

# A systematic RNAi screen identifies a critical role for mitochondria in *C. elegans* longevity

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**We report a systematic RNA interference (RNAi) screen of 5,690 *Caenorhabditis elegans* genes for gene inactivations that increase lifespan. We found that genes important for mitochondrial function stand out as a principal group of genes affecting *C. elegans* lifespan. A classical genetic screen identified a mutation in the mitochondrial leucyl-tRNA synthetase gene (*lrs-2*) that impaired mitochondrial function and was associated with longer-lifespan. The long-lived worms with impaired mitochondria had lower ATP content and oxygen consumption, but differential responses to free-radical and other stresses. These data suggest that the longer lifespan of *C. elegans* with compromised mitochondria cannot simply be assigned to lower free radical production and suggest a more complex coupling of metabolism and longevity.**

## Introduction

Genetic and environmental factors that affect metabolism and free-radical production have been shown to regulate longevity in a variety of systems<sup>1–3</sup>. The *daf-2* (transmembrane receptor protein tyrosine kinase)/insulin-like signaling pathway is a key regulator of longevity in *C. elegans*; reductions in the activity of the *daf-2* pathway increase lifespan by a factor of up to 4<sup>4–6</sup>. There are many insulin genes in *C. elegans*<sup>7</sup> that might mediate input to this pathway through environmental cues such as nutritional status or growth conditions. The longer lifespan associated with lower *daf-2* signaling is completely abrogated by loss-of-function mutations in *daf-16*, which encodes a forkhead transcription factor, indicating that *daf-16* is the major downstream effector of *daf-2* signaling<sup>8,9</sup>.

A handful of genes have been found to affect *C. elegans* lifespan through pathways downstream or parallel to the insulin signaling pathway. The feeding-defective *eat* mutants live slightly longer in a *daf-16*-independent manner. The *eat* mutations might extend lifespan through a mechanism resembling caloric restriction in mammals<sup>10</sup>. The *clk* (biological timing abnormality) mutations also extend lifespan, though not to the same extent as do mutations in *daf-2* pathway genes, and slow the rates of many physiological processes<sup>11</sup>. *clk-1* encodes a protein that is involved in the pathway of coenzyme Q synthesis, implicating the mitochondrion in lifespan determination<sup>12,13</sup>, and *clk-2* encodes a protein that is involved in DNA repair and, perhaps, telomere maintenance<sup>14–16</sup>.

To identify in a systematic manner all of the gene classes that control *C. elegans* lifespan, we used a set of more than 5,600 genes

in an RNAi screen to identify genes that extend lifespan when inactivated. This screen showed that a large number of genes essential for mitochondrial function have critical roles in determining *C. elegans* lifespan. Consistent with these results, in a classical genetic screen for increased lifespan, we found that a probable null mutation in a mitochondrial leucyl-tRNA synthetase gene (*lrs-2*) resulted in a markedly longer lifespan.

The long-lived worms with RNAi-induced or mutation-induced mitochondrial defects all had lower levels of ATP, and most had lower oxygen consumption and altered mitochondrial morphology, consistent with defective mitochondrial function. Whereas the long-lived worms with compromised mitochondria had normal or enhanced resistance to hydrogen peroxide and heat-shock treatment, some of them showed heightened sensitivity to the superoxide-inducing agent paraquat, suggesting that they might have had specific defects in the reactive oxygen species (ROS) pathway. In the RNAi screen, we also found that inactivation of two metabolic enzymes significantly extended lifespan. Thus, nematodes with dysfunctional mitochondria or specific impaired metabolic pathways might live longer owing to metabolic changes that prolong lifespan.

## Results

### Systematic RNAi screen for genes associated with longevity

To identify genes that extend lifespan when inactivated, we used feeding RNAi libraries<sup>17,18</sup> to systematically inactivate most of the annotated genes along two of the six *C. elegans* chromosomes and screened for longer lifespan. Although the screen was done

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blind to the identity of the gene in each well, the molecular identity of each gene could subsequently be decoded to reveal common themes in the genes identified.

We examined nearly all of the genes on Chromosomes I and II (5,690 genes), and found that RNAi inactivation of a large number of genes key to mitochondrial function markedly extended *C. elegans* lifespan (Fig. 1a). For example, the Chromosome I library contains RNAi clones corresponding to 2,663 genes, and we found that RNAi inactivation of 52 of these (1.8%) caused reproducible lifespan extension. Using large-scale screening conditions (with worms feeding on the same *Escherichia coli* expressing a dsRNA for the entire adult lifespan), these RNAi clones extended lifespan by 5–30%. The positive control *age-1* RNAi extended lifespan by 30%, compared with an *age-1* mutant that extended lifespan by 100%<sup>6,19</sup>. This difference is probably due to the fact RNAi does not generally yield as strong a phenotype as a mutated allele. When retested under more rigorous conditions

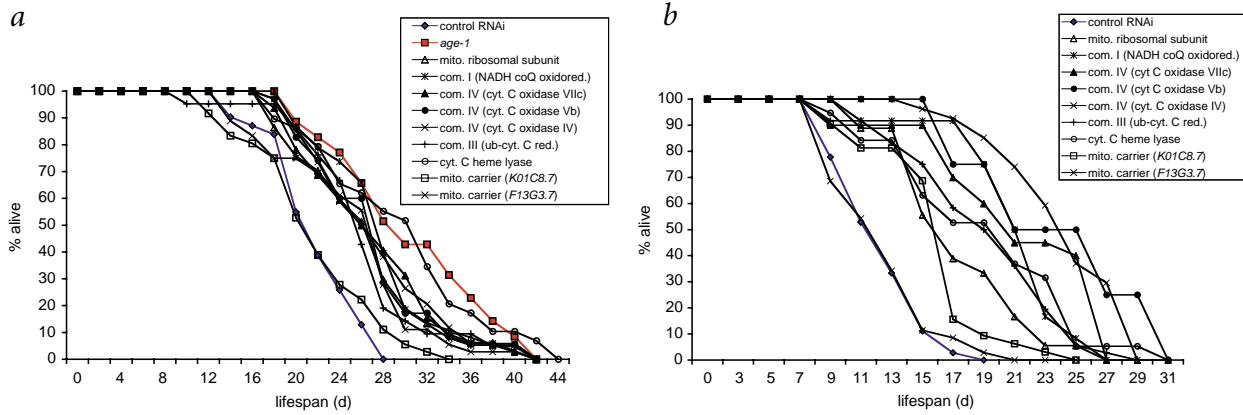
(with worms transferred to fresh *E. coli* expressing the dsRNA every four days) in at least four independent trials, a large number of these RNAi candidates induced more marked lifespan extension (Fig. 1a and Table 1).

Genes identified as influencing lifespan included those that are involved in gene expression, protein processing, signal transduction, stress response, energy metabolism and other uncharacterized systems (S.S.L. and G.R., unpublished data). Notably, 15% of the genes influencing lifespan were specific for mitochondrial function, including mitochondrial carriers, electron-transport chain components and a mitochondrial ribosomal subunit (Fig. 1a and Table 1). The lifespan extension induced by RNAi inactivation of these genes was most comparable to that induced by RNAi inactivation of insulin pathway genes. None of the 5,690 RNAi inactivations extended *C. elegans* lifespan more potently than RNAi inactivation of the insulin pathway genes.

