The Homeobox Protein CEH-23 Mediates Prolonged Longevity in Response to Impaired Mitochondrial Electron Transport Chain in *C. elegans*

Ludivine Walter, Aliswarya Baruah, Hsin-Wen Chang, Heather Mae Pace, Siu Sylvia Lee

Department of Molecular Biology and Genetics, Cornell University, Ithaca, New York, United States of America

**Abstract**

Recent findings indicate that perturbations of the mitochondrial electron transport chain (METC) can cause extended longevity in evolutionarily diverse organisms. To uncover the molecular basis of how altered METC increases lifespan in *C. elegans*, we performed an RNAi screen and revealed that three predicted transcription factors are specifically required for the extended longevity of mitochondrial mutants. In particular, we demonstrated that the nuclear homeobox protein CEH-23 uniquely mediates the longevity but not the slow development, reduced brood size, or resistance to oxidative stress associated with mitochondrial mutations. Furthermore, we showed that ceh-23 expression levels are responsive to altered METC, and enforced overexpression of ceh-23 is sufficient to extend lifespan in wild-type background. Our data point to mitochondria-to-nucleus communications to be key for longevity determination and highlight CEH-23 as a novel longevity factor capable of responding to mitochondrial perturbations. These findings provide a new paradigm for how mitochondria impact aging and age-dependent diseases.


**Academic Editor:** Andy Dillin, The Salk Institute for Biological Studies, United States of America

**Received** November 8, 2010; **Accepted** May 6, 2011; **Published** June 21, 2011

**Copyright:** © 2011 Walter et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** LW is supported by a grant from the United Mitochondrial Disease Foundation (UMDF, http://www.umdf.org/site/c.otJVJ7MMlqE/b.5472191/k.BDB0/Funding:C223) and by the AHA, aryl hydrocarbon receptor associated protein; ASI, amphid sensilla; ATP, adenosine triphosphate; BLAST, basic local alignment search tool; C. elegans, Caenorhabditis elegans; CAN, canal associated nerve; CEH, C. elegans homeobox; CEP, C. elegans P53 like protein; CLK, clock (biological timing) abnormality; CTB, cytochrome b; DAF, abnormal dauer formation; DVE, defective proventriculus homolog; EAT, eating: abnormal pharyngeal pumping; FOXO, forkhead box O; FSTR, faster; GFP, green fluorescent protein; HOM-C, cluster homeotic genes; HSF, heat shock protein; IIS, insulin/insulin like growth factor signaling; ISP, iron sulfur protein; LIN, abnormal cell lineage; METC, mitochondrial electron transport chain; MEV, abnormal methyl viologen sensitivity; mRNA, messenger RNA; NHR, nuclear hormone protein; PHA, defective pharynx development; qrt-PCR, quantitative reverse transcription PCR; RN1, RNA interference; ROS, Reactive Oxygen Species; RTG, retrograde genes; UTR, untranslated region.

**Competing Interests:** The authors have declared no competing interests exist.

**Abbreviations:** AHA, aryl hydrocarbon receptor associated protein; ASI, amphid sensilla; ATP, adenosine triphosphate; BLAST, basic local alignment search tool; C. elegans, *Caenorhabditis elegans*; CAN, canal associated nerve; CEH, *C. elegans* homeobox; CEP, *C. elegans* P53 like protein; CLK, clock (biological timing) abnormality; CTB, cytochrome b; DAF, abnormal dauer formation; DVE, defective proventriculus homolog; EAT, eating: abnormal pharyngeal pumping; FOXO, forkhead box O; FSTR, faster; GFP, green fluorescent protein; HOM-C, cluster homeotic genes; HSF, heat shock protein; IIS, insulin/insulin like growth factor signaling; ISP, iron sulfur protein; LIN, abnormal cell lineage; METC, mitochondrial electron transport chain; MEV, abnormal methyl viologen sensitivity; mRNA, messenger RNA; NHR, nuclear hormone protein; PHA, defective pharynx development; qrt-PCR, quantitative reverse transcription PCR; RN1, RNA interference; ROS, Reactive Oxygen Species; RTG, retrograde genes; UTR, untranslated region.

**E-mail:** sylvia.lee@cornell.edu

* Current address: Ecole Normale Superieure de Lyon - CNRS - Universite de Lyon Claude Bernard, Molecular Biology of the Cell Laboratory/UMRS239, 46, Lyon, France

**Introduction**

Alterations of mitochondrial function broadly impact animal physiology and physiopathology, including aging and age-related diseases. Correlative evidence has long demonstrated that mitochondrial function gradually declines with age, while oxidative damage and mitochondrial DNA mutations accumulate [1–4]. Interestingly, recent studies revealed that reduced mitochondrial electron transport chain (METC) function can cause substantial longevity increase in a wide range of organisms. In yeast, some respiration-deficient strains exhibit lifespan increase in [5]. In worms, particular METC mutations can greatly extend lifespan. These include mutations in *isp-1*, which encodes the iron sulfur protein of Complex III [6], and in *clk-1*, which encodes the hydroxylase protein necessary for the biosynthesis of the METC electron transporter coenzyme Q [7]. Furthermore, RNAi knockdown of several sub-units of the METC also results in greater longevity in worms [8–13] and in fruit flies [14,15]. In mice, heterozygous loss of the mouse *clk-1* homolog (*mclk-1*) [16] as well as defects in the assembly of the complex IV of the METC [17] extend lifespan. Therefore, the observation that reduced mitochondrial function can prolong lifespan appears highly conserved among evolutionarily diverse species and is likely to be relevant to human physiology.

Not surprisingly, METC mutations can also lead to deleterious manifestations such as developmental arrest or shorter lifespan. For instance, *mev-1* mutant worms, which harbor a mutation in the subunit C of the Complex II, are characterized by a drastic lifespan shortening compared to wild-type worms [18]. Additionally, genetic manipulations that reduce mitochondrial function are often associated with slower physiological rates, regardless of whether they cause a prolonged or shortened longevity phenotype [5–7,12,14,15,19]. In many instances, the severity of the mitochondrial perturbations correlates with their effects on lifespan [12,20]. A model has emerged in which moderate mitochondrial impairment positively impacts longevity until a threshold is reached, beyond which animal survival is compromised.
Author Summary

Mitochondria have long been associated with aging and age-related diseases. Recent research has shown that a slight dampening of mitochondrial function can dramatically increase the lifespan of a wide range of organisms, suggesting that a similar mechanism likely operates in humans. The molecular basis of this observation is largely unknown, however. Uncovering the genes that allow altered mitochondrial function to impact longevity will give us important new insights into how mitochondria affect the aging process and will pave the way for future therapeutic developments aiming to improve healthy aging and to treat age-related diseases. Here, we used an RNAi screen in the genetic model organism *C. elegans*, a nematode worm, to uncover how altered mitochondrial function can modulate longevity. We found that in order for mitochondria to affect lifespan, they must communicate with several unique transcription factors in the nucleus. Notably, we discovered that the putative homeobox transcription factor CEH-23, which has not previously been implicated in longevity determination, is able to respond to changes in mitochondrial function and in turn causes an extension in lifespan.

