

Sperm Loss Phenocopies Early Aging in *C. elegans*

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ABSTRACT Aging is a natural process that is associated with cellular and organismal degeneration through time. Although there has been extensive research into extreme aging and lifespan extension in *C. elegans*, there is comparatively little research focusing on the effects of aging in middle-aged animals. Here, we have studied a timepoint we call early aging, defined as the 3 days that occur after the L4 molt. We studied early aging in wild-type and spermless animals to understand whether egg-laying or sperm-status lead to different transcriptomic aging profiles. We find that spermless young adult animals partially phenocopy wild-type animals that have lost their sperm after 3 days of egg-laying, and that spermless animals also exhibit less transcriptomic changes associated with aging throughout these 3 days. Our results indicate that sperm loss triggers naturally occurring transcriptomic changes. These changes involve a variety of factors, and they are enriched in transcription factors canonically associated with neuronal development and differentiation.

KEYWORDS

C. elegans

fog-2

aging

longevity

transcriptome

INTRODUCTION

Aging is a natural phenomenon that affects most, if not all, animals.

While there has been a long-standing interest in *C. elegans* longevity and aging past 20 days of life, which has led to a rich literature (), there has not been such intense study into the changes that occur during a period we refer to as ‘early aging’. We define early aging in *C. elegans* as the period of time that occurs between the first day of adulthood (post-L4 molt) and 72 hours later. In this period of time, *C. elegans* worms undergo a transition between a hermaphroditic, self-fertile stage and its final form as a female worm.

We wanted to understand the transcriptomic changes that occur in early aging in the worm. We reasoned that early aging would have two components that ought to be separable in principle. Since the final stage of the worm is as a female, there might be transcriptomic changes associated with sperm status. A second component of aging, on the other hand, should be decoupled from sperm status. We designed a two factor experiment to test our hypothesis (see Fig. 1).

Wild-type worms naturally become female as they age. These worms should experience both components of early aging as time

proceeds. On the other hand, *C. elegans* defective in sperm formation will never transition to a hermaphroditic stage. As time moves forward, these spermless worms should not exhibit changes related to sperm or fertility status, and should only exhibit the genotype-independent changes. Additionally, we reasoned that we might be able to identify gene expression changes due to different life histories: whereas hermaphrodites lay almost 300 eggs over three days, spermless females likely engage in more mate-seeking behavior. The different life histories could affect gene expression and aging.

As a model for a spermless female worm, we selected the *fog-2* mutant for analysis. *fog-2* is involved in germ-cell sex determination in the hermaphrodite worm and is required for spermatogenesis (Schedl and Kimble 1988; Clifford *et al.* 2000). This protein is only expressed in the germline, which makes it a good target to generate spermless mutants with, since our perturbation would be targeted exclusively within the gonad.

Here, we show that early aging can be divided into two additive components. First, a component that is associated with loss of hermaphroditic sperm and which leads to increases in the levels of transcription factors that are canonically associated with development and cellular differentiation, and enriched in neuronal functions. We also describe a second transcriptomic module consisting of 5,592 genes that is probably associated with biological age in *C. elegans* and that is invariant between worms that undergo a hermaphroditic egg-laying stage and worms that are never fertilized. To facilitate exploration of the data, we have generated a website with interactive plots. This website is located at:

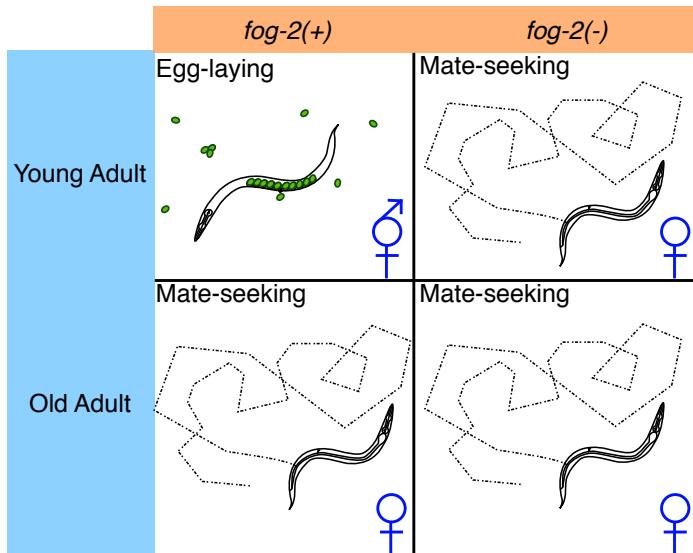


Figure 1 Experimental design to identify genes associated with life-history.

