 and then decrease in expression at one or two of the last time points (Figure 2C). Previous experiments using only two time points (young and old adults) would have missed many of the dynamic changes in expression with age. Moreover, the oldest worm populations in our studies were harvested at ages well past the mean life span of the population, while previous aging studies have used younger animals and thus would miss the changes seen at the latest age point [12–17, 19]. Heat shock proteins serve as chaperones to help fold proteins and prevent protein denaturation. Decreased basal expression of these heat shock proteins in old worms may increase levels of unfolded protein, possibly leading to increased toxicity and stress, impaired cell function, and organismic senescence; this is consistent with the high mortality rate seen at these ages [20].

One model for aging postulates that oxidative damage produced by mitochondria could contribute to cellular senescence [33]. We examined genes that encode mitochondrial proteins and genes involved in resistance to oxidative stress. We did not observe a consistent alteration in expression of either of these gene classes. Previous studies identified five genes that seemed to change expression during aging (*mup-2, act-4, unc-15*, and Y57G11C.12, *ost-1*) [19], and none of these showed strong expression changes in our experiments. This discrepancy could be due to the small sample size used in the previous experiment.

Other Gene Classes that Change during Aging

The set of aging-regulated genes includes three Tc3 transposons (*T02G5.5*, *F11D11.2*, and *F56A6.3*). Every Tc3 copy is nearly identical in sequence, and so it is not possible to determine expression from an individual Tc3 copy. Hence, we averaged the expression of all 18 of the Tc3 copies together to obtain an overview of Tc3 expression during aging (Figure 2D), and we found that expression for the entire Tc3 family tends to increase during aging (ANOVA, $p < 0.001$). In addition to Tc3, we found that the average expression for Mariner elements also increases in old worms ($p < 0.001$). Increased expression of Tc3 and Mariner mobile elements could lead to genomic instability and DNA mutations in old age, which could contribute to cellular and organismic senescence. Increased expression in old worms is not a general property of mobile elements, as Tc1, Tc4/Tc5 transposons and retrotransposons do not show significant expression changes during aging (data not shown).

Changes in Tissue-Specific Genes

Different tissues lose function and structural integrity at different times during aging. For example, the germline stops producing gametes at day 9, and there are alterations in muscle cells as early as day 7, but neuronal cells maintain their structural integrity throughout life (M. Driscoll, L. Herndon, D. Hall, P. Schmeissner, and Y. Sakano, personal communication). We used the microarray expression data to assess changes in tissue-specific aging at the molecular level; we selected genes known to be expressed in specific tissues and then determined whether they showed coordinate age-related changes. First, we looked at the expression of 44 known muscle and 112 known neuronal genes. Muscle and neuronal transcripts showed an apparent increase in expression during aging ($p < 0.002$ and $p < 0.001$, respectively; Figures 3A and 3B). Since the expression levels from each age are normalized as part of the data analysis, it could be that muscle and neuronal gene expression do not change during aging, but that expression of other gene groups decreases, yielding an apparent increase in muscle and neuronal RNAs relative to the rest of the genome.

Previous work has shown that the germline plays a role in determining longevity, as ablation of the germline lengthens life span [34, 35]. In order to investigate how the germline changes during aging at the molecular level, we analyzed changes in germline genes during aging. Previous microarray experiments had identified 508 germline intrinsic genes (enriched in both sperm-producing and oocyte-producing gonads) and 258 oocyte-enriched genes [36]. Figures 3C and 3D show that germline gene expression increases during days 4–16 and then decreases at the oldest age. The age when germline gene expression decreases (late in life) does not correlate with the time when oocyte production ceases (at about day 7 in hermaphrodites at 25°C [37, 38]), indicating that these are separate events. That is, decreased germline gene expression is not directly caused by a lack of oocytes, and decreased oocyte production is not directly caused by decreased gene expression. The observation that germline gene expression continues past the end of the reproductive period is consistent with a lack of selective advantage for turning off the expression of the germline after the end of