In yeast, impaired mitochondria can signal the nucleus through a retrograde signaling pathway that leads to nuclear gene expression changes that in turn extend longevity [21–23]. Similarly, in mammalian cells, changes in mitochondrial state trigger a retrograde signaling pathway that results in nuclear transcription factors activation and metabolic or stress-related responses [24,25] and [26] for review], but its effect on lifespan is unknown. In general, the molecular mechanisms that enable altered METC to positively impact longevity in multicellular organisms are still unclear. Emerging evidence indicates that longevity extension induced by METC impairment does not correlate with a decrease in oxidative stress response [1,3,27] or respiratory capacities [6,14,19,28]. Therefore, the mitochondrion is likely to play a causative role in longevity determination via novel mechanisms that are yet to be uncovered. We hypothesized that, similarly to what was shown in yeast, altered METC in *C. elegans* results in signaling that impinges upon specific transcription factor(s) to promote longevity. Our study revealed the identity of several nuclear transcription factors that are specifically important for the longevity increase associated with altered METC in *C. elegans*, and points to a novel molecular basis by which mitochondria impact longevity in animals.

Results

Targeted RNAi Screen for Putative Transcription Factors That Suppress the Longevity Phenotype of the Long-Lived *isp-1;ctb-1* Mitochondrial Mutant

To identify the putative transcription factor(s) that mediate the lifespan increase of worms with reduced METC function, we employed a targeted RNAi screening approach using the Transcription Factor RNAi Library (Gene Service Inc.), which covers ~41% of the predicted transcription factors in *C. elegans* [29]. We performed the RNAi screen using the *isp-1;ctb-1* mitochondrial mutant, which exhibits a robust lifespan increase with only mild delays in rates of developmental processes [6]. We systematically inactivated, by RNAi feeding [30] from the time of hatching, each of the 387 transcription factors in the library and looked for RNAi targets that suppress the prolonged lifespan of the *isp-1;ctb-1* mutant worms. From the primary screen, we identified 32 RNAi candidates that caused a decrease in the *isp-1;ctb-1* mutant lifespan by at least 15% when compared to empty vector control RNAi (p≤0.001, log-rank test). The 32 primary candidates were then retested in at least two independent trials (Figure S1). To select the RNAi candidates that consistently suppressed the prolonged lifespan of *isp-1;ctb-1*, we used two different methods of data analysis. The percentage of lifespan suppression caused by each RNAi clone was either calculated after pooling the data from the independent trials and comparing the mean lifespan by stratified log-rank test (p≤0.001) or averaged after a comparison of mean lifespan within each trial by log-rank-test (p≤0.001) (see Experimental Procedures). The results revealed that 17 RNAi candidates consistently shortened the lifespan of the *isp-1;ctb-1* mutant by more than 10% (Tables 1 and S1). Among these RNAi candidates, four correspond to transcription factors previously shown to be important for longevity maintenance in *C. elegans*: the heat shock factor HSF-1 [31], the nuclear hormone receptor NHR-49 [32], and the forkhead factors DAF-16 [33] and PHA-4 [34]. The other 13 candidate transcription factors have not been previously implicated in directly affecting *C. elegans* longevity. Many transcription factor families are represented within these 13 candidates, with an overrepresentation of the homeobox class (~40% of the candidate clones are homeobox proteins, whereas ~10% of all the clones tested in the RNAi screen are predicted to be homeobox proteins). Under the conditions of our lifespan assays, *isp-1;ctb-1* mutants typically exhibit a 1.5-fold increase in mean lifespan compared to wild-type (Figure 1). Thus, RNAi clones that decrease the lifespan of *isp-1;ctb-1* mutant worms by ~30% (nhr-119, nhr-265, ceh-37, abh-1, ceh-23, 2C123.3, ceh-29, and nhr-25; Figure 1) are of special interest since they represent RNAi knockdowns that restore the lifespan of the *isp-1;ctb-1* mutant to that of wild-type worms. Although cep-1, the *C. elegans* homolog of the tumor suppressor p53, has previously been shown to be required for the extended lifespan of *isp-1* mutant [35], cep-1 RNAi did not exhibit significant lifespan suppression in our screen. This might be due to cep-1 RNAi only partially knocked down cep-1 in our screen conditions or that cep-1 is not required for longevity increase of *isp-1;ctb-1* mutant.

Identification of Transcription Factors That Specifically Mediate the Effects of METC Mutations on Longevity

We reasoned that if the METC affects lifespan through specific transcription factors, RNAi knockdown of those transcription factors should decrease the *isp-1;ctb-1* mutant lifespan to an extent greater than that of wild-type worms or other longevity mutants thought to act independently of mitochondria. To assay the specificity of the transcription factors identified in our primary screen (Figure 1 and Table 1), we tested the 13 RNAi candidates for an effect on the lifespan of wild-type worms, the short-lived FOXO transcription factor daf-16 mutant [33,36], and the long-lived phosphatidyl inositol 3-kinase age-1 mutant [37,38] that exhibits a lifespan increase as robust as that of the *isp-1;ctb-1* mutant. Both daf-16 and age-1 represent components of the insulin/insulin-like growth factor signaling (IIS) pathway, which is thought to act mostly independently of the METC to affect lifespan in *C. elegans* [39]. As expected, several of the RNAi candidates (*dve-1; lin-40; nhr-49; ceh-20; lin-11; and nhr-77*) appeared to non-discriminately shorten lifespan in all strains tested (Tables 2 and S1), suggesting that the corresponding transcription factors are broadly required for survival. Interestingly, four RNAi clones (2C123.3; nhr-119; ceh-37; and abh-1) affected wild-type and *isp-1;ctb-1* mutant worms’ lifespan to the same extent but exerted only a moderate or no effect on daf-16 and
Figure 1. A targeted RNAi screen identifies 17 RNAi candidates that decrease the lifespan of the *isp-1;ctb-1* mutant. The mean fold change in lifespan relative to that of wild-type worms treated with empty vector (black bar, value set to 1) was calculated for *isp-1;ctb-1* mutant treated with empty vector or with each RNAi clone tested. The mean fold changes (± s.d.) presented are averaged from at least three independent experiments. Only the RNAi clones that decrease the lifespan of the *isp-1;ctb-1* mutant by at least 10% are represented (see Table 1). Among them, 13
age-1 mutant longevity (Tables 2 and S1). Those transcription factors are unlikely to specifically mediate the effects of METC mutations on longevity as their knock-down affected longevity of wild-type and isp-1;ctb-1 worms to a similar extent. Interestingly, four other RNAi clones, targeting the predicted transcription factors C52B9.2, NHR-25, CEH-23, and NHR-265, substantially shortened the lifespan of the isp-1;ctb-1 mutant, but either had a lesser or no effect on the other strains tested including wild-type worms (Tables 2 and S1), suggesting that the corresponding lesser or no effect on the other strains tested including wild-type worms. Among the four candidates tested, nhr-265 is the only one that is specifically required for isp-1;ctb-1 mutant worms to exhibit greater lifespan but not for the other long-lived mitochondrial mutants tested (Tables 2 and S1). The other three factors (C52B9.2, ceh-23, and nhr-25) appear to be important for the longevity effects of all three long-lived METC mutant strains (Tables 2 and S1).

We also tested whether RNAi of these four factors affected the lifespan of the short-lived mev-1 mitochondrial mutant worms. Three of the RNAi clones (nhr-25, ceh-23, and nhr-265) did not affect the lifespan of mev-1 mutants. C52B9.2 RNAi shortened mev-1 mutant lifespan (−12%) but to a lesser degree than that of wild-type worms (−23%) (Tables 2 and S1). All together, our data suggest that specific transcription factors play an important role in the longevity of long-lived mitochondrial mutants without affecting the lifespan phenotype of short-lived mitochondrial mutants.

Next, as the mitochondrial clk-1 mutation and the genetic mimic of dietary restriction eat-2 mutation have previously been shown to act in a similar genetic pathway [40], we examined the three transcription factors (C52B9.2, ceh-23, and nhr-25) that are important for the longevity of the clk-1 mutant (Tables 2 and S1) for an effect on the lifespan of the eat-2 mutant worms. RNAi knockdown of C52B9.2 and nhr-25 decreased the lifespan of the eat-2 mutant to a greater extent than that of wild-type worms.