**Table 1 • Summary of developmental and lifespan phenotypes following RNAi**

Gene (Chr.)	Homology <sup>a</sup>	Supp. by <i>daf-16</i>	Egg <sup>b</sup> RNAi phenotype	L1 <sup>b</sup> RNAi phenotype	N2 mean lifespan <sup>c</sup> (t-test, KS-test)	<i>daf-16</i> mean lifespan <sup>c</sup> (t-test, KS-test)
Control (vector)					19.2 ± 5.0	12.5 ± 1.8
D2030.4 (I)	NADH coQ oxidoreductaseB18 (complex I)	No	L4 arrest	Slight delay	27.4 ± 6.9 ( <i>P</i> = 1 × 10 <sup>-6</sup> , <i>P</i> = 4 × 10 <sup>-6</sup> )	21.5 ± 3.1 ( <i>P</i> = 2 × 10 <sup>-15</sup> , <i>P</i> = 5 × 10 <sup>-14</sup> )
T02H6.11 (II)	Ubiquinol-cytochrome b reductase subunit(complex III)	No	L3 arrest	1-d adult delay, sterile	28.1 ± 5.2 ( <i>P</i> = 2 × 10 <sup>-8</sup> , <i>P</i> = 4 × 10 <sup>-7</sup> )	23.5 ± 4.4 ( <i>P</i> = 1 × 10 <sup>-16</sup> , <i>P</i> = 8 × 10 <sup>-16</sup> )
F26E4.6 (I)	Cytochrome C oxidase Viic (complex IV)	No	L2 arrest	1-d adult delay, sterile	28.5 ± 5.8 ( <i>P</i> = 2 × 10 <sup>-8</sup> , <i>P</i> = 7 × 10 <sup>-7</sup> )	22.1 ± 4.3 ( <i>P</i> = 6 × 10 <sup>-15</sup> , <i>P</i> = 7 × 10 <sup>-15</sup> )
F26E4.9 (I)	Cytochrome C oxidase Vb (complex IV)	No	1-d adult delay, sterile	Slight delay, sterile	27.0 ± 5.9 ( <i>P</i> = 2 × 10 <sup>-6</sup> , <i>P</i> = 1 × 10 <sup>-6</sup> )	25.3 ± 4.2 ( <i>P</i> = 1 × 10 <sup>-15</sup> , <i>P</i> = 5 × 10 <sup>-14</sup> )
W09C5.8 (I)	Cytochrome C oxidase IV (complex IV)	No	1-d adult delay, sterile	Slight delay, sterile	31.1 ± 5.4 ( <i>P</i> = 5 × 10 <sup>-12</sup> , <i>P</i> = 2 × 10 <sup>-9</sup> )	25.9 ± 3.4 ( <i>P</i> = 3 × 10 <sup>-5</sup> , <i>P</i> = 1 × 10 <sup>-14</sup> )
B0261.4 <sup>d</sup> (I)	Mitochondria ribosomal subunit	No	1-d adult delay	Wild-type	22.7 ± 6.1 ( <i>P</i> = 0.0160, <i>P</i> = 0.0012)	16.5 ± 4.8 ( <i>P</i> = 0.0002, <i>P</i> = 1 × 10 <sup>-7</sup> )
T06D8.6 (II)	Cytochrome c heme lyase	No	L4 arrest	Slight delay	29.8 ± 7.6 ( <i>P</i> = 2 × 10 <sup>-7</sup> , <i>P</i> = 1 × 10 <sup>-5</sup> )	16.5 ± 4.8 ( <i>P</i> = 1 × 10 <sup>-5</sup> , <i>P</i> = 1 × 10 <sup>-6</sup> )
F13G3.7 (I)	Mitochondria carrier	Yes	Wild-type	Wild-type	24.0 ± 4.8 ( <i>P</i> = 0.0007, <i>P</i> = 2 × 10 <sup>-5</sup> )	13.6 ± 3.2 ( <i>P</i> = 0.0870, <sup>e</sup> <i>P</i> = 3 × 10 <sup>-5</sup> )
K01C8.7 <sup>d</sup> (II)	Mitochondria carrier	No	Wild-type	Wild-type	23.1 ± 4.2 ( <i>P</i> = 0.004, <i>P</i> = 5 × 10 <sup>-6</sup> )	16.4 ± 3.5 ( <i>P</i> = 3 × 10 <sup>-6</sup> , <i>P</i> = 5 × 10 <sup>-9</sup> )
F28B3.5 (I)	1-acyl-glycerol-3-phosphate acyltransferase	Yes	Wild-type	Wild-type	25.2 ± 6.7 ( <i>P</i> = 0.0016, <i>P</i> = 0.0003)	11.8 ± 2.6 ( <i>P</i> = 0.1897, <i>P</i> = 0.0303)
F57B10.3 (I)	Phosphoglycerate mutase	Yes	Wild-type	Wild-type	23.3 ± 6.9 ( <i>P</i> = 0.0185, <i>P</i> = 0.0012)	12.3 ± 3.3 ( <i>P</i> = 0.6725, <i>P</i> = 0.0142)

<sup>a</sup>As annotated in WormBase. <sup>b</sup>Egg: L4 worms were fed RNAi-expressing bacteria and allowed to egglay, and the progeny were scored for phenotypes. L1: synchronized L1 worms were fed RNAi-expressing bacteria, and their phenotypes were scored. <sup>c</sup>The mean lifespan ± s.d. (d) was obtained from 2 independent experiments. N2: wild-type worms were fed RNAi-expressing bacteria. *daf-16*: *daf-16(mgDf47)* worms were fed RNAi-expressing bacteria. Total number of worms tested for each RNAi clone was approximately 40. Statistical analysis was carried out using the lifespan of each worm of the entire population. <sup>d</sup>These RNAi clones induced modest lifespan extension in wild-type N2, but more noticeable extension in *daf-16(mgDf47)*. <sup>e</sup>Mean lifespan is suppressed by *daf-16(mgDf47)*, but few worms had lifespans significantly longer than did controls.



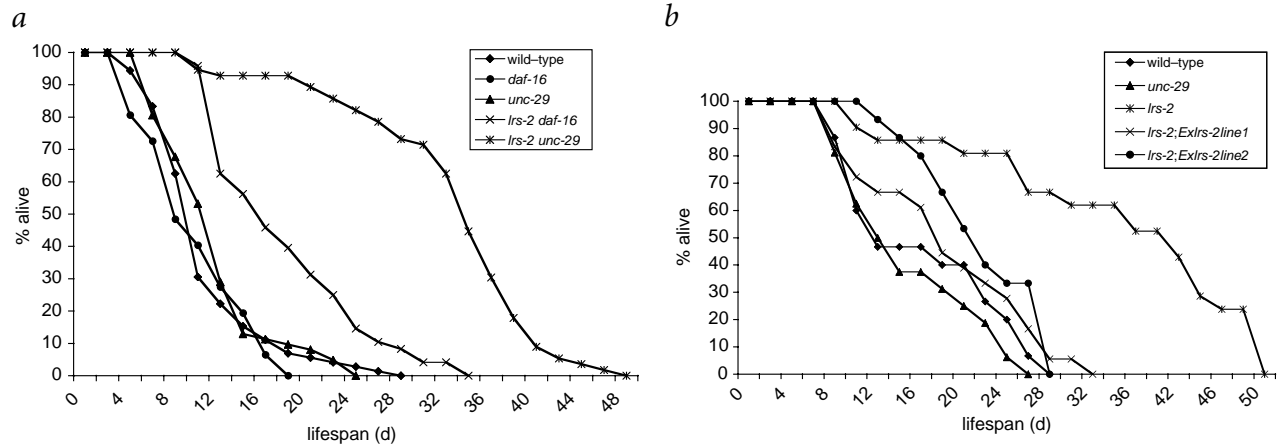
**Fig. 1** RNAi inactivation of genes specific for mitochondrial function markedly extended lifespan. The percentage of worms that were alive is plotted against adult age. Wild-type N2 worms (**a**) or *daf-16(mgDf47)* worms (**b**) were fed either bacteria not expressing any dsRNA (control RNAi) or bacteria expressing dsRNA that targeted the indicated mRNAs. The figure shows the results of one representative experiment carried out at 20 °C. See Table 1 for statistical analysis.