MATERIALS AND METHODS

RNA extraction

Synchronized worms were grown to either young adulthood or the 6th day of adulthood prior to RNA extraction. Synchronization and aging were carried out according to protocols described previously (Leighton *et al.* 2014). 1000–5000 worms from each replicate were rinsed into a microcentrifuge tube in S basal (5.85g/L NaCl, 1g/L K₂HPO₄, 6g/L KH₂PO₄), and then spun at 14,000rpm for 30s. The supernatant was removed and 1mL of Trizol was added. Worms were lysed by vortexing for 30s at room temperature and then 20 minutes at 4°. The Trizol lysate was then spun at 14,000rpm for 10 minutes at 4° down to allow removal of insoluble materials. Thereafter the Ambion TRIzol protocol was followed to finish the RNA extraction (MAN0001271 Rev. Date: 13 Dec 2012).

RNA-seq

RNA integrity was assessed using RNA 6000 Pico Kit for Bioanalyzer (Agilent Technologies #5067-1513) and mRNA was isolated using NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB #E7490). RNA-seq libraries were constructed using NEBNext Ultra RNA Library Prep Kit for Illumina (NEB #E7530) following manufacturer's instructions. Briefly, mRNA isolated from ~ 1μg of total RNA was fragmented to the average size of 200 nt by incubating at 94 °C for 15 min in first strand buffer, cDNA was synthesized using random primers and ProtoScript II Reverse Transcriptase followed by second strand synthesis using NEB Second Strand Synthesis Enzyme Mix. Resulting DNA fragments were end-repaired, dA tailed and ligated to NEBNext hairpin adaptors (NEB #E7335). After ligation, adaptors were converted to the 'Y' shape by treating with USER enzyme and DNA fragments were size selected using Agencourt AMPure XP beads (Beckman Coulter #A63880) to generate fragment sizes between 250 and 350 bp. Adaptor-ligated DNA was PCR amplified followed by AMPure XP bead clean up. Libraries were quantified with Qubit dsDNA HS Kit (ThermoFisher Scientific #Q32854) and the size distribution was confirmed with High Sensitivity DNA Kit for Bioanalyzer (Agilent Technologies

#5067- 4626). Libraries were sequenced on Illumina HiSeq2500 in single read mode with the read length of 50 nt following manufacturer's instructions. Base calls were performed with RTA 1.13.48.0 followed by conversion to FASTQ with bcl2fastq 1.8.4.

RNA interference

RNAi was performed as described in previous protocols (Kamath *et al.* 2001) using a commercially available RNAi library (Kamath *et al.* 2003). RNAi bacterial strains were grown in LB plus 100μg/mL ampicillin overnight. Fresh RNAi cultures were then plated onto NGM agar plates containing 25μg/mL carbenicillin and 1mM IPTG. N2 or *fog-2(q71)* hermaphrodites grown on *E. coli* OP50 were bleached onto sterile plates, and starved L1s transferred to recently seeded RNAi plates. All assays were performed on the offspring of these L1s. Worms grown on every strain were monitored for gross abnormalities, such as sterility, lethality and larval arrest. Control worms in all assays were fed with an anti-GFP RNAi strain. All RNAi worms were grown at 20° C.

Oocyte dropping assay

We performed a slightly modified oocyte dropping assay based on previously described protocols (White *et al.* 2012). Feminized *fog-2(q71)* hermaphrodites were picked as virgin L4s the day before the assay to a fresh RNAi plate. The following day, the adult animals were placed on assay plates (NGM agar seeded with 15μL of *E. coli* OP50 four days earlier), twenty worms per assay, and allowed to incubate at room temperature. Laid oocytes were counted after two hours. Plates were then left at room temperature overnight to serve as lawn-leaving assays.

Lawn-leaving assay

Lawn-leaving assays were performed as described previously (Lipton 2004). Young adult N2 hermaphrodites were selected the day of the assay and placed on assay plates (same as the oocyte dropping plates), twenty worms per assay, and allowed to incubate at room temperature overnight. Assays for *fog-2(q71)* hermaphrodites were performed on the same worms as used in the oocyte dropping assays. The following morning, plates were scored for leaving, with any worm touching any part of the bacterial lawn with any part of its body deemed to be "on" the lawn, and all others deemed to be "off".