### Table 1. A targeted RNAi screen identifies 17 RNAi candidates that decrease the prolonged lifespan of the METC mutant isp-1;ctb-1 by at least 10%.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Transcription Factor Family</th>
<th>Number of Independent Lifespan Assays</th>
<th>Stratified Log-Rank Test (p≤0.001) for Pooled Independent Lifespan Assay</th>
<th>Log-Rank Test (p≤0.001) for Each Independent Lifespan Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Decrease of isp-1;ctb-1 Mutant Mean Lifespan (%)</td>
<td>Average Decrease of isp-1;ctb-1 Mutant Mean Lifespan (Mean % ± S.D.)</td>
</tr>
<tr>
<td>dve-1</td>
<td>Homeobox</td>
<td>3</td>
<td>−55</td>
<td>−56±2</td>
</tr>
<tr>
<td>hsf-1*</td>
<td>Heat shock</td>
<td>2</td>
<td>−46</td>
<td>−46±1</td>
</tr>
<tr>
<td>lin-40</td>
<td>Histone deacetylase</td>
<td>3</td>
<td>−48</td>
<td>−46±2</td>
</tr>
<tr>
<td>nhr-49*</td>
<td>Nuclear hormone receptor</td>
<td>2</td>
<td>−46</td>
<td>−45±1</td>
</tr>
<tr>
<td>daf-16*</td>
<td>Forkhead</td>
<td>1</td>
<td>−44</td>
<td></td>
</tr>
<tr>
<td>C52B9.2</td>
<td>Ets domain</td>
<td>4</td>
<td>−38</td>
<td>−36±8</td>
</tr>
<tr>
<td>ZC123.3</td>
<td>Homeobox</td>
<td>3</td>
<td>−37</td>
<td>−34±14</td>
</tr>
<tr>
<td>ceh-20</td>
<td>Homeobox</td>
<td>2</td>
<td>−32</td>
<td>−33±12</td>
</tr>
<tr>
<td>nhr-25</td>
<td>Nuclear hormone receptor</td>
<td>5</td>
<td>−32</td>
<td>−28±21</td>
</tr>
<tr>
<td>ceh-23</td>
<td>Homeobox</td>
<td>3</td>
<td>−29</td>
<td>−26±8</td>
</tr>
<tr>
<td>pha-4*</td>
<td>Forkhead</td>
<td>2</td>
<td>−20</td>
<td>−20±17</td>
</tr>
<tr>
<td>nhr-119</td>
<td>Nuclear hormone receptor</td>
<td>2</td>
<td>−15</td>
<td>−13±4</td>
</tr>
<tr>
<td>nhr-265</td>
<td>Nuclear hormone receptor</td>
<td>2</td>
<td>−12</td>
<td>−13±3</td>
</tr>
<tr>
<td>ceh-37</td>
<td>Homeobox</td>
<td>4</td>
<td>−12</td>
<td>−14±3</td>
</tr>
<tr>
<td>lin-11</td>
<td>Homeobox</td>
<td>5</td>
<td>−11</td>
<td>−15±7</td>
</tr>
<tr>
<td>aha-1</td>
<td>Anhydridecarbonase receptor</td>
<td>1</td>
<td>−11</td>
<td>−16±8</td>
</tr>
<tr>
<td>nhr-77</td>
<td>Nuclear hormone receptor</td>
<td>2</td>
<td>−11</td>
<td>−12±6</td>
</tr>
</tbody>
</table>

The RNAi candidates identified in the primary screen were retested in the number of independent lifespan assays indicated. The lifespan data from the separate trials were either pooled, compared by a stratified log-rank test (p≤0.001), and the percentage of mean lifespan decrease calculated or compared by log-rank test (p≤0.001) within each trial and the percentage of mean lifespan decrease was averaged. Only the RNAi clones that decrease the lifespan of the isp-1;ctb-1 mutant by at least 10% in the two methods of analysis are represented. Among them, 13 represent transcription factors not previously known to function in aging and four (indicated with asterisks) are transcription factors already known to affect lifespan in C. elegans: HSF-1 [31], NHR-49 [32], DAF-16 [33], and PHA-4 [34]. Quantitative data and statistical analyses for the experiments shown here are included in Table S1.

doi:10.1371/journal.pbio.1001084.g001
suppressed the longevity of mitochondrial mutants. Type worms, worms to a similar extent and had no effect on the lifespan of wild- type C. elegans (respectively 8% in wild-type; p≤0.001, log-rank test; Table 2). In contrast, knockdown of ceh-23 had no significant consequence on the lifespan of eat-2 mutant worms (Table 2). Therefore, all together, our targeted RNAi screen data identified the homeobox protein CEH-23 as a candidate uniquely required for the extended longevity of mitochondrial mutants.

The Homeobox Transcription Factor CEH-23 Mediates the Longevity Effects of METC Mutations

Although based on sequence alignment (BLAST), the ceh-23 RNAi clone used in the screen is specific, we constructed three additional RNAi constructs targeting different regions of the ceh-23 gene to rule out possible off-target effects of the ceh-23 RNAi from the Gene Service Library (Figure S2). All three of the newly generated RNAi constructs significantly (p≤0.001, log-rank test) suppressed the longevity of clk-1, isp-1, ceh-23, and isp-1 mutant worms to a similar extent and had no effect on the lifespan of wild-type worms, age-1, daf-16, and eat-2 mutant worms (Figure S3 and unpublished data). Taken together, our data indicate that knockdown of ceh-23 specifically shortens the lifespan extension of the long-lived mitochondrial mutant worms and is not likely to compromise the general health of the worm. To avoid any pitfalls associated with the RNAi strategy [41], we next examined the ceh-23 (mt23) mutant, which harbors a deletion that covers 75% of the ceh-23 gene, including half of the homeobox domain ([42] and Figure S2). While the ceh-23 single mutant exhibited wild-type lifespan [43], we found that loss of ceh-23 decreased the lifespan of isp-1, ceh-23 mutant by ~10% and that of isp-1 by ~20% (Figure 2 and Table S2). The ceh-23 genetic mutation suppressed the METC mutants’ lifespan to a similar degree as the three ceh-23 RNAi constructs we generated in the lab (Figure S2), but to a lesser degree compared to ceh-23 RNAi construct from the screen library. We purposely performed the screen with a mixture of RNAi colonies for each RNAi construct; thus, it is possible that the ceh-23 RNAi bacteria used in the screen had unexpected off-target effects that caused greater shortening of METC mutant lifespan. Overall, the ceh-23 mutant data corroborated the ceh-23 RNAi results and indicated that ceh-23 inactivation specifically shortens the lifespan extension of the long-lived mitochondrial mutants without compromising the general health of the worm. Taken together, our data highlight a crucial role of CEH-23 in mediating the extended longevity caused by METC mutations.

CEH-23 Does Not Mediate the Development and Fertility Defects or the Paraquat Resistance Induced by isp-1 Mutation

Having established that C. elegans is often associated with stress resistance [44], an interesting characteristic of the long-lived mitochondrial mutant worms is that they do not exhibit consistent resistance to different stresses, especially oxidative stress [3,8]. In examining different oxidative stress conditions, we noticed that isp-1 mutant worms exhibited different responses to the superoxide-stress resistance [44], an interesting characteristic of the long-lived mitochondrial mutants and wild-type worms. We found that ceh-23 and ceh-23 RNAi treated worms exhibit developmental alterations and brood size indistinguishable from that of wild-type worms (Table S3). As previously published [6], isp-1 and isp-1 mutant worms develop substantially slower and produce a much lower brood compared to wild-type worms (Table 3). Importantly, ceh-23;isp-1 and ceh-23;isp-1 mutant has development rate and brood size similar to those of isp-1 or isp-1;isp-1 mutants, respectively. Similar results were obtained when ceh-23 was knocked down by RNAi in the isp-1;isp-1 mutant (Table 3). These data indicate that C. elegans is unlikely to participate in the regulation of development rate and brood size in mitochondrial mutants.