Several of the mitochondrial function genes that we identified as influencing lifespan in the RNAi screen had pleiotropic phenotypes, especially when exposed to RNAi early in development (Table 1). The lethality that we observed when some of the mitochondrial components were inactivated by RNAi might represent severe loss of mitochondrial function, whereas lifespan extension might result from a less severe loss of mitochondrial function. The sterility observed in worms undergoing RNAi inactivation of the mitochondrial complex III and IV subunits (Table 1) might be due to reduced mitochondrial function and insufficient energy production for germ-cell differentiation. Consistent with this explanation, a sixfold increase in the number of mitochondria occurred during the *C. elegans* transition from L4 to adult<sup>20</sup>, presumably owing to a surge in energy demand as the germ line developed.

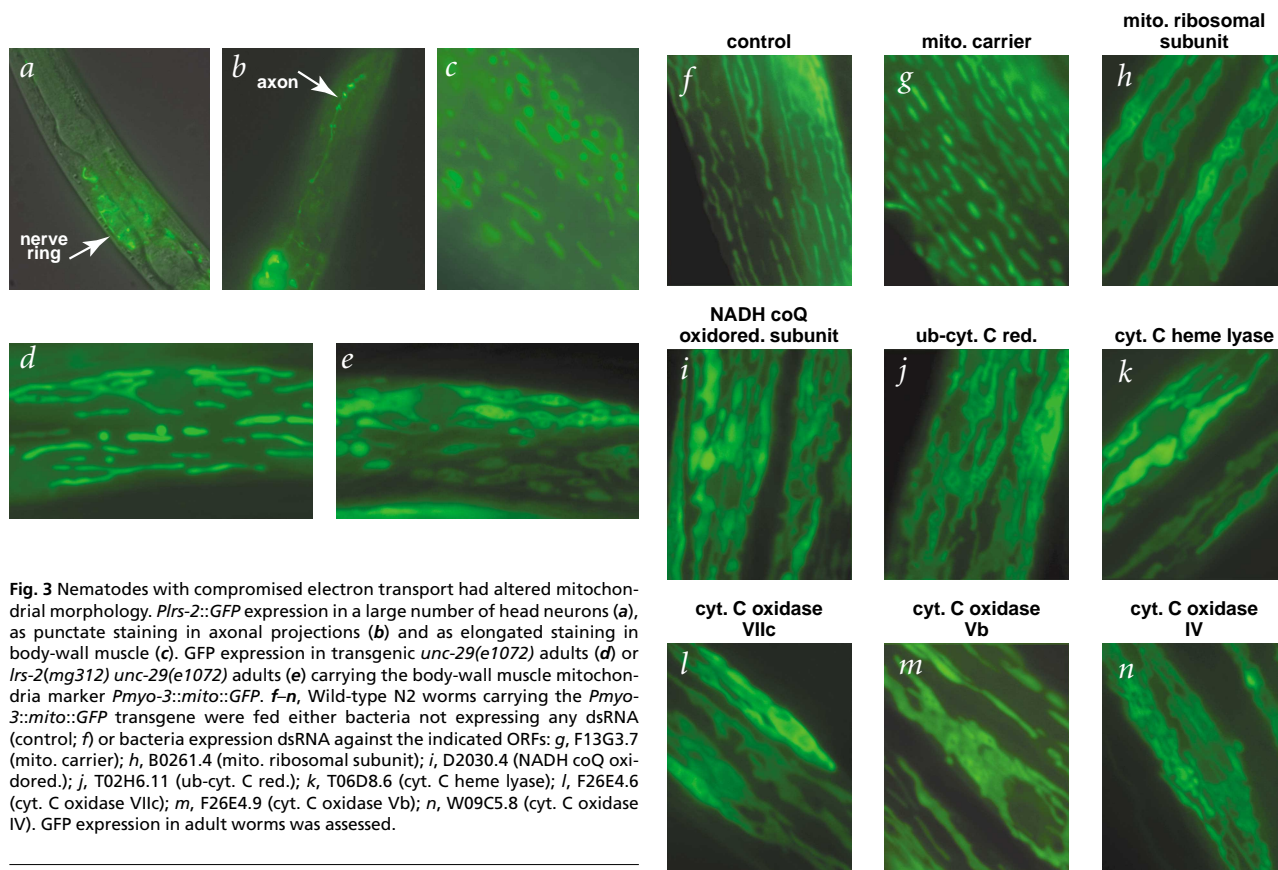
These mitochondrial components influence lifespan independently of the insulin signaling pathway. *daf-16(mgDf47)* null mutant worms, in which lifespan is decoupled from upstream insulin signaling, lived considerably longer when feeding on RNAi bacteria that targeted all but one of the mitochondrial

components (F13G3.7; Fig. 1b and Table 1). Although the absolute lifespan of the *daf-16* worms with mitochondrial components inactivated by RNAi was shorter than that of wild-type worms undergoing the same RNAi treatment, the percentage extension in lifespan was comparable. Thus, most of the mitochondrial components that influence lifespan must act downstream of *daf-16* or parallel to the insulin-like longevity-regulating pathway. Consistent with this possibility, a DAF-16::GFP fusion protein<sup>21</sup> that translocates from the cytoplasm into the nucleus when *daf-2* is inactivated by RNAi remained cytoplasmic when any of the mitochondrial function genes influencing lifespan were inactivated by RNAi (data not shown).

It is not clear why increased lifespan caused by RNAi inactivation of the probable mitochondrial carrier protein F13G3.7 depends on *daf-16*; one interesting possibility is that *daf-16*, the downstream transcription factor of the insulin pathway, might influence the availability of cellular metabolites that are substrates of that specific mitochondrial carrier.