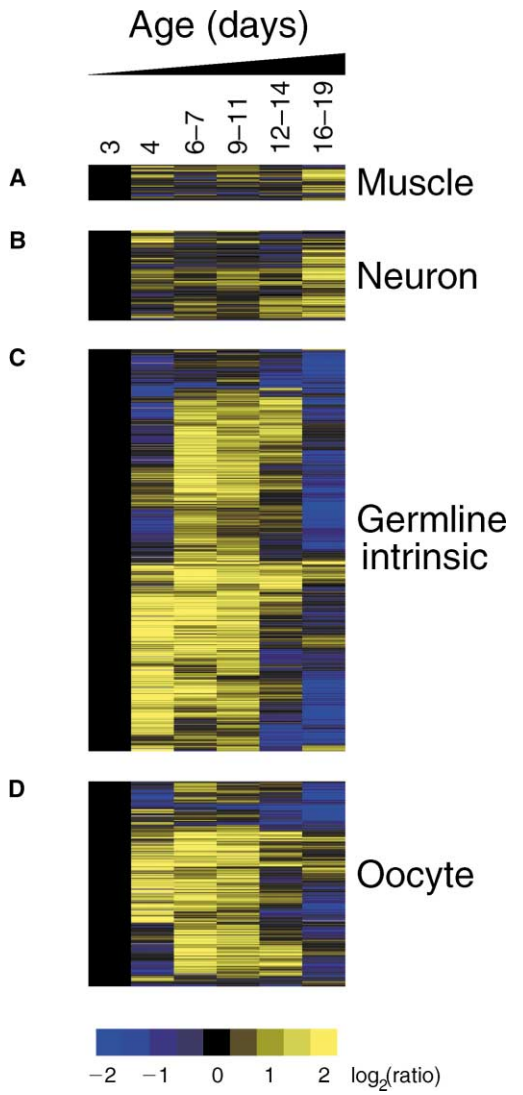

Figure 3. Expression Profile of Muscle, Neuronal, Germline-Intrinsic, and Oocyte Genes

Each row corresponds to a different gene. Gene identities and their expression are shown in Figure 3 at http://cmgm.stanford.edu/~kimlab/aging/. The scale shows the expression ratio.

(A) A total of 46 genes previously known to be expressed specifically in muscle.

(B) A total of 88 neuronal-specific genes.

(C) A total of 508 germline-intrinsic genes expressed in both sperm-producing and oocyte-producing animals.

(D) A total of 250 oocyte-enriched genes.

reproduction. This observation is also consistent with the ability of hermaphrodites to continue to reproduce depending on sperm availability [39]. Decreased germline expression occurs at an age with high mortality when many functions seem to be failing [20].

Overlap between Aging- and Dauer-Regulated Genes

In order to gain further insight into physiological processes that may change during aging, we compared the changes in gene expression observed in the aging microarray experiments to all other *C. elegans* DNA microarray experiments that have been done to date. To do this, we used a *C. elegans* gene expression map in which genes are placed into clusters based on gene expression similarities from 553 diverse DNA microarray experiments, involving many different growth conditions and mutant strains [40]. There are 44 distinct gene clusters (represented as mountains in the expression map) representing 17,661 genes (90% of the genome). So far, 34 mountains are enriched for genes expressed in a specific tissue (such as the germline, sperm, neurons, or muscle) or genes that have a common biological function (such as genes encoding histones, heat shock proteins, or cell cycle components). We compared the 164 aging-regulated genes to each of the mountains in the gene expression terrain map. As expected, the aging-regulated genes were significantly enriched in mounts 36 and 37; mount 36 is enriched for heat shock genes, mount 37 is enriched for Tc3 transposon copies, and both of these types of genes were previously noted among the set of aging-regulated genes.

In addition, the aging-regulated genes were significantly enriched in mount 15. There are 13 genes in common between mount 15 genes and aging-regulated genes, which is 5.6-fold higher than would be expected by chance ($p < 0.001$) (see the Experimental Procedures). Mount 15 contains 247 genes that have no obvious biological function in common. As a group, the expression level of genes in mount 15 increases during aging (Figure 4). We investigated the gene expression database to find out why these genes might cluster into a single gene expression mountain by determining which of the 553 DNA microarray experiments showed the strongest and most reproducible expression differences of the mount 15 genes. There were 14 experiments in which genes in mount 15 increased an average of more than 4-fold, and each of these DNA microarray hybridizations involved samples that likely contained dauer animals (data not shown).

