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p53/CEP-1 Increases or Decreases Lifespan, Depending on Level of Mitochondrial Bioenergetic Stress

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SUMMARY

Mitochondrial pathologies underlie a number of life-shortening diseases in humans. In the nematode *Caenorhabditis elegans*, severely reduced expression of mitochondrial proteins involved in electron transport chain-mediated energy production also leads to pathological phenotypes, including arrested development and/or shorter life; in sharp contrast, mild suppression of these same proteins extends lifespan. Here we show that the *C. elegans* p53 ortholog *cep-1* mediates these opposite effects. We find that *cep-1* is required to extend longevity in response to mild suppression of several bioenergetically relevant mitochondrial proteins, including frataxin - the protein defective in patients with Friedreich's Ataxia. Importantly we show that *cep-1* also mediates both the developmental arrest and life shortening induced by severe mitochondrial stress. Our findings support an evolutionarily conserved function for p53 in modulating organismal responses to mitochondrial dysfunction and suggest that metabolic checkpoint responses may play a role in longevity control and in human mitochondrial-associated diseases.

Keywords

p53/*cep-1*; mitochondria; frataxin; *C. elegans*; Mit mutants; aging; metabolic checkpoint

INTRODUCTION

Mutations in genes that affect mitochondrial functionality lead to a variety of life-shortening, degenerative disorders in humans (Wallace 2005). At the root of most of these diseases are reduced energy production and increased free radical production, resulting from direct or indirect impairment of the mitochondrial electron transport chain (ETC). Tissues

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AUTHOR CONTRIBUTIONS

N.V. designed and performed most of the experiments, analyzed the data and co-wrote the manuscript. S.R. participated with experimental design and execution, interpretation of results and co-wrote the manuscript. A.T. and A.S. performed some of the experiments. R.T. and T.J. equally helped support this work financially and edited the manuscript.

COMPETING INTEREST

The authors have declared that no competing interests exist.

most affected in these diseases are those with high oxidative energy requirements such as neuronal, cardiac, endocrine and renal systems. Friedreich's Ataxia (FRDA), the most frequently inherited ataxia, is one such disorder (Campuzano *et al.* 1996), and it is ascribed to severely defective expression of frataxin, a nuclear-encoded mitochondrial protein that plays a role in the biogenesis of Fe-S cluster containing proteins and thus in the functionality of mitochondrial proteins such as aconitase and complexes I, II and III of the ETC (Puccio *et al.* 2001).

In the nematode *Caenorhabditis elegans*, a collection of mutants defined by RNAi or genetic disruption of proteins involved in Mitochondrial ETC functionality (hereafter referred to as Mit mutants) are unexpectedly long-lived; they also have slower rates of development and decreased fertility (Rea 2005; Ventura *et al.* 2006). In our attempts to generate a nematode model of FRDA, we surprisingly found that decreasing the expression of the frataxin *C. elegans* ortholog, *frh-1*, also prolonged animal longevity (Ventura *et al.* 2005). Reduced expression of the NDUSF3 subunit of complex I of the ETC also leads to lifespan extension in *C. elegans*. In humans, mutation in complex I subunits including NDUSF3, cause Leigh Syndrome, another degenerative disorder. How can loss of genes critical for both cellular energy production and proper mitochondrial function, and which when mutated in humans lead to life-shortening debilitating diseases, result in life extension in *C. elegans*? To reconcile this human-nematode paradox we recently suggested that down to a certain threshold, cells have the ability to counteract a reduction in mitochondrial ETC function by invoking compensatory mechanisms, but beyond this threshold cell viability is severely compromised (Ventura *et al.* 2006). Consistent with this model, we previously showed that *C. elegans* Mit mutants exhibit lifespan extension only in a defined window of mitochondrial protein suppression; indeed when mitochondrial dysfunction becomes too severe and passes a critical threshold, these mutants exhibit pathological phenotypes including arrested development and lifespan reduction, perhaps similar to mitochondrial disease states in humans (Rea *et al.* 2007; Ventura & Rea 2007). The molecular mechanisms underlying this transition in phenotype in response to different levels of mitochondrial stress are still unknown.

