Methods

Microarray construction. We used Research Genetics “GenePairs” primers for 18,455 predicted genes to PCR amplify fragments from C. elegans N2 genomic DNA. PCR products were ethanol precipitated before printing onto polylysine slides. PCR product size was confirmed by ethidium bromide staining of agarose gels before printing.

Methods and strains. All strains used were maintained and handled as described previously. Mutations used in this study:
LG1: daf-16(mu86)
LGII: age-1(hx546), fer-15(b26), rrf-3(pk1426)
LGIII: daf-2(mu150), daf-2(e1368), daf-2(e1370)
LGIV: fem-1(hc17)
DAF-16::GFP strain: (muEx110 [pKL99-2 (daf-16::gfp/daf-16b (-))+pRF4(rol-6)]; daf-16(mu86) I; daf-2(e1370) III).

Gonad morphology was assayed to determine developmental stage of all strains.

RNAi. HT115 bacteria expressing dsRNA were grown at 37°C in LB with 10 mg/mL tetracycline and 50 mg/mL carbenicillin, then were seeded onto NG-carbenicillin HG plates and supplemented with 100 µL 0.1M IPTG, or were grown on NG-carbenicillin-IPTG plates of the same concentrations. Eggs were added to plates and animals were transferred to new plates every 4-7 days. Vectors used in this study: pAD12 (control vector Bluescript with opposing T7 promoters), pAD48 (2.3 kb of daf-2 coding sequence cloned into pAD12), pAD43 (1.1 kb of daf-16 coding sequence cloned into pAD12). Other dsRNA-producing bacteria were selected from an RNAi library kindly provided by J. Ahringer for lifespan tests. The identity of each RNAi clone was verified by PCR and subsequent sequence analysis.
**C. elegans growth and collection for microarray experiments.** 
daf-2 and age-1 mutants were synchronized by harvesting eggs after bleaching and arresting at L1, then grown on lawns of OP50 on 150 mm NG plates at 20°C or 25°C, as indicated; 30-50,000 worms were collected on the first day of adulthood. Microarray comparisons of mutants include daf-2(e1368), daf-2(mu150), and age-1(hx546) fer-15(b26) mutants versus wild type (N2 background or fer-15(b26); fem-1(hc17) background as appropriate), and daf-2(e1370) mutants versus daf-16(mu86); daf-2(e1370) double mutants, as well as a comparison of the expression profile of daf-16(mu86); daf-2(e1370) double mutant with the same strain carrying a rescuing daf-16::gfp fusion.

Synchronized fer-15(b26); fem-1(hc17) animals were grown on RNAi bacteria at 25°C, induced with IPTG on Day 1 of adulthood, and collected at the indicated time points (Fig. 1a); RNAi bacteria was supplemented as necessary for later time points. Worms were floated off lawns with M9 buffer, centrifuged, and washed again in M9. The pelleted worms were dissolved in Trizol (Gibco) and frozen in liquid nitrogen.

**Microarray hybrizations.** Standard Trizol purification was used to obtain RNA. Oligotex purification (Qiagen) was used to purify mRNA, from which cDNA was reverse transcribed. Labeling of cDNA with Cy dyes and hybridizations to microarrays were carried out as previously described; arrays were hybrizidized for 18 hours at 63°C, washed, and scanned. For time courses, one half of each sample was added to a pooled sample and every Cy5-labeled sample was compared to this Cy3-labeled mixed reference. Mutant comparisons were done both directly and in a pooled comparison.

**Microarray analysis:**

**Array normalization.** JPEG images of arrays were analyzed (GenePix, Axon) to determine ratios of Cy-labeled samples. Empty, weak, and saturated spots were flagged
and discarded. A normalization factor for the ratio of medians variable was calculated from the average spot intensity; this factor was used to correct all measurements for that array by multiplying it into all measurements for the Cy5 channel. A lower limit corresponding to the variance in local background pixel intensity was used to mute the extreme ratio measurements for faint spots; spots below a user-defined intensity were discarded. The ratio of the adjusted measurements for the two channels was the raw measure of differential hybridization. The log base two of this normalized, adjusted red/green ratio was used for clustering and analysis. NOMAD (www.derisilab.ucsf.org) was used to normalize, filter out genes with low intensity spots, and order a set of genes from daf-2 pathway mutant arrays prior to Cluster and SAM analysis (see below).