To determine whether the mount 15 genes show increased expression in dauer animals, we performed an additional DNA microarray experiment comparing RNA from dauer to that from non-dauer animals. We prepared four worm samples highly enriched for dauers and another four samples consisting of worms that had recently exited from the dauer stage (12 hr after feeding). The dauer and non-dauer RNAs were used to make Cy5-labeled cDNA, and these cDNAs were each hybridized to DNA microarrays along with Cy3-labeled cDNA prepared from polyA$^+$ RNA from mixed-stage hermaphrodites. We calculated the average $\log_2$(sample/reference) ratio for the dauer and non-dauer microarray hybridizations, and we then calculated the average $\log_2$(dauer/non-dauer) ratio. Figure 4 shows the average expression differences of the genes in mount 15 in dauer versus non-dauer samples. These genes show an average increase in expression of 3.6-fold in dauers. Of the 231 genes in mount 15 that were present on the DNA microarrays, 215 showed higher expression in dauers than non-dauers (87%). These results are consistent with prior results using SAGE to compare gene expression in dauers to mixed-stage hermaphrodites [41]; we selected the 247 genes contained in mount 15, analyzed their

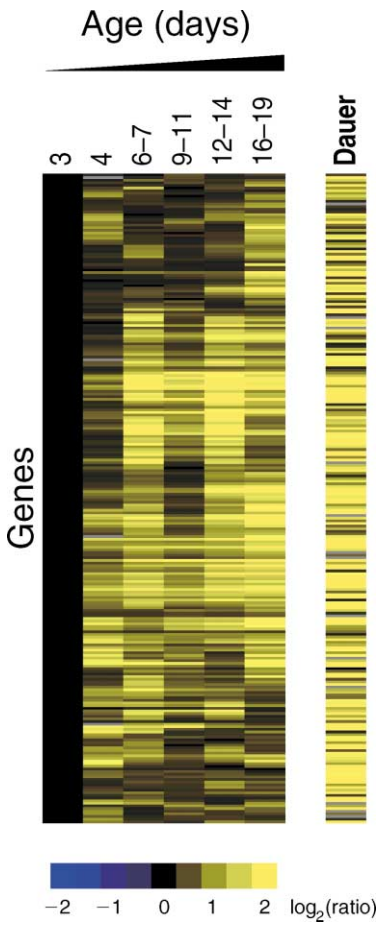

Figure 4. Expression Profile of Genes in Mount 15 during Aging and Dauer Exit

Each row corresponds to a gene from mount 15, which is one of the gene clusters from the gene expression terrain map [40]. The first six columns show expression ratios in the aging time course. The last column shows the expression ratio of dauer/non-dauer samples. The scale shows the level of expression.

expression levels in the SAGE data, and found that 71% have increased expression in dauers. Dauer animals have extended life spans and live more than seven times longer than normal worms [42]. The genes in mount 15 may form the basis for a common adaptive mechanism(s) specifying increased survival in normal aging and the dauer stage.

There are dramatic changes in behavior as worms age, including loss of movement, loss of egg-laying ability, and decreased rates of food ingestion [43–45]. At the morphological level, aging causes worms to accumulate lipofuscin, to stop producing eggs and oocytes, and results in a dramatic involution of the gonad. The data from the microarray experiments indicate that gene expression patterns are relatively stable during aging. Given the severe changes in morphology and physiology associated with aging and senescence [33, 45], it is remarkable to find only 164 genes with significant transcriptional changes (less than 1% of the genome). These data suggest that a substantial portion of the morphological differences seen between young and old worms is not accounted for by changes in gene expression, but rather is most likely due to cumulative stresses and damage from life itself. Similar studies in *Drosophila* have profiled expression differences during aging, and one study found that 6% of genes show significant age-related expression changes [17, 46]. These results suggest that aging could affect gene expression to a larger extent in flies than in worms. Another possibility is that the *C. elegans* microarray experiments might have missed a large number of aging-regulated genes because their changes in gene expression were too small to detect in this experiment. Alternatively, many of the age-dependent gene expression changes in *Drosophila* might be specific to one strain, and these genes would be filtered out from the *C. elegans* experiments by using multiple strains.