Here we show that, *cep-1*, the *C. elegans* p53 ortholog, modulates Mit mutant phenotypes. Our findings are in agreement with a growing body of evidence that reveal a role for p53 as a metabolic checkpoint sensor (Bensaad & Vousden 2007), and support an evolutionarily conserved function for the p53 family in specifying organismal response to mitochondrial stress. Importantly they suggest that p53 in humans may also play a role in controlling the presentation of FRDA, Leigh Syndrome and possibly other Human Mitochondrial Associated Diseases (HMADs).

RESULTS

Frataxin modulation of stress response genes and longevity

Previous findings suggest that the *C. elegans* Mit mutants experience endogenous stress (Rea *et al.* 2007; Ventura & Rea 2007). We tested if hormetic-like responses are responsible for life extension in these worms. Feeding *frh-1* RNAi to wild type animals for three consecutive generations results in a 75% decrease in frataxin mRNA level and a maximal increase in mean lifespan (Ventura *et al.* 2005). We found that this treatment also robustly induced the expression of multiple stress-responsive reporter genes (including *gst-4::GFP*, *hsp-16.2::GFP* (Link *et al.* 1999) and *hsp-6::GFP* (Yoneda *et al.* 2004)) when compared with animals fed empty vector (Fig. 1A). We quantified this induction by western blotting and found that, relative to control-treated animals, *frh-1* RNAi increased the expression of GST-4 and of HSP-6, 30 and 25 fold, respectively. HSP-16.2 is only expressed after heat shock. In control animals, HSP-16.2 was induced 30 fold by heat shock but almost 50 fold in

frh-1 RNAi-treated animals (Fig. 1B and Fig. S1A). *frh-1* RNAi progressively increased the expression of the GST-4 and the HSP-6 throughout the three generations of feeding (data not shown). *frh-1* RNAi also weakly induced the expression of the antioxidant gene *sod-3* over control (Fig. S1B). These data clearly indicate that mild reduction of frataxin expression induces a robust stress response.

We next sought to determine if known genetic modulators of reaction to stress and lifespan in worms, also controlled *frh-1* RNAi-mediated longevity. SKN-1 is the *C. elegans* functional ortholog of the mammalian redox transcription factor Nrf2. SKN-1 is activated following oxidative stress and, like Nrf2 in other species, it controls the induction of antioxidant responsive element (ARE)-containing genes, such as the Phase II enzymes *gcs-1* (An & Blackwell 2003), and *gst-4* (Kahn *et al.* 2008). Mild oxidative stress increases *C. elegans* lifespan ((Cypser *et al.* 2006) and Fig. S2A). We tested whether *frh-1* RNAi-induced oxidative stress might extend lifespan through *skn-1*. While a strain carrying a *skn-1(zu67)* mutant allele is short-lived compared to wild-type (An & Blackwell 2003), we found that *frh-1* suppression by RNAi still increased its lifespan (Fig. 2A and Table I). Moreover, while the weak increase in lifespan induced by mild oxidative stress is completely blocked in *skn-1* mutants, these animals were still long-lived after treatment with *frh-1* RNAi, even when exposed to oxidative stress (Fig. S2B and Table I). The *skn-1* gene encodes three different SKN-1 isoforms, SKN-1A, SKN-1B and SKN-1C, which are expressed in different tissues. The *skn-1(zu67)* mutant allele does not knock out isoform B (Fig. S2C), which is primarily expressed in the ASI neurons and which was recently shown to specifically mediate lifespan extension by caloric restriction, via effects on mitochondrial respiration (Bishop & Guarente 2007). Nonetheless, even in a *skn-1(zu135)* mutant background, which disrupts the expression of all three SKN-1 isoforms, *frh-1* RNAi was still able to evoke a lifespan increase (Table I).