Brood size counting

Worms were selected for this assay as L4 hermaphrodites to ensure that all progeny could be counted. For each replicate of each assay, a single worm was placed on a fresh RNAi plate and incubated at 20°. Every 1–2 days, the test worm was moved to a fresh RNAi plate, until it stopped laying eggs. Progeny were counted on each plate before they reached adulthood to ensure that only a single generation was counted.

Statistical Analysis

RNA-Seq Analysis. RNA-seq alignment was performed using Kallisto (Bray *et al.* 2015) with 200 bootstraps. The commands used for read-alignment are in the S.I.. Differential expression analysis was performed using Sleuth (Pimentel *et al.* 2016). The following Generalized Linear Model (GLM) was fit:

$$\log(y_i) = \beta_{0,i} + \beta_{G,i} \cdot G + \beta_{A,i} \cdot A + \beta_{A::G,i} \cdot A \cdot G, \quad (1)$$

where y_i are the TPM counts for the i th gene; $\beta_{0,i}$ is the intercept for the i th gene, and $\beta_{X,i}$ is the regression coefficient for variable X for the i th gene; A is a binary age variable indicating

young adult (0) or old adult (1) and G is the genotype variable indicating wild-type (0) or *fog-2* (1); $\beta_{A::G,i}$ refers to the regression coefficient accounting for the interaction between the age and genotype variables in the i th gene. Genes were called significant if the FDR-adjusted q-value for any regression coefficient was less than 0.1. Our script for differential analysis is available on GitHub.

Regression coefficients and TPM counts were processed using Python 3.5 in a Jupyter Notebook (Pérez and Granger 2007). Data analysis was performed using the Pandas, NumPy and SciPy libraries (McKinney 2011; Van Der Walt et al. 2011; Oliphant 2007). Graphics were created using the Matplotlib and Seaborn libraries (Waskom et al. 2016; Hunter 2007). Interactive graphics were generated using Bokeh (Bokeh Development Team 2014).

Tissue Enrichment Analysis was performed using WormBase's TEA tool (Angeles-Albores et al. 2016) for Python.

Brood Size Analysis. Brood size results were analyzed using Welch's t-test to identify genes that were significantly different from a GFP RNAi control. RNAi control results were pooled over multiple days because we could not detect systematic day-day variation. We did not apply FDR or Bonferroni correction because, at a p-value threshold for significance of 0.05, we expected 1 false positive on average per screen.

Lawn-leaving Analysis. Lawn-leaving results were analyzed using a χ^2 test for categorical variables. Results were considered statistically significant if $p < 0.05$. No FDR or Bonferroni correction was applied because the size of the screen was too small, with 1 false positive expected on average per screen. However, the lawn-leaving results suffered from high variance, which can lead to false positive results. To safeguard against false positive discovery, we used a non-parametric bootstrap to estimate the true χ^2 value. Using a bootstrap on this dataset does not lead to statistical acceptance of any gene that was not accepted without a bootstrap; however, applying a bootstrap does lead to statistical rejection of a large number of results.

Oocyte Dropping Analysis. Oocyte dropping results were analyzed using a non-parametric bootstrapped Mann-Whitney U-test because the GFP control variance was very large relative to the variance of the RNAi treatments. Results were considered statistically significant if $p < 0.05$.

Data Availability

Strains are available upon request. All of the data and scripts pertinent for this project except the raw reads can be found on our github repository https://github.com/WormLabCaltech/Angeles_Leighton_2016. File S1 contains Kallisto commands used to process the read alignments as well as the accession numbers for the raw-reads, which are available at GenBank. File S2 contains the list of genes that were altered in aging regardless of genotype. File S3 contains the list of genes that change with sperm loss.

RESULTS

Transcriptomics

We used a linear generalized model (see Statistical Analysis) with interactions to identify a transcriptomic profile associated with the *fog-2* genotype, a transcriptomic profile of *C. elegans* aging common to both genotypes. We identified an aging transcriptomic phenotypic consisting of 5,592 genes that were differentially expressed in 6 day old animals relative to 3 day old animals. This constitutes almost one quarter of the genes in *C. elegans*. Tissue Enrichment