**Fig. 2** A mutation of leucyl-tRNA synthetase extended lifespan. **a**, Mutant *lrs-2(mg312)* worms had markedly extended lifespans. The figure shows the results of one representative experiment carried out at 20 °C. Mean lifespan for wild-type N2 was 10.8 d ( $n = 72$ ), for *daf-16(mgDf47)* was 9.9 d ( $n = 62$ ), for *unc-29(e1072)* was 11.5 d ( $n = 62$ ), for *mg312 daf-16(mgDf47)* was 18 d ( $n = 48$ ) and for *mg312 unc-29(e1072)* was 32 d ( $n = 56$ ). Similar results were obtained when lifespan assays were carried out using plates with no FUDR (data not shown). **b**, Wild-type *lrs-2* expression rescued the lifespan extension phenotype of *lrs-2(mg312)*. *Exlrs-2* is a transgenic array carrying a PCR-amplified genomic fragment of *lrs-2*. Worms of at least three independent transgenic lines were tested, and they behaved similarly to each other. The figure shows the results of one representative experiment carried out at 20 °C. Mean lifespan for wild-type N2 was 16.4 d ( $n = 15$ ), for *unc-29(e1072)* was 15 d ( $n = 16$ ), for *lrs-2(mg312) unc-29(e1072)* was 35.4 d ( $n = 21$ ), for *lrs-2(mg312) unc-29(e1072); Exlrs-2line1* was 18.4 d ( $n = 18$ ) and for *lrs-2(mg312) unc-29(e1072); Exlrs-2line2* was 21.7 d ( $n = 15$ ).



**Fig. 3** Nematodes with compromised electron transport had altered mitochondrial morphology. *Plrs-2::GFP* expression in a large number of head neurons (a), as punctate staining in axonal projections (b) and as elongated staining in body-wall muscle (c). GFP expression in transgenic *unc-29(e1072)* adults (d) or *lrs-2(mg312) unc-29(e1072)* adults (e) carrying the body-wall muscle mitochondria marker *Pmyo-3::mito::GFP*. f–n, Wild-type N2 worms carrying the *Pmyo-3::mito::GFP* transgene were fed either bacteria not expressing any dsRNA (control; f) or bacteria expression dsRNA against the indicated ORFs: g, F13G3.7 (mito. carrier); h, B0261.4 (mito. ribosomal subunit); i, D2030.4 (NADH coQ oxidored.); j, T02H6.11 (ub-cyt. C red.); k, T06D8.6 (cyt. C heme lyase); l, F26E4.6 (cyt. C oxidase VIIc); m, F26E4.9 (cyt. C oxidase Vb); n, W09C5.8 (cyt. C oxidase IV). GFP expression in adult worms was assessed.

### Mutation in a mitochondrial tRNA synthetase extends lifespan

A classical genetic screen for *C. elegans* long-lifespan mutants also identified a role for mitochondrial function in influencing aging. To identify genes that act downstream of *daf-16* or in parallel with the *daf-2* pathway to control lifespan, we carried out a genetic screen using the *daf-16(mgDf47)* null mutant to isolate long-lived mutants. The recessive allele *mg312* extended lifespan by 200% at 20 °C and 30% at 25 °C (Fig. 2a and data not shown). Lifespan extension by *mg312* was not dependent on *daf-16(mgDf47)*. Worms of genotype *mg312 unc-29*, which carry a *daf-16(+)* locus, had greater lifespan extension (almost 300% at 20 °C) than did control *unc-29* worms (Fig. 2a). In addition to longer lifespan, homozygous *mg312* worms had multiple pleiotropies: rates of growth, pumping and defecation were lower, and the adults remained the size of early L4 worms and were sterile, with an arrested gonad that exhibited no germ-cell differentiation (data not shown).

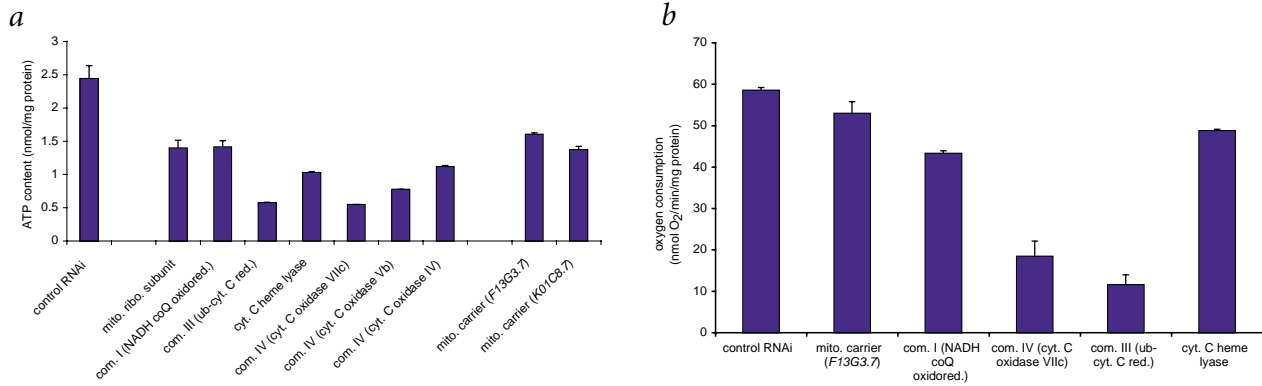
Positional cloning using single-nucleotide polymorphisms (SNPs) and visible markers and cosmid transformation showed that *mg312* is an allele of the mitochondrial leucyl-tRNA synthetase (*lrs-2*) gene. *lrs-2(mg312)* contains a G→A transition that is predicted to change tryptophan to a stop codon at codon 247, truncating LRS-2 protein upstream of conserved domains, and therefore represents a probable null mutation. Extrachromosomal arrays containing a PCR product of the genomic fragment of *lrs-2* (15 kb upstream of the start codon, all exons and introns and 0.5 kb downstream of the stop codon) co-injected with complex DNA to inhibit transgene silencing in the germ line<sup>22</sup> efficiently rescued the extended lifespan and the pleiotropic phenotypes associated with *lrs-2(mg312)* (Fig. 2b and data not shown).

tRNA synthetases catalyze the aminoacylation of tRNAs to their appropriate amino acids; each amino acid has a corresponding tRNA synthetase. Cytoplasmic tRNA synthetases charge tRNAs for the translation of nuclear-encoded mRNAs, whereas mitochondrial tRNA synthetases charge mitochondrial tRNAs for the translation of the few mitochondrial mRNAs<sup>23</sup>. The mitochondrial genome in *C. elegans* encodes 12 polypeptides, all of which are components of the electron-transport chain and ATP synthase<sup>24</sup>. Thus, *lrs-2(mg312)* is predicted to specifically compromise mitochondrial electron transport and ATP synthesis through its effects on the 12 mitochondrially encoded genes.

We examined the expression pattern of *lrs-2* using a translational fusion with GFP fused downstream of codon 31 of LRS-2 (*Plrs-2::GFP*). This fusion gene was ubiquitously expressed, with prominent expression in body-wall muscle and neurons (Fig. 3a–c and data not shown). *Plrs-2::GFP* showed mitochondrial subcellular localization, reflected in the neatly arranged elongated structures in body-wall muscle and the punctate staining in neurons and axonal projections (Fig. 3a–c), consistent with a mitochondrial localization sequence motif in the N-terminal region of LRS-2. A GFP fusion to the full length *lrs-2* gene, *lrs-2::GFP*, containing the same upstream sequences and the entire *lrs-2* open reading frame (ORF), rescued the longevity phenotype of *lrs-2(mg312)* and had similar expression and localization patterns (data not shown).