Whereas increased longevity in C. elegans is often associated with stress resistance [44], an interesting characteristic of the long-lived mitochondrial mutant worms is that they do not exhibit consistent resistance to different stresses, especially oxidative stress [3,8]. In examining different oxidative stress conditions, we noticed that isp-1 mutant worms exhibited different responses to the superoxide-inducing agent paraquat depending on the developmental stage at which they were exposed to the chemical (Figure S4). We found that isp-1 mutant worms exhibited a better mean survival than wild-type worms when paraquat treatment was initiated at the L4 stage (Figure S4 and Table 3). Under this condition, loss of ceh-23 had no effect on the mean survival of wild-type or isp-1 mutants worms (Table 3), indicating that C. elegans is not required for the paraquat resistance of isp-1 mutant worms under this assaying condition.

Taken together, the results suggest that C. elegans is specifically important for the prolonged longevity, but not the slow rates of development and reproduction, or the oxidative stress resistance associated with the isp-1 mutation.

Table 2. Genetic interaction analyses of the RNAi candidates in several longevity mutant strains.

<table>
<thead>
<tr>
<th>Gene</th>
<th>isp-1;ctb-1</th>
<th>Wild-type</th>
<th>daf-16</th>
<th>age-1</th>
<th>isp-1</th>
<th>clk-1</th>
<th>mev-1</th>
<th>eat-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>dve-1</td>
<td>−55</td>
<td>−53</td>
<td>−35</td>
<td>−53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lin-49</td>
<td>−48</td>
<td>−18</td>
<td>−18</td>
<td>−57</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nhr-77</td>
<td>−11</td>
<td>−7</td>
<td>−10</td>
<td>−15</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZC1233</td>
<td>−37</td>
<td>−45</td>
<td>−12</td>
<td>n.s.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nhr-119</td>
<td>−15</td>
<td>−10</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ceh-37</td>
<td>−12</td>
<td>−8</td>
<td>n.s.</td>
<td>n.s.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>aha-1</td>
<td>−11</td>
<td>−10</td>
<td>−5</td>
<td>n.s.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C52B9.2</td>
<td>−38</td>
<td>−23</td>
<td>−12</td>
<td>n.s.</td>
<td>−30</td>
<td>−35</td>
<td>−12</td>
<td>−30</td>
</tr>
<tr>
<td>nhr-25</td>
<td>−32</td>
<td>−8</td>
<td>n.s.</td>
<td>+14</td>
<td>−46</td>
<td>−29</td>
<td>n.s.</td>
<td>−44</td>
</tr>
<tr>
<td>ceh-23</td>
<td>−29</td>
<td>+12</td>
<td>+23</td>
<td>n.s.</td>
<td>−29</td>
<td>−32</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
<tr>
<td>nhr-265</td>
<td>−12</td>
<td>−4</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

The effects of the RNAi candidates identified by our screen on the lifespan of different C. elegans mutant strains are shown as a percentage variation of the mean adult lifespan when compared to empty vector RNAi (stratified log-rank, p≤0.001). The mean adult lifespan for each RNAi candidate and for empty vector RNAi was obtained by pooling at least two independent lifespan assays. RNAi clones of C52B9.2, nhr-25, and ceh-23 specifically shortened the lifespan of all METC mutants tested (isp-1, ceh-1, and clk-1 mutants). Among them, ceh-23 RNAi is the only one that does not affect the lifespan of eat-2 mutants. Quantitative data and statistical analyses for the experiments shown here are included in Table S1. indicates a percentage decrease and + indicates a percentage increase of the mean adulthood lifespan when the mean lifespan of worms fed with the RNAi candidate and those fed with the empty vector RNAi were statistically significantly different (stratified log-rank, p≤0.001). n.s. indicates that the mean lifespan of worms fed with the RNAi candidate and those fed with the empty vector RNAi were not statistically significantly different (stratified log-rank, p>0.001). Underlined gene names indicate genes that, when down-regulated, have a stronger effect on isp-1; ctb-1 mutant worms’ lifespan than on wild-type worms’ lifespan.

doi:10.1371/journal.pbio.1001084.t002

Mitochondria Prolong Lifespan through CEH-23

A

Fraction of nematodes alive

Wild-type
ceh-23 mutant

Time (days)

0 10 20 30

B

Fraction of nematodes alive

isp-1;ctb-1
ceh-23;isp-1;ctb-1

Time (days)

0 10 20 30 40

C

% variation in adulthood lifespan compared to wild-type worms

p<0.005

D

Fraction of nematodes alive

isp-1
ceh-23;isp-1

Time (days)

0 10 20 30 40

E

% variation in adulthood lifespan compared to wild-type worms

p<0.005
The Expression of the Homeobox Transcription Factor CEH-23 Is Enriched in the Nuclei of Intestinal and Neuronal Cells

To further characterize CEH-23, we examined its expression pattern in wild-type and mitochondrial mutant worms. Because an antibody capable of recognizing endogenous CEH-23 is not available, we established multiple independent transgenic lines overexpressing an N-terminal GFP-fused CEH-23. This construct is likely to reflect the authentic CEH-23 expression pattern as it contains the entire coding region of ceh-23 (including introns), as well as its predicted promoter and 3’-UTR (Figure S2). Interestingly, the expression of CEH-23::GFP was restricted to a handful of neurons and the intestine of the worm (Figure S3A and D represent the data obtained in two independent lines). Although we have not identified the neurons in which CEH-23 is expressed, the observed neuronal expression of our CEH-23::GFP construct (Figure 3B and 3C) resembles that of a previously published GFP construct fused to a partial CEH-23 (gmIs18[ceh-23::gfp]; [47] and Figure 3F), which was reported to express in the pair of CAN neurons in the central body and 12 sets of sensory neurons in the head (10 pairs) and the tail (2 pairs). While the neuronal expression of CEH-23 was found in all the transgenic worms, the intestinal expression was only detected in ~50% of the transgenic worms with a stronger expression in the posterior intestine (Figure 3D). We currently do not understand what determines the mosaicism of the intestinal expression. However, it is of note that intestinal localization of ceh-23 has been previously observed using a promoter(ceh-23)::GFP fusion [47]. Enriched nuclear localization of CEH-23 was detected in both the neurons and the intestine, consistent with a putative role of CEH-23 as a transcription factor. No systematic differences in CEH-23 expression pattern were detected in isp-1;ctb-1 or isp-1 mutant worms compared to wild-type animals (unpublished data), suggesting that METC mutations are unlikely to affect the tissues nor the cellular compartments in which CEH-23 is expressed.

CEH-23 Expression Is Increased in Long-Lived Mitochondrial Mutants

We next compared the expression levels of ceh-23 in wild-type and in long-lived METC mutants using quantitative reverse-transcription PCR (qRT-PCR). Interestingly, we found that ceh-23 mRNA levels were elevated in the isp-1;ctb-1, isp-1, and clk-1 mutant worms compared to wild-type worms (Figure 3E, p<0.05, Student’s t test). Our finding is consistent with a recent microarray study indicating that ceh-23 mRNA level is elevated upon certain mitochondrial perturbations [48]. To further corroborate our qRT-PCR data, we compared ceh-23::gfp expression in wild-type and in the isp-1;ctb-1 mutant. We used the gmdIs18[ceh-23::gfp] strain (see above) for this imaging experiment due to its stable and homogenous expression among worm populations of the same genotype. Using confocal microscopy, we detected a substantially higher level of ceh-23::gfp expression in the isp-1;ctb-1 mutant compared to wild-type worms throughout different developmental stages of the worms (Figure 3F–G and Figure S5). Our data indicate that METC mutations can lead to increased CEH-23 expression and suggest that CEH-23 is able to respond to altered mitochondrial function (Figure 4C).