Daf-16, a *C. elegans* homolog of the human forkhead redox transcription factor FOXO family, is part of the insulin/IGF-1-like pathway and is required for increased stress resistance and longevity in many *C. elegans* Age mutants (Henderson *et al.* 2005). *Daf-16* modulates lifespan in parallel with *skn-1* (Tullet *et al.* 2008), and along with other Daf genes is involved in the hormetic response to heat shock (Cypser *et al.* 2006). Compared to control-fed animals, *frh-1* RNAi increased lifespan of the *daf-16(mgDf50)* short-lived mutant (Fig. 2B and Table I). This result is in agreement with previous work showing other mitochondrial mutants increase longevity independently of *daf-16* (Dillin *et al.* 2002). Moreover, consistent with *frh-1* RNAi specifying longevity independently of the IGF/insulin like pathway, we also found that RNAi against *frh-1* further increased the lifespan of the long-lived PI3K mutant *age-1(hx546)* (Table I).

SIRT1 and FOXO interact to modulate longevity and gene expression in response to oxidative stress and caloric restriction both in mammalian cells and in *C. elegans* (Wang & Tissenbaum 2006; Wang *et al.* 2007). We found that *frh-1* RNAi still increased longevity in two different sirtuin knock-out mutants, *sir-2.1(ok434)* and *sir-2.3(ok444)* (Fig. 2C and Table I). Our findings indicate that known lifespan-modulating pathways, which are often activated in response to stress, do not mediate the lifespan extension induced by reduced expression of frataxin.

Mit mutant longevity is dependent upon p53/*cep-1*

The tumor suppressor p53 is activated in response to a broad range of stressors, including genotoxic, metabolic and oxidative challenges (Vousden & Lane 2007). In *C. elegans*, there is one p53 homolog, *cep-1*, already known to be required for germ-line apoptosis in response to DNA damage and for normal resistance in the soma to environmental stressors such as hypoxia and starvation (Derry *et al.* 2001). Consistent with the role of p53 in

modulating cell senescence and aging in different species (Bauer & Helfand 2006), it was recently shown that loss of *cep-1* in *C. elegans* increases lifespan (Arum & Johnson 2007). We have now confirmed that a different *cep-1* allele, *cep-1(lg12501)* (Schumacher *et al.* 2001) is also long lived (Table I).

We tested if p53/*cep-1*-modulated *C. elegans* longevity following reduced *frh-1* expression. We fed the *C. elegans* p53 knock-out strain carrying the *cep-1(gk138)* allele, previously out-crossed ten times (Arum & Johnson 2007), with control or frataxin RNAi for three consecutive generations, and found that the absence of *cep-1* significantly suppressed the increase in lifespan induced by *frh-1* RNAi (Fig. 2D and Table I). We next investigated whether our observation with p53/*cep-1* could be translated to other Mit mutants. Many of these mutants display life extension only in a discrete range of mitochondrial protein suppression. When mitochondrial protein expression is reduced too severely, lifespan begins to shorten and other pathological phenotypes appear. We previously demonstrated this phenomenon using an RNAi feeding dilution strategy ((Rea *et al.* 2007) and Fig.S3). We therefore followed the lifespan of wild-type and *cep-1(gk138)* strains cultured on RNAi to one of three Mit targets, at concentrations found to maximally increase lifespan: we decreased the expression of the following mitochondrial components - ATP-3, the ATP5O/OSCP subunit of the F₁F_oATPase; ISP-1, the Rieske iron-sulfur protein of ubiquinol-cytochrome c oxidoreductase; CCO-1, the COX5B subunit of cytochrome c oxidoreductase. Wild-type animals fed with a 1/10 dilution of *atp-3*, *isp-1* and *cco-1* RNAi are all long lived compared to control RNAi-treated animals (Rea *et al.* 2007). Interestingly, the lifespan extension induced by the same Mit RNAi concentration was significantly suppressed in the *cep-1(gk138)* strain (Fig. 3A–C and Table II). Nevertheless, these Mit mutants remained significantly longer-lived than vector-treated animals. These results indicate a role for *cep-1* in sensing mitochondrial alterations and consequently modulating *C. elegans* longevity.