**Significance Analysis.** We hierarchically clustered the genes whose expression level changed at least four-fold\(^{10}\) (see below) across the set of mutant arrays. We also used SAM\(^{11}\) to analyze statistical significance of the changes in gene expression from repeated permutations. SAM analysis of mutant arrays without fold-change elimination identified genes with small but consistent changes, establishing a q-value (a p-value for multiple testing) for the difference in expression of each gene (Table 1s). Data from nine mutant arrays were imported from NOMAD and Cluster to normalize and eliminate poor spots, and SAM analysis was performed (one-class response, 100 permutations, K nearest neighbor, 10 neighbors) on the remaining set. The delta value was adjusted until the number of expected false positives < 1, producing a list of genes with the reported q-value, the lowest false discovery rate at which that gene is called significant. In this set of arrays, 70 upregulated and 100 downregulated genes were found to be significant (q-value = 0.0011194) with 0.6207 median false significant genes (Table 1s; calculation for a delta value of 1.47).
**Correlation coefficient analysis.** We calculated a vector comprising the entirety of log ratio comparisons for all the genes with a valid, reliable signal at a single time point, so that each array could be described in a single value. We compared each array in the two time courses to all other arrays in that time course. For each pairwise comparison of array experiment, the Pearson correlation of the log base-two of these expression ratios was calculated. We used this technique for quality control: five arrays from the set of 60 time points did not correlate with neighboring timepoints, and were deleted prior to further analyses to avoid biasing the results.

**Cluster analysis.** After normalization, log transformation, and quality confirmation through correlation coefficient analysis, data from 55 RNAi arrays and 5 mutant arrays were imported into Gene Cluster\textsuperscript{10} for fold-cutoff analysis and hierarchical clustering. Genes were filtered to obtain only those that were present in 80% of the 60 arrays in the data set and which met a Max-Min=2 (4-fold), 3 (8-fold), or 4 (16-fold) criterion. The filtered set was hierarchically clustered, a self-organized map was constructed with 300,000 iterations, and the gene set was displayed in TreeView\textsuperscript{10}. We found that 7380 genes met a 4-fold cutoff, 2734 genes met an eight-fold cutoff, and 1280 genes met a 16-fold cutoff over the entire set of 55 RNAi arrays and five mutant arrays (Fig. 1d). Our RNAi analysis focused on a smaller subset of genes whose expression profiles changed in opposite ways under *daf*-2(-) and *daf*-16(-) conditions.

**Upstream sequence analysis.** The sequence 1 kb upstream of the translation start site of each ORF was assembled and subjected to two algorithms to search for potential transcription factor binding sites. The first, Mobydick\textsuperscript{12}, uses a statistical model of segmentation to delineate biologically meaningful “words” that are likely to be regulatory elements from the potential regulatory regions of genes. Exact repeats of length 14 or longer were removed before building the dictionary; words were then screened by
contrasting the frequency of occurrences in the cluster to that in the upstream regions of all the genes in the genome. A second algorithm searches exhaustively for oligonucleotides that are over-represented in the cluster\textsuperscript{13}. The occurrence of the identified sequences in the 5 kb upstream of each gene was then determined\textsuperscript{14}.

**Survival analyses.** Lifespan analyses were performed on 60-70 worms per experiment as described previously\textsuperscript{15,16}. Genes were prioritized by fold expression change (Fig. 1d), and in some cases, by interesting gene function (e.g., \textit{ins-7}), and the corresponding bacteria were selected from the Ahringer RNAi library\textsuperscript{6}. We tested 58 genes (Table 1). In all experiments, the first day of adulthood was used as \(t=0\), and the log-rank (Mantel-Cox) method was used to test the hypothesis that the lifespans were equal (StatView 5.01, SAS Software). \textit{fer-15(b26); fem-1(hc17)} animals were grown at 25\(^\circ\) C on RNAi bacteria and lifespans were measured at this temperature unless otherwise indicated. \textit{daf-2(mu150)} worms were raised and measured at 25\(^\circ\) C in one trial, and in subsequent tests were raised until L3 at 20\(^\circ\) C then shifted to 25\(^\circ\) C for the remainder of their lives. \textit{rrf-3(pk1426)} worms, which display enhanced RNAi effects\textsuperscript{4}, were treated at 20\(^\circ\) C in one experiment; in subsequent experiments, the worms were shifted to 25\(^\circ\) C at L2 through young adulthood to induce sterility, and adult lifespan was measured at 20\(^\circ\) C. \textit{rrf-3(pk1426); daf-2(e1370)} lifespans were done at 20\(^\circ\)C.

**Dauer tests.** \textit{daf-2(e1370)} worms were grown on RNAi-bacteria at 20\(^\circ\) C, and F1 eggs were incubated at 22.5\(^\circ\) C; at this temperature, about 60\% of \textit{daf-2(e1370)} mutants enter the dauer state. These worms were scored for dauer arrest 72 hours later.