The Nuclear Homeobox Transcription Factor CEH-23 Modulates C. elegans Longevity

Since mitochondrial mutant worms require the presence of ceh-23 to maintain their lifespan (Figure 2) and exhibit higher levels of ceh-23 (Figure 3), we hypothesized that overexpression of ceh-23 might confer a long-lived phenotype, possibly by mimicking induced ceh-23 expression in the mitochondrial mutants. To test this, we established multiple independent transgenic lines overexpressing ceh-23 (ceh-23(o/e)) using a construct identical to the one we used for examining CEH-23 expression above except that it lacks the GFP fusion (Figure S2). We tested the lifespan of six independent extrachromosomal ceh-23(o/e) lines and observed that three of the lines showed a consistent and significant lifespan increase compared to the co-injection marker only transgenic controls (Figure 4A and Table S3, p<0.001 log-rank test). In the three other lines tested, overexpressing CEH-23 significantly increased longevity only in half of the experiments (Table S3). Mosaicism among transgenic lines is commonly observed in C. elegans. The differences in longevity of the different transgenic lines assayed may be due to silencing of the transgene in some of the lines upon propagation [49] and/or different levels of CEH-23 expression in the different lines. To further address this point, we generated transgenic worms expressing lower levels of CEH-23 by transforming worms with 10 times less DNA (1 ng/µl instead of 10 ng/µl). Overall, the 1 ng/µl lines exhibited only marginal lifespan increase, indicating that the effect of CEH-23 overexpression on the longevity of otherwise wild-type worms appears to be proportional to the quantity of DNA injected into worms (Figure 4A and Table S3). To further ensure that the lifespan increase phenotype we observed was due to elevated CEH-23 expression, we performed two additional control experiments. First, we treated one ceh-23(o/e) line (rwEx16, line 5) that consistently showed significant lifespan extension with ceh-23 RNAi and showed that the lifespan increase phenotype was abrogated when ceh-23 was knocked down in the transgenic worms (Figure S6A). Second, we introduced one ceh-23(o/e) line (rav21), which showed no obvious lifespan increase in wild-type background, into the ceh-23(isp-1) mutant background. Overexpression of ceh-23 in this scenario was able to significantly prolong the normally shortened lifespan of the ceh-23(isp-1) mutant worms, suggesting that re-expression of functional ceh-23 was able to revert the lifespan suppression caused by ceh-23 mutation (Figure S6B).

Because overexpressing ceh-23 is sufficient to increase wild-type lifespan (Figure 4A), we hypothesized that increased ceh-23 levels contribute to the longevity increase of mitochondrial mutants and that elevated ceh-23 expression and METC mutations increase lifespan through common mechanisms. With such a model, one...
might expect that overexpressing CEH-23 in METC mutants will not cause further extension of lifespan. To test our hypothesis, we examined the effect of CEH-23 overexpression in isp-1;ctb-1 mutant worms using three independent isp-1;ctb-1;ceh-23(o/e) lines. Two isp-1;ctb-1;ceh-23(o/e) lines further increased lifespan when compared to the isp-1;ctb-1;mec-7::rfp control lines (Figure 4B and Table S3, isp-1(qm150);ctb-1(qm189);rxaEx16[ceh-23+;mec-7::rfp], lines 4 and 3 versus control isp-1(qm150);ctb-1(qm189);rxaEx18[ceh-23;+;mec-7::rfp]; line 3, p>0.001, log-rank test). Another isp-1;ctb-1;ceh-23(o/e) line either exhibited the same or a shorter lifespan than the isp-1;ctb-1;mec-7::rfp control line (Table S3, isp-1(qm150);ctb-1(qm189);rxaEx16[ceh-23+;mec-7::rfp], line 1 versus isp-1(qm150);ctb-1(qm189);rxaEx18[ceh-23;+;mec-7::rfp]; line 3). Thus, our data showed that overexpressing ceh-23 can provoke a lifespan extension that is greater than that normally produced by overexpressing ceh-23 in wild-type worms. This suggests that the effect of overexpressing CEH-23 on longevity can be enhanced in isp-1;ctb-1 mutants. One intriguing hypothesis is that there is an optimal elevated level of ceh-23 for maximal longevity increase that was not reached in wild-type worms overexpressing CEH-23. Since METC mutants

<table>
<thead>
<tr>
<th>Table 3. Effects of knocking down ceh-23 on the rate of development, self-brood size, and resistance to paraquat.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Development and Brood Size</strong></td>
</tr>
<tr>
<td><strong>Number of Hours to Reach Adulthood</strong></td>
</tr>
<tr>
<td>Mean ± SD (Hours)</td>
</tr>
<tr>
<td><strong>Self-Brood Size</strong></td>
</tr>
<tr>
<td>Mean ± SD (Number of Worms)</td>
</tr>
<tr>
<td><strong>Strain</strong></td>
</tr>
<tr>
<td>Strain (Sample size)</td>
</tr>
<tr>
<td>wild-type</td>
</tr>
<tr>
<td>ceh-23(ms23)</td>
</tr>
<tr>
<td>isp-1(qm150);ctb-1(qm189)</td>
</tr>
<tr>
<td>ceh-23(ms23);isp-1(qm150);ctb-1(qm189)</td>
</tr>
<tr>
<td>isp-1(qm150)</td>
</tr>
<tr>
<td>ceh-23(ms23);isp-1(qm150);ctb-1(qm189)</td>
</tr>
<tr>
<td>wild-type+empty vector</td>
</tr>
<tr>
<td>wild-type+ceh-23 RNAI</td>
</tr>
<tr>
<td>isp-1(qm150);ctb-1(qm189)+empty vector</td>
</tr>
<tr>
<td>isp-1(qm150);ctb-1(qm189)+ceh-23 RNAI</td>
</tr>
</tbody>
</table>

| Survival on Paraquat                                          |
| **Survival**                                                  |
| Mean ± SD (Days)                                              |
| Strain (Sample Size)                                         | Strain (Sample Size) |
| wild-type                                                     | 2.47±0.08 (82)       | 2.47±0.08 (82)       |
| ceh-23(ms23)                                                  | 2.57±0.06 (85)       | 2.57±0.06 (85)       |
| isp-1(qm150)                                                  | 3.85±0.09* (109)     | 3.85±0.09* (109)     |
| ceh-23(ms23);isp-1(qm150)                                    | 4.13±0.12* (101)     | 4.13±0.12* (101)     |

Data shown are the average of two independent experiments. *Significantly different when compared to wild-type worms, p<0.05 Student’s t test. †Significantly different when compared to wild-type worms, p<0.001 log-rank test.

doi:10.1371/journal.pbio.1001084.t003
Mitochondria Prolong Lifespan through CEH-23

Figure A: Wild-type worm.
Figure B: Immunofluorescence staining of wild-type worms showing mitochondrial localization.
Figure C: Wild-type worm showing mitochondrial localization.
Figure D: Wild-type worm showing mitochondrial localization.
Figure E: Bar graph showing fold change relative to wild-type worms and normalized to actin mRNA levels.
Figure F: Wild-type and isp-1;ctb-1 worms stained with GFP.
Figure G: Bar graph showing GFP mean intensity (arbitrary units) for head and mid-body areas.