### Abnormal mitochondria in worms with defects in mitochondrial function genes

Using a muscle-specific GFP fusion gene that targets mitochondria to visualize the mitochondrial morphology in live worms<sup>25</sup>, we observed marked defects in mitochondrial structure of *lrs-2* mutant worms or worms with RNAi-inactivated mitochondrial



**Fig. 4** ATP content and oxygen consumption rates in mitochondrially compromised long-lived *C. elegans*. **a**, Wild-type N2 worms were fed either bacteria not expressing any dsRNA (control RNAi) or bacteria expressing dsRNA against the indicated ORFs. ATP content was measured in young adult worms using a luciferase-based assay. **b**, Wild-type N2 worms were fed either bacteria not expressing any dsRNA (control RNAi) or bacteria expressing dsRNA against the indicated ORFs: F13G3.7 (mito. carrier), D2030.4 (NADH coQ oxidored.), F26E4.6 (cyt. C oxidase VIIc), T02H6.11 (ub-cyt. C red.), T06D8.6 (cyt. C heme lyase). Oxygen consumption rates were measured in young adult worms using an oxygen electrode. ATP content and oxygen consumption rate of each population was normalized to total protein. Results shown are the mean  $\pm$  s.d. of two replicates.

components (Fig. 3*d–n*). In control worms, the muscle mitochondria were elongated and well-organized<sup>25</sup>. In *lrs-2(mg312)* worms, the mitochondria were noticeably disorganized, swollen and sometimes fused (Fig. 3*d,e*). We observed similarly distorted mitochondria in worms undergoing RNAi inactivation of the various electron-transport chain subunits, the mitochondrial ribosomal subunit and the cytochrome C heme lyase (Fig. 3*f–n*). Worms undergoing RNAi against the mitochondrial carriers had normal mitochondrial morphology (Fig. 3*f–n* and data not shown). It is not clear whether this difference occurred because RNAi inactivation of the mitochondrial carriers did not reduce mitochondrial function to a threshold that triggers morphology alterations or because mitochondrial morphology is severely affected only by perturbing electron and proton transport.

To investigate whether slower metabolism might account for the lifespan extension in the mutant and RNAi-inactivated worms, we measured the ATP content and oxygen consumption rates of worms undergoing RNAi inactivation of the various longevity genes important for mitochondrial function. As predicted given that mitochondria are the major sites of ATP production, ATP levels were substantially lower in worms undergoing RNAi inactivation of mitochondrial components (Fig. 4*a*). The reduction in ATP content correlated with the developmental phenotypes caused by RNAi inactivation of each gene. Worms with RNAi-inactivated complex III and IV subunits were most severely affected developmentally (Table 1); they had the highest percentage of slow growth and larval arrest and the largest reduction of ATP content (25% of control). Worms undergoing RNAi inactivation of the mitochondrial carriers developed at similar rates as did control worms and showed only modest reduction in ATP content (65% of control).

Using an oxygen electrode, we assayed oxygen consumption rates of worms undergoing RNAi inactivation of mitochondrial function genes. The oxygen consumption rate in the different RNAi-treated worms (Fig. 4*b*) generally correlated well with their ATP content (Fig. 4*a*). For example, worms undergoing RNAi inactivation of the complex III and IV subunits had markedly lower oxygen consumption rates than did control worms (5 times and 3 times less, respectively), which might account for the lower ATP content and the slower rate of development in these worms. Worms with RNAi-inactivated mitochondrial carrier F13G3.7 had essentially normal rates of oxygen consumption, suggesting that their metabolic rate was not measurably altered. These worms nevertheless stored

noticeably lower levels of ATP. It is possible that these worms might have had normal oxygen intake and electron transport, but had other defects that affected mitochondrial membrane potential and ATP production.

#### Altered stress responses in long-lived *C. elegans* with mitochondrial dysfunction

Long-lived *C. elegans* with mutations affecting coenzyme Q synthesis (*clk-1*)<sup>12,13</sup> or electron-transport complex III (*isp-1*) have been identified<sup>26</sup>. It has been proposed that the reduced metabolic rates of these worms lead to decreased ROS production and slower aging<sup>27</sup>. This prediction is consistent with the rate-of-living and free-radical theories of aging, which state that a higher metabolic rate leads to greater the production of ROS and ROS-induced cellular damage and shorter lifespan<sup>28,29</sup>.

Because direct measurement of endogenous ROS production in each of the mutant/RNAi worms is challenging, we used several surrogate phenotypes as an index of how the long-lived nematodes coped with oxidative stress. For example, long-lived *daf-2* mutants are highly tolerant to multiple stress treatments, including paraquat, hydrogen peroxide and heat shock<sup>30–32</sup>, and this heightened stress resistance is generally thought to be key to the extended lifespan in *daf-2* worms.

We examined the response of the *lrs-2(mg312)* mutant and worms undergoing RNAi inactivation of mitochondrial components to an acute treatment of paraquat or hydrogen peroxide. Notably, most of the long-lived worms with compromised mitochondria did not show greater resistance to paraquat (Fig. 5*a*). In fact, the *lrs-2(mg312)* mutant and worms undergoing RNAi inactivation of several of the electron-transport chain components, especially those of complex III and IV, showed greater sensitivity to paraquat (Fig. 5*a*). In contrast, worms with reduced *daf-2* signaling were highly resistant to paraquat in this experiment, consistent with previous findings<sup>31</sup>.

To address whether the sensitivity to paraquat was a reflection of compromised health and general sensitivity to stress, the same mutant and RNAi-inactivated worms were subjected to another stress, heat-shock treatment at 35 °C. In this assay, most of the long-lived worms had normal to slightly enhanced resistance to heat shock (Fig. 5*b*). Furthermore, most of the long-lived worms showed increased tolerance to hydrogen peroxide (Fig. 5*c*), a different type of free radical, and some had marked resistance, comparable to that observed in worms with reduced *daf-2* signaling.

## Specific metabolic pathways influence *C. elegans* lifespan

Because long-lived *C. elegans* with defective electron transport have lower ATP production, it is conceivable that they undergo a variety of metabolic changes that eventually might be beneficial for long life. Consistent with this, our RNAi screen identified that *F28B3.5*, a gene with weak homology to the triacylglycerol