Janus-faced role of p53 is evolutionary conserved

p53 responds to different levels of stress by inducing opposite cellular outcomes. This effect is mediated, in part, by the action of differential gene activation and p53 modification (Vousden & Lane 2007). We wondered whether *cep-1* would have opposite effects on the specification of Mit mutant longevity, depending on the severity of mitochondrial bioenergetic stress. We thus fed wild-type and *cep-1(gk138)* strains with more severe preparations (undiluted) of RNAi against *atp-3*, *isp-1* or *cco-1* and found that *cep-1* mutants displayed longer lifespan than wild-type animals (Fig. 3 D–F and Table II). Specifically, undiluted *atp-3* RNAi decreased the lifespan of the wild-type strain below that of control RNAi-treated animals ((Rea *et al.* 2007) and Fig. S3A) while in the *cep-1* mutant strain lifespan was increased with the same RNAi treatment (Fig. 3D and Fig. S4A). We also tested a 1:2 *atp-3* to empty-vector RNAi ratio and consistently found the same results (Fig. S4A). Undiluted *isp-1* RNAi shortened lifespan of the wild-type strain when compared to the 1/10 dilution, though to control-treated animals this treatment still resulted in some life extension ((Rea *et al.* 2007) and Fig. S3B). Undiluted *cco-1*, more severely affected animals' phenotype, which appeared more fragile and almost sterile, but it did not shorten animal lifespan compared to the 1/10 dilution. Nonetheless, consistent to what we saw with more severe mitochondrial damage induced by undiluted *atp-3* RNAi, treatment of *cep-1* mutant strain with undiluted *isp-1* and *cco-1* RNAi increased lifespan relative to wild-type animals fed with the same RNAi concentration (Fig. 3 E, F and Fig. S4B).

One trivial explanation for the differential effects of *cep-1(gk138)* on Mit mutant longevity is that the efficacy of RNAi in this line is reduced relative to wild type (N2) animals. RT-PCR was used to compare *atp-3* mRNA levels in *cep-1* and N2 animals following RNAi treatment (Fig 4A). No difference in RNAi knockdown efficacy was observed. Importantly,

we obtained similar lifespan outcomes upon feeding different *atp-3* RNAi dilutions to a different *cep-1* allele, *cep-1(lg12501)* (Fig. 4B, Fig. S4C and Table III).

In summary, our results are consistent with a Janus-faced role of p53 in response to different level of stress (Vousden & Lane 2007) and suggest that *cep-1* may sense mitochondria alterations and subsequently induce opposite effects on animal longevity depending on the level of mitochondrial stress.

***cep-1* modulates Mit mutant development**

Besides changes in lifespan, Mit mutants display other phenotypic alterations that correlate with degree of mitochondrial disruption (Rea *et al.* 2007). Severe reduction of either *atp-3* or *isp-1* by feeding RNAi is sufficient to arrest development in wild-type animals (Rea *et al.* 2007). Developmental arrest is also observed in animals fed for two consecutive generations with RNAi against *nuo-2* (which encodes the NDUFS3 subunit of NADH-ubiquinone oxidoreductase) (Ventura & Rea 2007). *cep-1(gk138)* mutant strain had no effect on the developmental rate of animals fed control vector but it clearly prevented the developmental arrest induced by either undiluted *atp-3* or *isp-1* RNAi (Fig. 5A), although this effect was not fully penetrant. Similar results were obtained using the *cep-1(lg12501)* allele fed undiluted *atp-3* (Fig. S6), and when undiluted *nuo-2* RNAi was fed for two consecutive generations (Fig. 5B). These findings suggest *cep-1* can modulate development under conditions of bioenergetic stress.

The DNA intercalating agent ethidium bromide (EtBr) inhibits mitochondrial (mt) DNA replication and transcription at low concentrations without causing detectable effects on nuclear DNA (Nass 1972). For this reason it is widely used in mammalian cells to mimic human mitochondrial diseases (Biswas *et al.* 1999). Previous studies have also shown that EtBr induces both developmental arrest and increase lifespan in *C. elegans* (Tsang & Lemire 2002). We found that the developmental arrest induced by 75 µg/ml of EtBr is partially reverted by loss of *cep-1* (Fig. 5A), lending further support to a role for CEP-1 in sensing mitochondrial bioenergetic alterations and modulating the coordinate expression of multiple Mit mutant phenotypes. This finding again underscores the notion that the effect of *cep-1* in our above experiments is not due simply to subtle differences in RNAi efficacy.