Wild-type vs. isp-1;ctb-1:
- Head area: Wild-type > isp-1;ctb-1
- Mid-body area: Wild-type > isp-1;ctb-1
already elevated levels of ceh-23, the transgene introduced into isp-1(xib-1) mutants could promote further lifespan increase due to higher levels of ceh-23. We also noticed that worms overexpressing CEH-23 exhibited similar development rates and brood size as their respective control lines (unpublished data). Thus, CEH-23 plays a role in longevity determination that is independent of any obvious effects on the pace of development of the worms.

**Discussion**

Signals from the mitochondrion to the nucleus are of crucial importance in the establishment of cellular adaptations resulting from altered METC function [5,21–25,50]. In yeast, mitochondrial dysfunction can induce nuclear expression changes that are key to lifespan determination through a signaling pathway that involves the RTG genes [21]. However, it is unknown whether similar retrograde signaling from the mitochondrion to the nucleus plays a role in longevity maintenance of animals. Through a targeted RNAi screen, we revealed three putative nuclear transcription factors that are broadly required for the longevity increase of METC mutants regardless of the specific lesions. Because Yang et al. recently showed that inactivation by RNAi or genomic mutation of the same subunit of the METC trigger different mechanisms of longevity [19], it will be interesting to test in the future whether any of the transcription factors identified in our screen as mediators of the long-lived phenotype of mitochondrial mutants will also be required for worms that live long due to mitochondrial dysfunction induced by RNAi knockdown.

Our findings implicate specific transcription factors in the extended longevity associated with mitochondrial mutations and strongly support the model that signaling from the mitochondria to the nucleus can modulate longevity in an animal. Our results nicely complement two recent C. elegans studies showing that mitochondrial perturbations are associated with major alterations in nuclear gene expression [48,51]. While it is tempting to speculate that the transcription factor candidates we uncovered in this study might act to regulate the gene expression changes reported in the previous microarray studies, our data also highlight differential molecular outputs downstream of METC alterations. For instance, Cristina et al. proposed that changes in the expression levels of fis-1/2 contribute to the phenotypic outcomes of the clk-1 mutation, as RNAi knockdown of fis-1/2 suppressed both the lifespan extension and the slow behaviors of clk-1 mutant worms [48]. On the contrary, our data indicate that CEH-23 is specifically important for longevity but not the developmental phenotype of isp-1 and isp-1(xib-1) mutants. Moreover, while fis-1/2 is uniquely required for the prolonged lifespan of clk-1 mutant, but not that of isp-1 mutant, we showed that ceh-23 contributes to the extended longevity associated with clk-1, isp-1, and isp-1(xib-1) mutations, suggesting that the retrograde signaling pathway involving ceh-23 can be triggered by different METC mutations regardless of the specific lesions. These observations are consistent with a model that fis-1/2 and ceh-23 act in two different pathways to affect longevity. Along a similar line, the nuclear hormone receptor NHR-49 has recently been shown to mediate a number of compensatory metabolic responses upon METC dysfunction in C. elegans, but NHR-49 was not found to affect the longevity increase associated with METC impairment [52]. These findings together suggest a model, similar to that proposed in yeast [23], in which multiple distinct mitochondrial retrograde signaling pathways, each inducing specific responses, modulate worm lifespan and physiology upon mitochondrial dysfunction.

Our RNAi screen revealed additional interesting insights into the molecular mechanisms that enable mitochondria to modulate longevity. First, we found that the long-lived phenotype of the several mitochondrial mutants tested greatly depends on three candidate transcription factors (C52B9.2; NHR-25; and CEH-23) that appear to have no substantial effect on the lifespan of the short-lived METC mutant mev-1. A previous study has shown that long-lived and short-lived mitochondrial mutants share a number of common changes in their nuclear transcriptional profiles when compared to wild-type worms [51]. One possibility is that those common gene expression changes account for the phenotypes shared between long-lived and short-lived METC mutants, such as slow development rate. Another hypothesis is that mitochondrial perturbations induce overlapping nuclear gene expression changes in both the long-lived and the short-lived mutant worms to affect lifespan, but additional detrimental effects in the short-lived mitochondrial mutants, for instance highly elevated levels of ROS [18,33], contribute to the opposite phenotypes observed. Since the candidate transcription factors we identified specifically suppress the long-lived phenotype of several mitochondrial mutants but did not cause further shortening of the mev-1 mutant lifespan, we propose that distinct molecular mechanisms likely underlie the differential longevity phenotypes of METC mutants in C. elegans. Another interesting finding from the screen is that three out of the four candidate transcription factors (with the exception of ceh-23) we identified to be important for the isp-1(xib-1) mutant longevity also appeared to be required for the prolonged lifespan of the genetic mimic of caloric restriction, eat-2. Our findings are consistent with previous observations [40] and suggest that eat-2 and METC mutants employ common as well as distinct mechanisms to modulate lifespan.

Our RNAi screen identified ceh-23 as specifically important for the longevity of both isp-1 and isp-1(xib-1) mutant worms and we confirmed our findings using a ceh-23 genetic mutation. One puzzling observation is that knocking down ceh-23 by mutation has a stronger suppressor effect on the longevity of the isp-1 mutant

**Figure 3. ceh-23 expression is enriched in the nuclei of intestinal and neuronal cells and is increased in response to altered mitochondrial function.** (A) DIC image of a rwEx19[gfp::ceh-23+mec-7::rfp] transgenic worm. (B) Overlay of a GFP-channel image (shown in green) and a RFP-channel image (shown in red) of a rwEx19[gfp::ceh-23+mec-7::rfp] transgenic worm. mec-7::rfp transgene was used as a co-injection marker. The green fluorescence likely represents the authentic expression pattern of CEH-23. The white arrows point to the nuclei of intestinal cells. The image shown is a representative of data obtained with two independent lines. (C) Enlarged overlay (GFP and RFP) image of the head of a rwEx19[gfp::ceh-23+mec-7::rfp] transgenic worm showing some of the neurons expressing CEH-23. (D) Enlarged overlay (GFP and RFP) image of the posterior intestine of a rwEx19[gfp::ceh-23+mec-7::rfp] transgenic worm showing enriched GFP expression in the nuclei of some of the intestinal cells (white arrows). (E) mRNA expression level of ceh-23 in wild-type worms and clk-1(e2519), isp-1(qm150);ctb-1(qm189), and isp-1(qm150) mutant worms. The mean ceh-23 mRNA levels normalized to actin mRNA levels are shown. The mean normalized mRNA levels in wild-type worms were set as 1. Data shown were pooled from three independent experiments, p<0.05, Student t test. (F) GFP expression in gmls-18[ceh-23::gfp] and in isp-1(ctb-1)gmls-18[ceh-23::gfp] transgenic mutant worms. Data shown were obtained from four representative worms.
Mitochondria Prolong Lifespan through CEH-23

A

Wild-type worms

Fraction of nematodes alive

Time (days)

rwEx18[mec-7::rfp]
rwEx17[ceh-23+mec-7::rfp] (1 ng/ul)
rwEx16[ceh-23+mec-7::rfp] (10 ng/ul)

B

isp-1;ctb-1 mutant worms

Fraction of nematodes alive

Time (days)

isp-1;ctb-1;rwEx18[mec-7::rfp]
isp-1;ctb-1;rwEx16[ceh-23+mec-7::rfp]