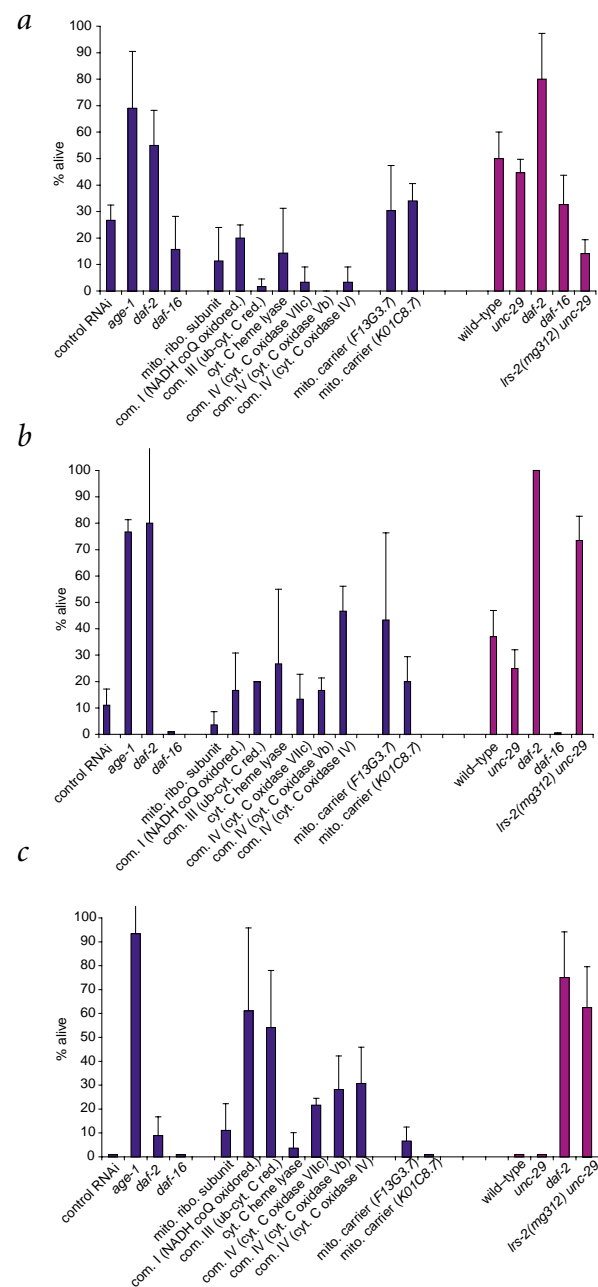
synthesis pathway enzyme 1-acyl-glycerol-3-phosphate acyl-transferase, and *F57B10.3*, a phosphoglycerate mutase required in the glycolysis pathway, significantly influenced *C. elegans* lifespan (Fig. 6). Unlike worms with reduced mitochondrial function, worms with these metabolic enzyme genes inactivated by RNAi showed no developmental pleiotropies or defects in mitochondrial morphology (data not shown). Furthermore, these worms showed relatively normal responses to paraquat, hydrogen peroxide and heat shock (data not shown). Notably, the lifespan extension associated with RNAi inactivation of these genes was suppressed by *daf-16* (Table 1), suggesting that these metabolic genes might function upstream of *daf-16* to influence lifespan. These findings suggest that modulation of metabolism, either by reducing ATP production or by inactivating certain metabolic pathways, might have a role in determining lifespan.

## Discussion

According to annotations of the *C. elegans* genome sequence, only about 1.5% of genes in the Chromosome I RNAi library have mitochondrial functions. We found that 15% of the RNAi clones that extended lifespan were mitochondrial (see Web Table A online), representing a tenfold over-representation of mitochondrial function genes affecting lifespan. We obtained similar results for Chromosome II (Fig. 1a and Table 1). In fact, apart from the insulin-pathway components, RNAi inactivation of the mitochondrial function genes caused the largest increase in lifespan. The current annotations of mitochondrial function genes in *C. elegans* may not be complete; for example, recent results estimated that 13% of yeast proteins are localized to the mitochondria<sup>33</sup>. Thus, it is probable that more of the genes that impact aging are important for mitochondrial function. These findings point to mitochondrial energetics as a key determinant of *C. elegans* lifespan.

RNAi inactivation of about 20% of all ORFs annotated to function in the mitochondria on Chromosome I extended lifespan, whereas RNAi inactivation of most of the remaining mitochondrial function genes gave no detectable phenotypes (see Web Table A online). It is important to consider that the effectiveness of RNAi in suppressing gene expression could be variable. Because verification of RNAi efficiency on each gene tested is impractical in a large-scale comprehensive screen, we cannot confirm a complete abrogation of gene function for RNAi clones that did not cause a detectable phenotype. But systematic analysis of the RNAi library has shown that 90% of genes in which mutations were previously identified by classical genetics to cause embryonic lethality also cause the same phenotype when inactivated by feeding RNAi, pointing to the quality of the library and the effectiveness of feeding RNAi<sup>18</sup>. The efficiency of RNAi in detecting other generally visible worm phenotypes is roughly 50%. Assuming that successful RNAi clones are randomly distributed, we predict that it is the general modulation of mitochondrial function that leads to long lifespan, although it is possible that a particular aspect of mitochondrial dysfunction (for example, electron transport or ATP synthesis) might have the greatest impact on longevity.

It is notable that a probable null mutation in the only mitochondrial leucyl tRNA synthetase in *C. elegans* did not produce an early-larval arrest phenotype, as is induced by deletions of other essential respiration chain components<sup>13,34</sup>. Other mitochondrial amino-acid tRNA synthetases might be able to complement some of the leucine-loading function, or a low level of read-through of the stop codon in the *mg312* allele might allow synthesis of enough LRS-2 to sustain development. Although it is possible that the maternal *lrs-2* is able to sustain the mutant to adulthood, microinjection of *lrs-2* dsRNA into wild-type worms, expected to eliminate both maternal and zygotic *lrs-2* mRNA, again caused a sterile adult phenotype (data not shown).



**Fig. 5** Stress response in long-lived *C. elegans* with compromised mitochondria. **a**, 5-d-old N2 adults feeding on bacteria expressing the indicated RNAi or 5-d-old adults of wild-type N2, *unc-29(e1072)*, *daf-2(e1370)*, *daf-16(mgDf47)* or *lrs-2(mg312) unc-29(e1072)* were exposed to paraquat, and survival was scored 6 h after treatment. **b**, 5-d-old N2 adults feeding on bacteria expressing the indicated RNAi or 5-d-old adults of wild-type N2, *unc-29(e1072)*, *daf-2(e1370)*, *daf-16(mgDf47)* or *lrs-2(mg312) unc-29(e1072)* were transferred to 35 °C, and survival was scored 24 h after treatment. **c**, 5-d-old N2 adults feeding on bacteria expressing the indicated RNAi or 5-d-old adults of wild-type N2, *unc-29(e1072)*, *daf-2(e1370)*, *daf-16(mgDf47)* or *lrs-2(mg312) unc-29(e1072)* were exposed to hydrogen peroxide, and survival was scored 5 h after treatment. Results shown are the mean  $\pm$  s.d. of three independent experiments. Approximately 30 worms were tested for each strain or RNAi clone.

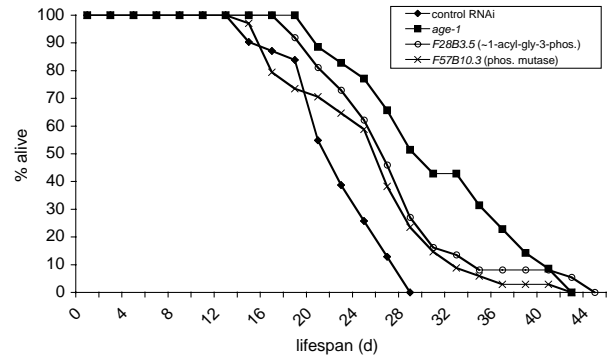
Mitochondrial function and ATP synthesis are critical for coupling glucose sensing and insulin secretion in the pancreatic  $\beta$ -cells<sup>35</sup>. Thus, it is notable that a mutation in the mitochondrial leucine tRNA gene is frequently associated with diabetes in humans<sup>36</sup>. This suggests that mitochondrial function in *C. elegans* might be coupled to lifespan through its effects on insulin-like signaling. Although both insulin-like signaling and mitochondrial components are potent modulators of lifespan in *C. elegans*, our work and the work of others suggest that the mitochondrial regulation of lifespan acts downstream of or in parallel to insulin-like signaling (Figs. 1*b*, 2*a*). Alternatively, there might be a coupling of mitochondrial leucine levels to metabolic control in the insulin-producing cells. Even though our genetic data points to mitochondrial leucine loading defects, the systematic RNAi screen suggests that a wide range of mitochondrial dysfunctions can extend *C. elegans* lifespan.