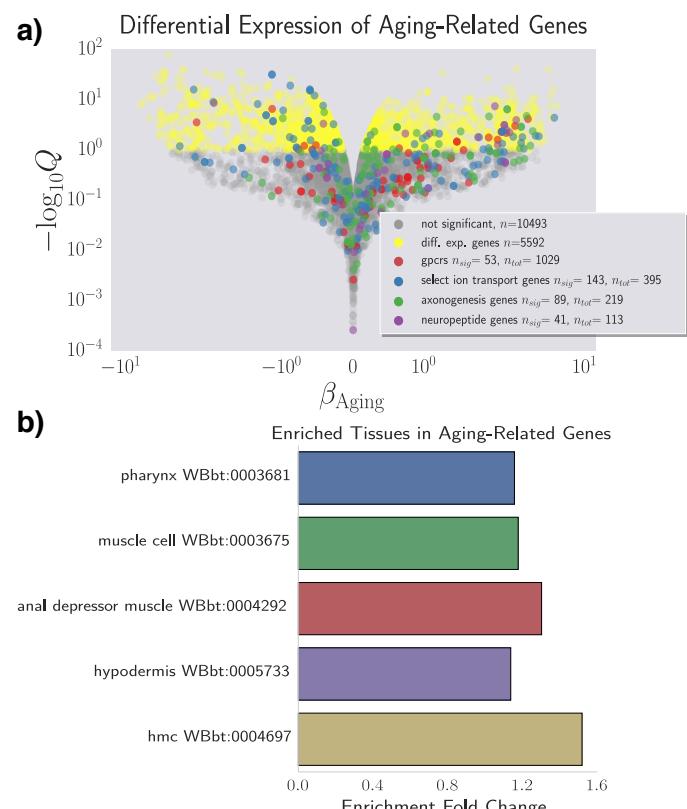


Figure 2 **a** We identified a common aging transcriptome between N2 and *fog-2* animals, which showed 5,592 altered genes. The volcano plot was randomly down-sampled 30%. **b** Tissue Enrichment Analysis showed that genes associated with muscle tissues and hypodermis are enriched in this dataset. An interactive version of this graph can be found on our [website](#).

Analysis (TEA) showed that muscle-associated and hypodermis-associated genes are particularly enriched in this dataset (see Figure 2).

To verify the quality of our dataset, we generated a list of genes expected to be altered in 6 day old worms using previous literature reports including downstream genes of *daf-12*, *daf-16*, and aging and lifespan extension datasets (). Out of 1056 genes in our golden standards, we found 506 genes. This results was statistically significant with a p-value < 10^{-38} .

We extracted all the transcription factors that were altered in the aging gene-set. We found 145 transcription factors. We expected these transcription factors to reflect the same tissue enrichment as the bulk dataset, but the results showed enrichment of hypodermic tissues and neuronal tissues, not muscle (see Table 1). Many of these transcription factors have been associated with developmental processes, and it is unclear why they would change expression in adult animals. Interactive volcano plots for each gene-set can be found in our [website](#).

We were also able to identify 1,881 genes associated with the *fog-2* genotype, including 60 transcription factors. Gonad-related tissues were enriched in this gene set, consistent with the function of *fog-2* as a sperm specification factor. As before, tissue enrichment analysis of these transcription factors reveals that they are involved in neural development. Of the 1,881 genes that we identified in the *fog-2* transcriptome, 1,040 genes were also identified in our aging set. Moreover, of these 1,040 genes, 905 genes changed in the same direction in age and genotype.

We were surprised at the large fraction of genes that overlapped between these two categories. We built our model to explicitly avoid overlap between variables. Our original expectation had been that certain genes would show a common aging phenotype regardless of genotype; that *fog-2* would exhibit a specific set of changes; and that a small set of genes would age differently between genotypes. However, the large fraction of genes that exhibit shared changes between both variables suggests that almost all genes that are involved in sperm loss through mutation of *fog-2* have an aberrant aging behaviour.

This aberrant behaviour can be most clearly observed by plotting the β regression coefficients for each variable against each other. Doing so reveals a clear trend along the line $y = x$. However, our model is built to specifically disallow this. The only situation in which $\beta_{\text{aging}} = \beta_{\text{genotype}}$ is a valid statement in our model is when $\beta_{\text{aging}:\text{genotype}} \neq 0$. Therefore, we also plotted $\beta_{\text{aging}:\text{genotype}}$ against β_{aging} . This revealed a strong inverse relationship: The interaction term cancels the aging (or genotype) term. An old, *fog-2* worm would be represented as $\beta_{\text{aging}} + \beta_{\text{genotype}} + \beta_{\text{aging}:\text{genotype}} = \beta_{\text{genotype}}$. Therefore, genes that are associated with sperm loss through mutation in the *fog-2* gene do not change through early aging in these animals. However, in animals that are wild-type, these same genes will eventually change to the same levels as in the *fog-2* mutants. In other words, *fog-2* partially phenocopies the aging process in wild-type animals 3 days post-adulthood (see Fig. 3).