Life shortening and developmental arrest induced by severe electron transport chain disruption involves *egl-1*

Mitochondrial ETC disruption can lead to DNA damage, which is a major p53-activating stressor in human cells and, depending on its severity, can in turn lead either to transient cell-cycle arrest or apoptosis. In worms, *atm-1* (the *C. elegans* ortholog of the human Ataxia Teleangiectasia gene), and *egl-1* (the *C. elegans* Bcl-2 homology domain-3 (BH3)-only protein), act upstream and downstream, respectively, of *cep-1* to induce germline apoptosis in response to DNA damage (Hofmann *et al.* 2002; Garcia-Muse & Boulton 2005). EGL-1 is also required to induce apoptosis and mitochondrial fragmentation of the 131 out of 1090 somatic cells that normally die during *C. elegans* development (Conradt & Horvitz 1998; Jagasia *et al.* 2005), and it is required in the embryo to induce autophagy in response to starvation (Maiuri *et al.* 2007). We tested if either *atm-1* or *egl-1* were essential to the life-lengthening or the life-shortening effects of mitochondrial ETC disruption.

We found that *atm-1(gk186)* is long lived while *egl-1(n487)* is short lived compared to wild-type animals (Table IV). We observed that both *atm-1* and *egl-1* play no apparent role in the life extension that occurs following mild *frh-1* RNAi treatment (Fig. S5 A, B and Table IV), or following feeding with 1/10 diluted *atp-3* RNAi (Fig. S5C, Fig. 6A and Table IV). Furthermore, *atm-1* plays no obvious role in the life shortening that occurs following

treatment with undiluted *atp-3* RNAi (Fig. S5D and Table IV). Loss of *egl-1*, on the other hand, shortens the lifespan of control fed animal but partially reverted the lifespan shortening effect caused by growth on undiluted *atp-3* RNAi (Fig. 6B and Table IV). This effect was never complete, illustrating that other factors await identification or that *egl-1(n487)* is not fully penetrant. Consistently, we observed that *egl-1* knock-out animals suppressed the developmental arrest induced by undiluted *atp-3* RNAi, but this phenotype was also not fully penetrant (data not shown). We conclude that mild mitochondrial disruption extends lifespan in a *cep-1* dependent manner but independently of the DNA-damage apoptotic pathway, while severe reduction of mitochondrial function requires, in part, the *cep-1/egl-1* pathway to decrease lifespan and arrest development in the Mit mutants.

In agreement with our above observations showing a functional requirement for *egl-1* only at strong *atp-3* RNAi concentrations, we observed that growth of wild type animals on treatments that increased lifespan, namely mild *frh-1* RNAi or a 1/10 dilution of *atp-3* RNAi, only marginally induced *egl-1* mRNA (Fig. 6C). Neither treatment affected the expression of an *egl-1::gfp* translational reporter strain (data not shown). On the contrary, growth of wild type animals on a treatment that shortens lifespan, namely undiluted *atp-3* RNAi, strongly induced *egl-1* mRNA (Fig. 6C), although not at the classical levels shown in response to DNA-damage inducing agents (Hofmann *et al.* 2002; Garcia-Muse & Boulton 2005). Induction of *egl-1* by *atp-3* RNAi at 1/10 and undiluted potency are significantly suppressed by loss of *cep-1*. These findings reveal a new role for the *cep-1/egl-1* pathway acting during *C. elegans* development to regulate animal lifespan.

DISCUSSION

Friedreich's Ataxia (FRDA), as well as many other disorders associated with mitochondrial dysfunction, is a chronic, degenerative disease that often becomes clinically apparent later in life. It is probable that protective pathways are activated in the pre-symptomatic phase of FRDA to adapt and cope with dysfunctional mitochondria, thus delaying its clinical appearance. Similar pathways may be induced in *C. elegans* by a mild decrease of the frataxin protein (Ventura *et al.* 2006). We have now shown that protective stress-response genes, such as heat shock proteins and antioxidants, are strongly induced in worms in response to mild frataxin silencing. In addition we found that the tumor suppressor protein p53 has a key role in modulating the phenotypic effects resulting from mitochondrial disruption. Our findings hence invoke new players in the etiology of human mitochondrial associated diseases such as FRDA and Leigh Syndrome.