Mitochondria are dynamic organelles, with their structure and number changing in response to diverse cellular cues. Our results suggest that normal electron transport is not only necessary for mitochondrial function, but also for the normal dynamics of mitochondrial biogenesis. Mutations affecting mitochondrial fission or fusion profoundly impact mitochondrial morphology<sup>37</sup>, and mutations in the electron-transport complex V ATP synthase affect mitochondria cristae formation<sup>38</sup>. The alteration in mitochondrial morphology observed in *C. elegans* with compromised electron transport and ATP synthesis supports a coupling of mitochondrial function to cristae formation and global dynamic regulation of mitochondrial biogenesis.

Our results indicate that *C. elegans* with compromised mitochondria were able to withstand certain environmental stresses as well as or better than control worms but were hypersensitive to paraquat. This suggests a specific compromise in the ROS pathway in the mitochondrially challenged long-lived worms. The nematodes with compromised mitochondria might have different stress responses than those of worms with reduced *daf-2* signaling, even though both have extended lifespans.

The RNAi clones that target elements in the electron-transport chain upstream of ATP synthase would be expected to have lower mitochondrial membrane potential and thus lower free-radical production. Owing to the lower level of free-radical production, long-lived worms with compromised electron-transport systems would be expected to be resistant to additionally generated free radicals. This is the case for hydrogen peroxide but not for paraquat. Paraquat triggers the production of superoxide in a NADPH-dependent reaction. The paraquat hypersensitivity of worms with electron-transport deficiencies might be due to an increase in NADPH associated with a metabolic response to reduced electron transport that in turn facilitates paraquat activation and free-radical production. Alternatively, the paraquat hypersensitivity is consistent with the idea that worms with electron-transport deficiencies produce more endogenous superoxide and are less capable of dealing with additional oxidative stress imposed on them. From the point of view of mitochondrial physiology, this is not unexpected. During normal respiration, electron leaks at complexes I and III are thought to be principal sites of ROS generation. Incomplete reduction of O<sub>2</sub> to H<sub>2</sub>O at complex IV also contributes to additional ROS production. A block in each step of the electron-transport chain can either increase the pre-existing electron leak or foster the production of novel free radicals<sup>39</sup>.

A complex pathway of antioxidant defense controls the eventual ROS-induced cellular damage and perhaps the aging process. Therefore, it is possible that when facing increased ROS production, the long-lived worms with compromised electron transport sense the free radicals and induce defense mechanisms



**Fig. 6** RNAi inactivation of two metabolic genes extended lifespan. Wild-type N2 worms were fed either bacteria not expressing any dsRNA (control RNAi) or bacteria expressing dsRNA against the indicated ORFs. The figure shows the results of one representative experiment carried out at 20 °C. See Table 1 for statistical analysis.

to counteract ROS damage and extend lifespan. It is possible that only a subset of antioxidant defense was induced and, therefore, only specific oxidative stress agents were effectively resolved, which might account for the differential responses to paraquat and hydrogen peroxide observed with the mitochondrially compromised long-lived worms. Our results indicate that although stress resistance might contribute in part to the extended lifespan of the nematodes described here, the mechanism by which compromised mitochondria extend lifespan might not be as simple as a heightened ability to combat oxidative stress.

In several species of fungi, such as *Neurospora crassa* and *Podospira anserina*, chemical inhibitors and mutations affecting mitochondrial function extend lifespan<sup>40</sup>, probably through a mechanism related to ROS production. In *Saccharomyces cerevisiae*, lack of mitochondrial DNA also extends lifespan in certain genetic backgrounds. Notably, the lifespan extension associated with impaired yeast mitochondria depends on a retrograde intracellular signaling pathway involving at least three transcriptional factors, which adjust nuclear gene expression and induce a shift of metabolism from the Krebs cycle to the glyoxylate cycle<sup>41,42</sup>. The fact that compromised mitochondria extend lifespan in fungi and nematodes suggests a conserved role of mitochondrial energetics in lifespan specification. The observation that a global metabolic shift is required for mitochondria to influence lifespan in yeast suggests that similar metabolic changes might occur in nematodes and other animals to modulate lifespan. In the long-lived dauer stage of *C. elegans*, lower activity of the Krebs cycle and higher dependence of the glyoxylate cycle have also been observed<sup>43</sup>. It is possible that a shift of metabolism from the Krebs cycle towards the glyoxylate cycle contributes to extended lifespan in *C. elegans*.

Identifying the metabolic genes that regulate lifespan in *C. elegans* might pinpoint the specific metabolic pathways that are most critical for lifespan control. For instance, lifespan extension associated with RNAi inactivation of phosphoglycerate mutase in *C. elegans*, which is expected to affect glycolysis, might be analogous to the extended lifespan caused by mutations in yeast hexokinase 2 (*HXK-2*), one of the first enzymes in the glycolysis pathway. The changes in gene expression in *HXK-2*(-) mutant yeast are quite similar to those observed in calorically restricted yeast<sup>44</sup>, suggesting that they might share common mechanisms of lifespan extension. Another parallel is the long-lived *Drosophila melanogaster* mutant *Indy*, which carries a mutation in a sodium dicarboxylate cotransporter, a membrane protein that transports Krebs cycle intermediates. The *Indy* mutation is

thought to modulate nutrient utilization or to lower intermediate metabolism, thus extending lifespan through a mechanism similar to that of caloric restriction<sup>45</sup>. Therefore, lifespan extension caused by reduced metabolic enzyme activity in *C. elegans* might induce molecular changes similar to those observed in calorically restricted long-lived worms. Future gene expression profiling experiments might provide insights into this possibility.

This comprehensive RNAi screen provides the first genome-wide view of animal longevity genes. Our results reinforce the idea that energy metabolism is critical for the determination of animal lifespan. This RNAi screen also identifies two metabolic genes that are important for lifespan determination. Inactivations of other genes that extended *C. elegans* lifespan but are not annotated to be mitochondrial or metabolic enzymes may identify pathways that either respond to the metabolic shift caused by decreased energy production or impact lifespan in parallel to energetics.

## Methods

**Strains.** We used the following *C. elegans* strains: wild-type N2 Bristol, *daf-16(mgDf47)*, *dpy-5(e61) unc-29(e1072)*, *unc-13(e51) ozDf5 I; nDp4(I;V)/+*, *unc-29(e1072)*, *unc-13(e51); nDp4(I;V)/+*.

**RNAi lifespan screen.** We carried out a large-scale RNAi screen as described<sup>17</sup> with slight modifications. Each RNAi colony was grown overnight in Luria broth with 50  $\mu\text{g ml}^{-1}$  ampicillin and then seeded onto 24-well RNAi agar plates containing 5 mM isopropylthiogalactoside to induce dsRNA expression overnight at room temperature. We added about 30 synchronized L1s to each well, allowed worms to develop to adults and then added 5-fluorodeoxyuridine (FUDR) solution to RNAi plates and scored lifespan. All annotations of RNAi clones were according to WormBase.