Conclusions

The global molecular profile of aging from these microarray experiments helps refine models for aging. The small number of genes that change with age is consistent with models suggesting that the basis for organismic aging is damage at the cellular and molecular level that has accumulated over a lifetime [27, 29, 33].

Our data also support the view that some changes in gene expression may play a role in specifying life span. The microarray data show coordinate expression changes in several functional gene groups during aging, including increased expression of dauer-regulated genes, decreased heat shock gene expression, and increased expression of insulin-like genes. *ins-2* and *ins-18* are thought to antagonize the *daf-2/age-1* insulin-signaling pathway [47]. One possibility is that expression of these insulin-like genes might increase in response to decreased feeding in old worms. Increased expression of *ins-2* and *ins-18* in old worms would lead to decreased insulin signaling, increased expression of dauer genes, and increased resistance to late life stressors.

It seems unlikely that decreased expression of the heat shock genes is merely a consequence of organismic changes associated with aging, since one might expect that aging would lead to an increased expression of heat shock genes (from increased levels of unfolded proteins). Rather, it could be that decreased expression of heat shock genes could alter cell physiology and be a cause of organismic senescence.

Another observation relevant to the specification of life span is that the set of 164 aging-regulated genes contains several genes that encode regulatory proteins. These genes include 12 that are involved in signaling (including *ins-2*) as well as 7 that encode transcription factors (see Table S1 at http://cmgm.stanford.edu/~kimlab/aging/). These regulatory genes could alter the expression of genes that affect survival or aging.

In addition to providing insight into the molecular mechanisms involved in aging, this work lays the foundation for the use of molecular probes to measure physiologic age. For example, aging-regulated genes could be used as molecular markers to track aspects of aging in individual worms. As aging is a complex process, it would be preferable to use a large set of molecular

markers to score physiologic age, in addition to merely scoring time of death. This study could be extended to an analysis of gene expression changes in longevity mutants and could enable one to understand the molecular basis of life extension specified by diverse mutations in genes extending life span.

Experimental Procedures

Worms were grown as in [20–22]. RNA preparation, cDNA synthesis, microarray hybridization, and microarray scanning were performed as previously described [18].

The fluorescence intensities of the Cy3 and the Cy5 signals were normalized such that the sum of all the signals from each microarray was the same for the Cy3 and Cy5 probes. A data filter was then applied such that only spots with intensities that were 1.5 times greater than the background intensity in both channels were used in the analysis. The samples were grouped by age into six time points, and single factor ANOVA analysis was performed on each gene ($p < 0.001$). We also evaluated the data by using a general linear model (GLM), which uses a type III sum of squares and doesn't require a balanced experimental design. The GLM revealed essentially the same genes as one-way ANOVA ($p < 0.001$). A post-hoc test was used to identify maturity genes that had expression level changes only between day 3 and day 4 (Tukey's HSD test; $p < 0.05$).

To examine strain effects, the variance under a two-way model considering strain and age was calculated for the ANOVA ($p < 0.001$) genes. Three genes had more variance due to strain than age and were not analyzed further (Table S1). To further evaluate whether expression varies as a function of age more than between strains, Pearson correlation coefficients between each pair of microarray hybridizations were calculated. For 21 of the 26 microarrays, the best correlation was with a microarray experiment involving the same sample age. For the remaining five microarrays, the best correlation was from another microarray in the same time course but at a different age. The greatest gene expression differences in these five microarrays may reflect variation between time courses or variation due to strain. In no case was the correlation stronger by strain than by age between different time courses.

The average gene expression values indicate the expression of each gene relative to all other genes at that time and do not show mRNA levels per worm. Expression for each time point was used as input for the clustering programs Cluster and TreeView [48]. The significance of the overlap between the genes in mount 15 and the aging-regulated genes was calculated by using hypergeometric probability according to [40].

Supplementary Material

Supplementary Material including the microarray gene expression data and additional figures and tables is available at http://images.cellpress.com/supmat/supmatin.htm.

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