C

Mitochondrion

Nucleus of neuronal and/or intestinal cells

CEH-23

Longevity
It is intriguing that CEH-23 localization is confined to a subset of neurons, most of which are sensory neurons, and the intestine. Importantly, strains carrying the gfp::ceh-23 transgene also exhibited increased lifespan when compared to control animals (unpublished data), suggesting that overexpression of ceh-23 in a subset of neurons, most of which are sensory neurons, and/or the intestine is sufficient to modulate longevity. Interestingly, one of the sets of neurons expressing CEH-23 [42] is the ASI neurons, which are implicated in a model of caloric restriction-mediated longevity increase in C. elegans [57]. The restricted expression pattern of CEH-23 suggests that retrograde signaling from mitochondria to CEH-23 in specific neuron(s) and/or the intestine is sufficient to cause longevity changes. It is also possible that particular tissues, for instance highly ATP-dependent tissues like neurons, are more affected by METC mutations and are therefore key to mitochondrial signaling that modulates longevity. Supporting this hypothesis, neuronal-specific knockdown of METC subunits is sufficient to extend longevity in Drosophila [14]. An alternate but not mutually exclusive possibility is that CEH-23 acts in a select subset of cells to integrate signals from dysfunctional mitochondria from the whole worm and modulate longevity in a non-cell autonomous manner. If CEH-23 acts as a transcription factor, one or more of the CEH-23 transcriptional targets may constitute a signal that can travel through the worms to coordinate aging rate of the entire organism. Future investigation of CEH-23 downstream targets will go a long way in revealing the mechanism of action of CEH-23 in mediating the lifespan extension of METC mutants.

Our study revealed a novel longevity determinant that specifically responds to altered mitochondrial function to affect lifespan in a non-cell autonomous manner, pointing to a new paradigm of the molecular pathways that enable mitochondria to impact aging and age-dependent diseases in an animal.

**Experimental Procedures**

*C. elegans strains.* Most of the strains used were obtained from the Caenorhabditis Genetic Center. The gmIs18[ceh-23::gfp] strain [45] was a kind gift from the Bargmann and Garriga laboratories. The ceh-23(m23);isp-1(qm150);ctb-1(qm189);ceh-23(m23);isp-1(qm150), and the isp-1(qm150);ctb-1(qm189);gmIs18[ceh-23::gfp] strains were constructed using standard genetic methods. All strains were out-crossed into wild-type N2 in our lab at least four times and cultured using standard methods [50].

**Generation of ceh-23 Construct and Transgenic Lines**

All constructs were verified by sequencing or restriction fragment length. Sequences of the primers used for PCR are available upon request. The genomic sequence of ceh-23 and the transgenes are illustrated in Figure S2.

*ceh-23 transgene under control of endogenous ceh-23 promoter.* A 10.3 Kbp genomic fragment containing the 7.3 Kbp sequence upstream of the ceh-23 coding region, the 1.8 Kbp ceh-23 coding region, and the 1.1 Kbp sequence...
downstream of the ceh-23 coding region was amplified from genomic DNA by PCR. 

**ceh-23-GFP transgene under control of endogenous ceh-23 promoter.** A GFP coding sequence without stop codon was amplified by PCR from the plasmid pPD95-81. The amplified GFP sequence was then fused in frame at the N-terminal of the ceh-23 coding region into the 10.3 kb genomic fragment described above [59].

For generation of transgenic animals carrying extrachromosomal arrays of ceh-23 or gfp:ceh-23, 10 ng/μl or 1 ng/μl of the ceh-23 transgene (nixEx16[ceh-23+mec-7:zfpy] and nixEx17[ceh-23+mec-7:zfpy], respectively) or 10 ng/μl of the gfp:ceh-23 transgene (nixEx19[gfp:ceh-23+mec-7:zfpy] and 50 ng/μl of mec-7:zfpy as a co-injection marker and 50 ng/μl Bluescript plasmid filler DNA were microinjected into the gonads of adult wild-type animals using standard methods [60]. F1 progeny were selected on the basis of RFP fluorescence. Individual F2 worms were isolated to establish independent lines. 

**RNAi experiments.** Transgenic worms were allowed to lay progeny for 100% transgene (left) and induced overnight at room temperature. Duplicate plates of RNAi bacteria were freshly transformed before each trial and the bacteria were induced with 4 mM IPTG after reaching an optical density at 600 nm value of 0.8. In some experiments, the worms were fed as needed by adding 150 μl bacteria solution to the plates. In some experiments, the worms were transferred every other day into plates seeded with freshly induced bacteria. Using a feeding or a transfer protocol did not significantly change the results. The worms were scored every 1 or 2 d. Each lifespan assay was tested on duplicate or triplicate plates in at least two independent experiments.

**Lifespan assays with OP50 bacteria.** The lifespan assays performed on wild-type worms, ceh-23(m23) mutant, isp-1(qm150), and isp-1(qm150);zib-1(qm189), ceh-23(m23);zib-1 (qm150);xb-1(qm189), and transgenic worms were performed on OP50 bacteria. The optical density at 600 nm of the bacteria cultures was adjusted to the same value (1× bacteria; values at 0.175 for 1/10 dilution). Bacteria were seeded at 1× or 10× concentration (the concentration of the bacteria did not affect the differences between the lifespan of the different strains tested). Worms were transferred onto fresh plates every other day and scored as described above. Each lifespan assay was tested on in duplicate or triplicate plates in at least three independent experiments. For the lifespan assays involving transgenic worms, transgenic parents (based on RFP fluorescence) were used for the egg lay. At day 0 of adulthood, 30 transgenic worms (based on RFP fluorescence) from the progeny were transferred onto fresh plates.

**Statistical Analysis of the Lifespan Assays**

The independent trials were either analyzed separately as described above or, when appropriate, the data from the independent trials were pooled and compared using stratified log rank test after testing homogeneity among strata (individually controlled experimental replicates). In some cases, the mean variation in lifespan for each strain tested was calculated and compared to wild-type worms for each experiment. The averaged mean variations were analyzed using mixed model analysis with ceh-23 mutation as the fixed effect and experiment as the random effect.

**Assays for Developmental Rate and Brood Size Phenotypes**

Developmental rate and brood size were monitored as previously described [61]. Worms were grown on plates seeded either with OP50 or with RNAi bacteria induced with 4 mM IPTG. Self-brood size Young adults were singled onto fresh plates incubated at 20°C and transferred onto fresh plates every 12 h to prevent overcrowding until egg laying ceased. The progeny produced on each plate was counted 36 h after removal of the parent. The mean self-brood size obtained for worms of each strain was compared using Student’s t test. Time to reach adulthood: 60 synchronized eggs were plated and incubated at 20°C until they reached L4 stage and then scored for vulva formation every 6–8 h. The time elapsed from the egg stage until the stage of complete vulva formation was considered as the time necessary to reach adulthood. The mean number of hours required to reach adulthood obtained for worms of each strain was compared using Student’s t test. Similar results were obtained when animals were scored for adult alae appearance.

**Paraquat Assays**

Thirty worms synchronized at the L4 stage or at the first day of adulthood or at day 4 of adulthood were transferred into plates seeded with OP50 bacteria and containing 16 mM paraquat and
50 μg/ml of 5-fluoro-2'-deoxyuridine (FUDR). Worms were grown at 25°C for 3 d (to avoid vulva bursting) and then transferred to 20°C. Worms were scored every day. Survival on paraquat was defined as the time elapsed from when paraquat was added until the worms are scored as dead. The survival function of each worm population was estimated using the Kaplan Meier estimator (SPSS software), and statistical analysis was done using log rank test. p<0.001 was considered as significantly different from the control population.