**Lifespan assay.** We carried out aging assays at 20 °C or 25 °C, with agar plates containing 0.1  $\text{g ml}^{-1}$  FUDR to prevent growth of progeny. We grew worms from single-day egglays on nematode-growth medium plates until they reached young adult stage, transferred them to FUDR plates and then scored them every 2 d. Worms were scored as dead when they no longer responded to a gentle prodding with a platinum wire. Lifespan is defined as the time elapsed from when worms were put on FUDR plates (lifespan = 0) to when they were scored as dead. Worms that crawled off the plates during the assay were excluded from calculations. All lifespan assays were repeated in at least four independent experiments.

For lifespan of animals undergoing RNAi, we fed synchronized L1s with RNAi-expressing bacteria, grew them to young adults, transferred them to fresh RNAi plates with FUDR every 5 d and scored lifespan as described. Alternatively, we fed gravid adults with RNAi-expressing bacteria and allowed them to egglay overnight. We grew their progeny on RNAi-expressing bacteria until they were young adults, transferred them to fresh RNAi plates with FUDR every 5 d and scored lifespan as described. Lifespan assays were repeated at least twice under each condition as completely independent experiments. We carried out statistical analyses using the lifespan distribution of each population of worms from two independent experiments.

**Long-lived mutant genetic screen.** We treated *daf-16(mgDf47)* worms with the mutagen ethylmethane sulfonate and obtained mutant F1 and F2 worms by normal egg-preparation procedures. We placed a cohort of ten F2 worms on each nematode-growth medium plate and allowed them to lay eggs overnight. We then transferred the ten hermaphrodites from each plate to a separate holding plate. We allowed progeny from the overnight egglay to grow to young adult stage, transferred them to FUDR plates and assayed their lifespan at 25 °C. We monitored each plate for the presence of any mutant worms that lived longer than control *daf-16(mgDf47)* worms. A mutant was scored as long-lived when its lifespan exceeded that of the control by >2 d. We then recovered the mutated allele from the appropriate holding plate. Because each holding plate contained progeny of ten different genotypes, we singled out at least 40 worms from each candidate holding plate and assayed the lifespan of a population of progeny from each. We screened approximately 2,000 haploid genomes and identified three alleles associated with longevity. The *mg312* mutant is completely sterile, and was originally identified as small, long-lived segregants from an apparently wild-type hermaphrodite.

**Genetic mapping and cloning.** We crossed heterozygous *mg312* worms with the Hawaiian strain and selected homozygous mutant worms on the basis of slow growth and sterility. We used SNP markers, visible markers (*dpy-5(e61)* and *unc-29(e1072)*) and deficiency strain (*unc-13(e51) ozDf5 I; nDp4(I;V)/+*) for mapping. We injected overlapping cosmids spanning a small region on LG1 and two PCR-amplified genomic fragments (21,096–25,647 of cosmid ZK524 and 21,096 of cosmid ZK524 to 7,250 of cosmid T28F4) encompassing the *lrs-2* gene into mutant worms to rescue them. We injected *lrs-2(mg312) unc-29(e1072); nDp4(I;V)/+* worms with 5  $\text{ng } \mu\text{l}^{-1}$  of PCR fragment and 50  $\text{ng } \mu\text{l}^{-1}$  of *Pmec-7::GFP*. Using 5' RACE PCR, we found an ATG codon 90 bp upstream of the ATG codon predicted by GeneFinder (as originally reported in WormBase) and a SL2 leader sequence at the 5' end of the mRNA, indicating that *lrs-2* resides in an operon and is probably transcribed as a single transcript with the upstream gene ZK524.4, encoding a predicted serine/threonine kinase.

***lrs-2* expression analysis.** We used genomic sequences 15 kb upstream of *lrs-2*, encompassing the sequences upstream of *lrs-2* to the end of the second upstream gene, to generate *Plrs-2::GFP*. We amplified the genomic sequences from N2 genomic DNA by PCR and the GFP sequences from pPD95.81. GFP was fused immediately after the ATG codon of *lrs-2* predicted by GeneFinder (90-bp internal of the start codon). We injected GFP fusion fragments into N2 animals at 5  $\text{ng } \mu\text{l}^{-1}$  with 50  $\text{ng } \mu\text{l}^{-1}$  of pRF4 *rol-6(d)*. We examined stably transformed strains for fluorescence using an Axioplan microscope (Zeiss). Duplicate constructs from independent PCR reactions were injected, and multiple transgenic lines were analyzed.

**Mitochondrial morphology.** Mitochondria were visualized using *Pmyo-3::mito::GFP* construct<sup>25</sup>. We injected the *Pmyo-3::mito::GFP* plasmid into N2, *unc-29(e1072)*, or *lrs-2(mg312) unc-29(e1072); nDp4(I;V)/+* worms at 1  $\text{ng } \mu\text{l}^{-1}$  with 50  $\text{ng } \mu\text{l}^{-1}$  of pRF4 *rol-6(d)*. Multiple stably transgenic lines were examined for fluorescence as above. For mitochondrial component RNAi, we fed the wild-type strain carrying *Pmyo-3::mito::GFP* fusion gene with RNAi-expressing bacteria and surveyed the morphology of mitochondria by fluorescence microscopy.

**ATP content.** We measured the ATP content of each population of RNAi-inactivated worms as described previously<sup>46</sup>. We grew approximately 3,000 hatched L1 worms to the young adult stage on large plates containing each RNAi-expressing bacteria and then washed them free of bacteria and collected them in S-basal buffer. We boiled each frozen aliquot of worms, diluted it in double-distilled water, prepared serial dilutions of each and analyzed them for ATP levels using an ATP assay kit (Roche). We analyzed a matched aliquot from each RNAi-inactivated group for protein content using BCA reagent (Pierce) as described<sup>46</sup>. The ATP levels for each population of RNAi animals were normalized to protein content.

**Oxygen consumption rate.** We measured the oxygen consumption rate of each population of RNAi-inactivated worms as described previously<sup>46</sup>. We grew approximately 10,000 hatched L1 worms to young adult stage on large plates containing each RNAi-expressing bacteria, washed them free of bacteria and collected them in S-basal buffer. We monitored changes in oxygen concentration of a suspension of worms in S-basal buffer using Clark type electrode (Oxytherm, Hansatech). We delivered 1 ml of suspended worms into the chamber, which was maintained at 25 °C, and measured oxygen concentration for usually 5–20 min, depending on the oxygen consumption rate. The slope of the straight portion of the plot was used to derive the oxygen consumption rate. We recovered worms from the chamber after oxygen measurements and collected them for protein quantification. Oxygen consumption rate for each population of RNAi animals was normalized to its protein content.

**Stress assays.** We used single-day egglays to generate a semi-synchronized population for each strain, exposed 5-d-old adults to S-basal buffer containing paraquat (300 mM) or hydrogen peroxide (10 mM) and then scored the worms for survival at the indicated time points after treatment. We used 5-d-old adults to eliminate the complication in which hatching of progeny leads to premature death of hermaphrodites. The stress assays were modified from previous methods<sup>30–32</sup>. For paraquat and hydrogen peroxide, we tested a titration of different concentrations and chose 300 mM of paraquat and 10 mM of hydrogen peroxide because control

worms exposed to these concentrations died in a relatively short amount of time (4–8 h). Because of the short assaying time, worms were assayed in S-basal buffer containing the respective chemical in the absence of bacteria. For heat-shock assay, 5-d-old adults were shifted to 35 °C and scored at the indicated time points for survival.

*Note: Supplementary information is available on the Nature Genetics website.*

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**Competing interests statement**

The authors declare that they have no competing financial interests.

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