Screens

We performed a brood size screen, selecting as targets genes that were upregulated in N2 but downregulated in *fog-2*. We identified 9 genes that altered brood size (see Figure 4). Of these 7 genes, XXX were previously known. XXX/7 were genes that were associated specifically with the *fog-2* phenotype. XXX/7 were associated with aging or life history.

Some of the genes in our genotype dataset could be associated

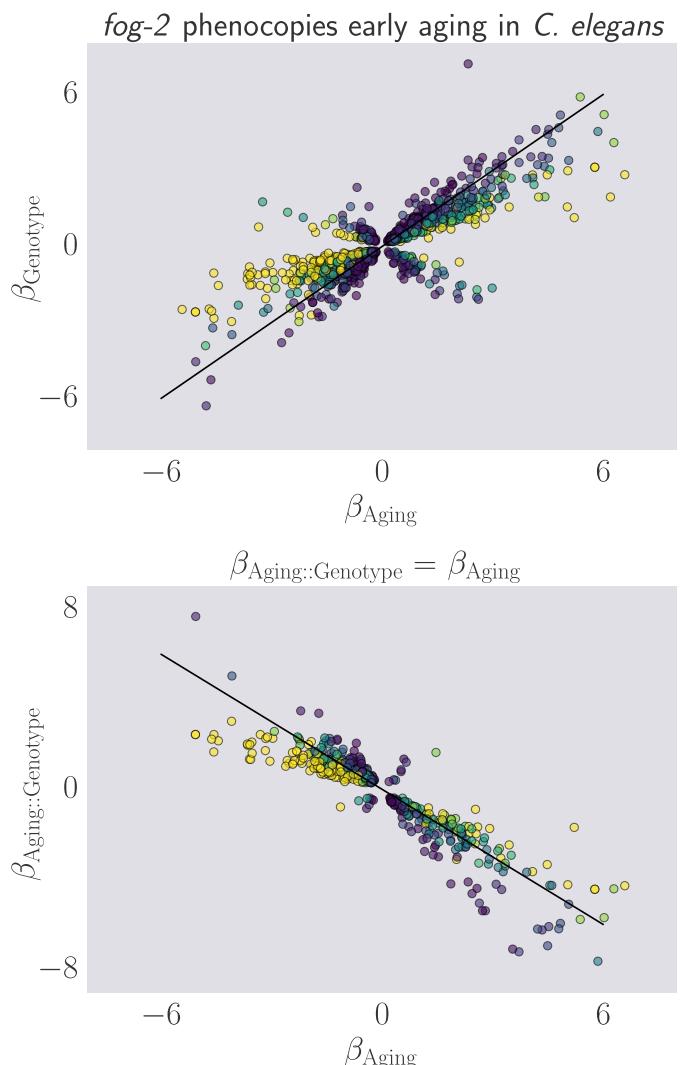


Table 1 Transcription Factor TEA Results. We ran TEA on the transcription factors associated with aging. Raw results showed a large list of terms associated with embryonic tissues that are neuronal precursors (AB lineage), so we trimmed the results by removing any terms that were expected to show up less than once in our list (if a term is expected to show up 10^{-3} times in a list, 1 occurrence is enough to show enrichment in this list). The best 4 results by Q-value are shown below.

Tissue	Expected	Observed	Q-value
P11	1	9	0.000006
ventral nerve cord	9	26	0.000006
dorsal nerve cord	5	19	0.000006
head muscle	3	13	0.000040
P7.p	1	6	0.001382