RNA Isolation and Quantitative Reverse Transcription PCR (qRT-PCR)

Synchronized populations of ~4,000 eggs for each strain tested were either grown to late L4 staged worms (confirmed by Nomarski images of the gonad arms) at 20°C and harvested or grown to the young adult stage, treated with FUDR, and harvested 4 d later. Total RNA was isolated using Tri-reagent (Molecular Research Center, Inc.) [62]. cDNAs were synthesized with random hexamers using SuperScript III First-Strand Kit (Invitrogen). qRT-PCR reactions were performed using iQ SYBR Green Supermix (BIO-RAD) and the MyiQ Single Color Real-Time PCR Detection System (BIO-RAD). Melting curve analysis was performed for each primer set at the end to ensure the specificity of the amplified product. act-1 was used as the internal control so that the RNA level of each gene of interest was normalized to the level of act-1. As an additional control, in each experiment, mRNA expression of tbb-2 was measured to ensure that tbb-2 expression normalized to act-1 did not change between the strains tested. The qRT-PCR experiments were repeated at least three times using independent RNA/cDNA preparations. Quantitative PCR primer sequences are available upon request.

GFP Localization and Quantification

For GFP localization, worms at L1–L3 stage were paralyzed with levamisole on an agar pad. The GFP and RFP expressions were visualized at 60× magnification using a Leica DM 5000B microscope. Images were captured using OpenLab software. For GFP quantification, worms at L1–L2 or L4 stage were paralyzed with levamisole on an agar pad. The GFP expression was visualized at 60× magnification using a Leica SP2 confocal microscope. Images were acquired using Leica software. In the GFP channel, images were collected using a z-stack acquisition at 1 μm step interval, with each frame averaged 4 times, and projected in a 20 μm z-stack covering the entire worm. Some of the experiments were performed as double-blind assays. The mean GFP intensities were quantified using Image J and compared between strains using Student’s t test.

Note: While this paper was in review, several studies were published [63–65] that greatly advanced our understanding of the molecular mechanisms by which METC mutations prolong lifespan in C. elegans. Future investigations of how CEH-23 integrates with the new pathways revealed by these studies will provide important new insights into how mitochondria impact animal physiology and longevity.

Supporting Information

Figure S1 Targeted RNAi screen strategy to identify putative transcription factors that specifically suppress the long-lived phenotype of mitochondrial mutant worms. Using the C. elegans Transcription Factor RNAi Library (Gene Service Inc.) and feeding RNAi, we identified CEH-23 as a specific mediator of the longevity phenotype of isp-1;cb-1, isp-1, and clk-1 mutant worms. (EPS)

Figure S2 Schematic of ceh-23 (worm base). Exons are shown in blue and untranslated regions in gray. Z652.6 and Z652.8 are, respectively, the genes upstream and downstream of ceh-23. Dotted lines indicate DNA segments used to generate the four RNAi constructs (ceh-23A, ceh-23B, ceh-23C, and ceh-23 shringher constructs) used in this study. The mec-7 deletion that covers half of the predicted homeobox of ceh-23 is shown in red. The solid black lines indicate the segment of DNA that was amplified by PCR for injection to generate reEx16[ceh-23+;nec-7:rfp], reEx17[ceh-23+;nec-7:rfp], and reEx19[isp-1;ceh-23+;nec-7:rfp] transgenic worms. The insertion site of the GFP sequence in the transgene is also indicated. (EPS)

Figure S3 Effects of three ceh-23 RNAi clones on the lifespan of wild-type and mitochondrial mutant worms. The data presented are from two pooled independent experiments. (A) Wild-type worms treated with ceh-23 RNAi construct A (blue curve) or ceh-23 RNAi construct B (red curve) or ceh-23 RNAi construct C (green curve) exhibited the same lifespan as wild-type worms treated with empty vector control (black curve). (B) clk-1(e2519) worms treated with ceh-23 RNAi construct A (blue curve) or ceh-23 RNAi construct B (red curve) or ceh-23 RNAi construct C (green curve) exhibited a lifespan shorter than that of clk-1(e2519) worms treated with empty vector control (black curve). (C) isp-1(qm150);ctb-1(qm189) worms treated with ceh-23 RNAi construct A (blue curve) or ceh-23 RNAi construct B (red curve) or ceh-23 RNAi construct C (green curve) exhibited a lifespan shorter than that of isp-1(qm150);ctb-1(qm189) worms treated with empty vector control (black curve). (D) isp-1(qm150) worms treated with ceh-23 RNAi construct A (blue curve) or ceh-23 RNAi construct B (red curve) or ceh-23 RNAi construct C (green curve) exhibited a lifespan shorter than that of isp-1(qm150) worms treated with empty vector control (black curve). (EPS)

Figure S4 Survival on paraquat. The data presented are from two pooled independent experiments. (A) isp-1 (red curve, mean survival 3.85±0.10 d) and ceh-23;isp-1 (green curve, mean survival 4.13±0.13 d) mutants exhibited a better mean survival than wild-type (black curve, mean survival 2.48±0.08 d) and ceh-23 mutants (blue curve, mean survival 2.58±0.06 d) when paraquat treatment was initiated at L4 stage. (B) Wild-type (black curve, mean survival 4.22±0.11 d), ceh-23 mutant (blue curve, mean survival 4.10±0.18 d), isp-1 (red curve, mean survival 4.94±0.23 d), and ceh-23;isp-1 (green curve, mean survival 5.00±0.19 d) mutant worms exhibited the same mean survival when paraquat treatment was initiated at day 0 of adulthood. (C) Wild-type (black curve, mean survival 7.90±0.25 d), ceh-23 mutant (blue curve, mean survival 7.71±0.29 d), isp-1 (red curve, mean survival 7.94±0.26 d), and ceh-23;isp-1 (green curve, mean survival 8.44±0.28 d) mutant worms exhibited the same mean survival when paraquat treatment was initiated at day 4 of adulthood. (D) Mean survival of wild-type (black), ceh-23 mutant (blue), isp-1 (red), and ceh-23;isp-1 (green) mutant worms exposed to paraquat starting at stage L4, day 0, or day 4 of adulthood. (EPS)

Figure S5 ceh-23 expression is elevated in mitochondrial mutants. (A) GFP expression in gmls18[ceh-23;gfp] and in isp-1;cb-1;gmls18[ceh-23;gfp] transgenic mutant worms at L1 stage was visualized using confocal microscopy. CEH-23::GFP expression is shown in green and fluorescent image was merged with DIC image. The worms were mounted on the same agar pad. (B) Quantification of the GFP expression levels in the gmls18[ceh-23;gfp] mutant (mean ± SEM) with respect to wild-type (red). (EPS)
23::gfp] and in isp-1::tub-1[ems18/ceh-23::gfp] transgenic mutant worms shown in (A).

**Table S1** Quantitative data and statistical analyses of mean adult lifespan presented in Figure 1 and Tables 1 and 2.

**Table S2** Quantitative data and statistical analyses of mean adult lifespan of rwaEx16/ceh-23/+ worms shown in (A).

**Acknowledgments**

We thank the Caenorhabditis Genetics Center (University of Minnesota) and the Bargmann and Garriga laboratories for strains used in this study. We are very grateful to the members of the Lee Laboratory and the Cornell C. elegans groups (Kemphues, Lin, Schroeder, Vatamananik labs) for discussions during the course of this work. We thank Carol Bayles of the Microscopy and Imaging facility at Cornell University, Françoise Vermeylen of the Cornell University Statistical Consulting, and Rada Omanovic and Esther Mollerme for technical support.

**Author Contributions**

The author(s) have made the following declarations about their contributions: Conceived and designed the experiments: LW SSL. Contributed reagents/materials/analysis tools: SB. Performed the experiments: LW AB HMP HWC. Analyzed the data: LW. Wrote the paper: LW SSL.

**References**