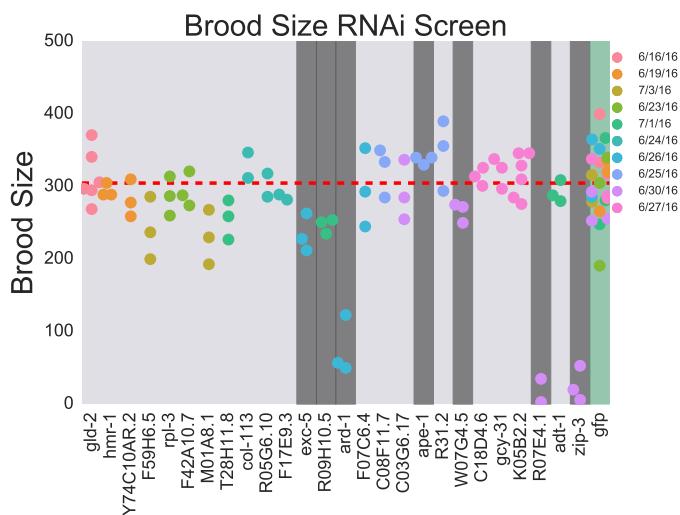


Figure 4 Brood size screen results. GFP control RNAi is shaded in green. Results that are statistically significantly different from GFP are shaded in dark grey. Dotted red line shows the mean of the pooled GFP controls. Points are colored by the date the assay was started on.

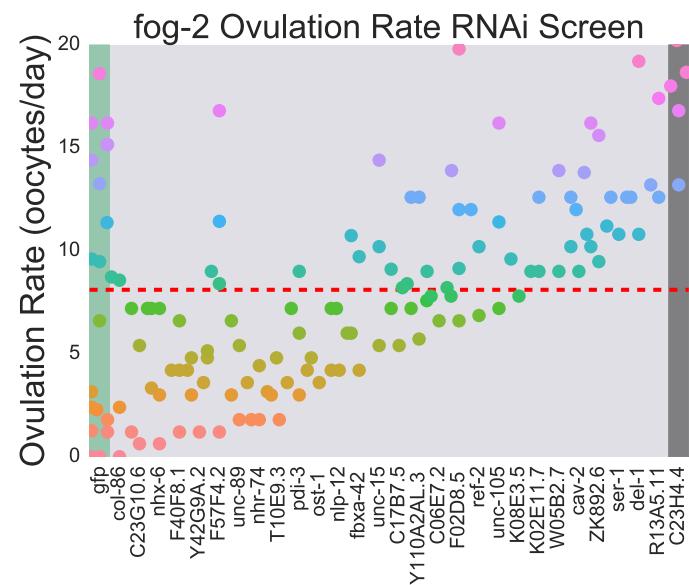


Figure 5 Ovulation Rate Assay. GFP control RNAi is shaded in green. Results that are statistically significantly different from GFP are shaded in dark grey. Dotted red line shows the mean of the pooled GFP controls. Points are colored proportionally to their y-coordinate

with ovulation rate in *fog-2*. We selected genes that showed upregulation in *fog-2* animals and placed them on a lawn for two hours. We observed large variation in the ovulation rate for the control RNAi, and as a result no genes were associated with alterations in ovulation rate. However, the pooled variance across the screen was very similar to the control variance, suggesting that our failure to identify genes was not a result of poor conditions or experimental variance. Our screen identified a single hit: C23H4.4, a carboxyl ester lipase with unknown function.

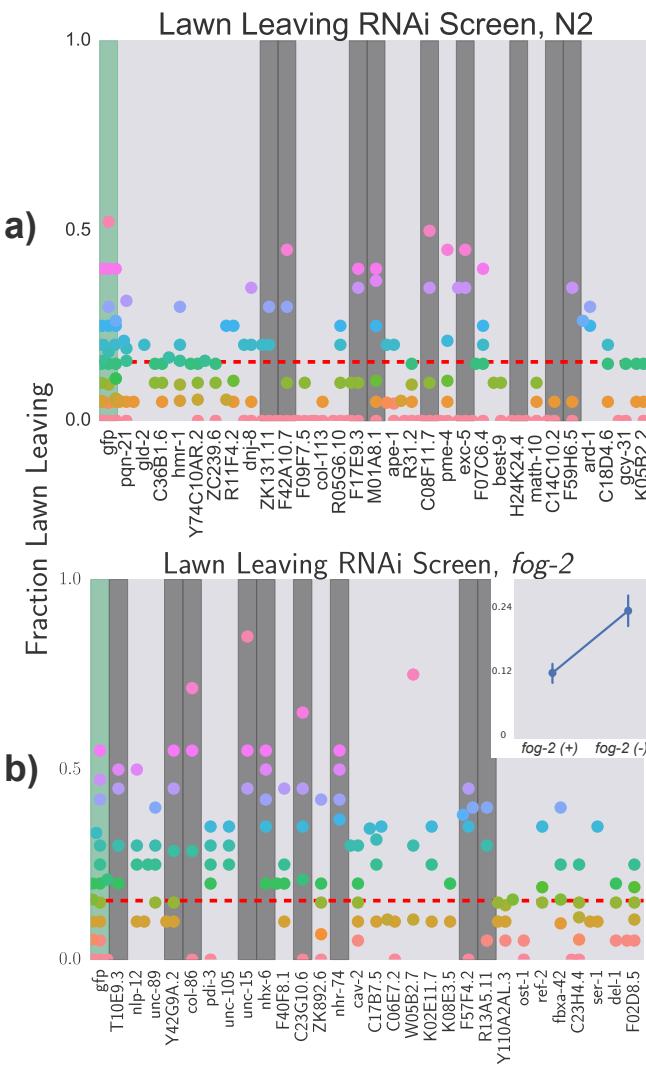


Figure 6 lawn-leaving Screen Results. GFP control RNAi is shaded in green. Results that are statistically significantly different from GFP are shaded in dark grey. Dotted red line shows the mean of the pooled GFP controls. Points are colored proportionally to their y-coordinate. **a** N2 lawn-leaving assay. **b** *fog-2* lawn-leaving assay. Inset shows the screen-wide average lawn-leaving rate of N2 and *fog-2*, which shows that the screen-wide lawn-leaving rate of *fog-2* worms is twice the rate of lawn-leaving of N2 worms, in agreement with previous literature ()�.

We also performed lawn-leaving assays because previous research has reported differences in N2 and *fog-2* leaving rates (). We tested genes that increased in *fog-2* animals in a *fog-2* background, expecting that decreasing these genes should lead to a

reversal of the lawn-leaving assay. Likewise, we tested genes that were decreased in *fog-2* animals in an N2 background, expecting that knockdown of these genes would cause lawn-leaving. We identified 9 genes that had an altered lawn-leaving profile in an N2 background. We also identified 9 genes that had an altered lawn-leaving profile in a *fog-2* background. Oddly, both screens identified genes that mainly stimulate lawn-leaving. Initially, we had expected that RNAi knockdown would allow us to identify genes that inhibit lawn-leaving behaviour in an N2 background; whereas RNAi knockdown in a *fog-2* background would identify genes that promote lawn-leaving. However, our screen only identified genes that suppress lawn-leaving in an N2 background. All other hits stimulated lawn-leaving.

DISCUSSION

Defining an Early Aging Phenotype

Our experimental design enables us to decouple the effects of egg-laying from aging. As a result of this, we identified a set of almost 4,000 genes that are altered similarly between wild-type and *fog-2* mutants. Due to the read depth of our transcriptomic data (20 million reads) and the number of samples measured (3 biological replicates for 4 different life stages/genotypes), this dataset constitutes a high-quality description of the genetic changes that occur in natural *C. elegans* early aging independently of genotype.

Developmental Factors Change During Early Aging

Our transcriptomic explorations revealed a host of transcription factors that changed during early aging in *C. elegans*. Many of these transcription factors have been associated with embryonic development via cellular differentiation and specification. For example, we identified the transcription factor *lin-32*, which has been associated with neuron development (); the Six5 ortholog *unc-39* has been associated with axonal pathfinding; *cnd-1*, a homolog of the vertebrate NeuroD transcription factors, is expressed in the early embryo and its expression is reported to disappear by the end of the first larval stage ()�.

Our explorations shown that the loss of the *fog-2* transcription factor phenocopies early aging in *C. elegans*. The reason for phenocopying is unclear at this point, but we speculate that such a result could happen if an animal could sense its own fertilization status. Indeed, prior work has established that *C. elegans* is capable of detecting its fertilization state, and that this state has consequences for pheromone production and mating behaviours. Given the enrichment of neuronal transcription factors that are associated with sperm loss in our dataset, we believe this dataset should contain some of the transcriptomic modules that are involved in these pheromone and behavioural pathways. Currently, we cannot judge how many of the changes induced by loss of hermaphroditic sperm are developmental (i.e., irreversible), and how many can be rescued by mating to a male. While an entertaining thought experiment, establishing whether these transcriptomic changes can be rescued by males is a daunting experimental task, given that the timescales for robust, whole-animal transcriptomic changes could reasonably be the same as the timescale of onset of embryonic transcription.

Interpretation of Screen Hits

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