Protective Responses to Mitochondrial Dysfunction

Hormetic responses to mild heat shock or oxidative stress have been associated with prolonged longevity in *C. elegans*. When we looked at the contribution of *daf-16* and *skn-1* (which encode transcription factors that modulate heat shock and oxidative stress responses, respectively), to the longevity increase induced by *frh-1* RNAi, we found that loss of neither transcription factor prevented life extension. Our results indicate that oxidative stress response, at least that controlled by these two transcription factors, is not the limiting factor regulating Mit mutant lifespan. Importantly if, as we have previously hypothesized (Ventura *et al.* 2006), similar pathways counteract mitochondrial dysfunction in both long-lived animals and in the pre-symptomatic phase of FRDA, our result imply that oxidative stress responses may also play a role in the human disease, but that it might not be the only player in eliciting protective pathways to help delay the established pathology.

Caloric Restriction (CR) extends *C. elegans* longevity and increases respiratory rate in a SIR2.1 and SKN-1 dependent manner (Wang & Tissenbaum 2006; Bishop & Guarente

2007). We found that *frh-1* RNAi increased *C. elegans* lifespan independently of *sir2.1* and, as mentioned, *skn-1*. Our findings do not, however, discount a role for increased mitochondrial respiration resulting from a compensatory induction of mitochondrial biogenesis in response to *frh-1* RNAi. Indeed, induction of mitochondrial biogenesis in response to mild mitochondrial dysfunction in the pre-symptomatic phase of FRDA could help sustain cellular viability and thus act to clinically delay disease onset. In humans, p53 is known to regulate both mitochondrial biogenesis and mitochondrial respiration (Donahue *et al.* 2001; Matoba *et al.* 2006) and we have now found that p53/*cep-1* is required for life extension in the Mit mutants. Derry and colleagues have reported that loss of *cep-1*, *per se*, causes significant transcriptional alterations (Derry *et al.* 2007), although these findings were not confirmed in a subsequent study (Greiss *et al.* 2008). Nevertheless, among the purportedly altered genes of the former analysis, there were many encoding for mitochondrial proteins (listed in Table S1). We have previously shown that mitochondrial ETC protein expression is pliant and responds uniquely in different Mit mutants (Ventura & Rea 2007). Additional work is warranted in order to understand the role played by p53 in the regulation of mitochondrial biogenesis, mitochondrial proteins composition and its relationship with longevity specification and disease prevention in response to mitochondrial dysfunction.

The Janus-faced role of p53

Considerable evidence supports a dual role for p53 in eliciting different responses depending on level of cellular stress (Vousden & Lane 2007). Mild or transient cellular damage, such as mild increase in free radical production, minor DNA damage, and transitory glucose or ATP depletion, induce protective p53 pathways that improve antioxidant defenses and DNA repair mechanisms, and fulfill energy requirements. In these instances p53 temporarily arrests the cell-cycle until the stress has been resolved. More severe oxidative stress, irreparable DNA damage, or complete ATP deprivation leads to p53-dependent cell death or irreversible cell-cycle arrest (replicative cell senescence). Both p53-dependent responses account for its tumor suppressor activity in humans. Consistent with the Janus-faced activity of p53 (Vousden & Lane 2007), and with p53's role as a sensor and mediator of mitochondrial metabolism (Jones *et al.* 2005; Mandal *et al.* 2005), we have shown that *cep-1*, the *C. elegans* p53 ortholog, modulates Mit mutant lifespan in an opposite manner, depending on the level of mitochondrial stress experienced. *cep-1* is required for increased longevity under mild mitochondrial disruption, and for mediating the detrimental effect on lifespan when mitochondrial damage is more severe.

Recently, two Mit mutations that increase lifespan, *isp-1(qm150)* and *clk-1(qm30)*, were shown to protect against tumor growth in the *C. elegans gld-1* tumor-like mutant, in a *cep-1*-dependent fashion (Pinkston *et al.* 2006). In conjunction with our present findings, these results imply that modulating mitochondrial function, by way of p53, can both regulate longevity and concurrently serve as an anti-tumor strategy. A similar situation seems to exist in mice where increased, but otherwise normally-regulated, levels of p53 lead to both tumor suppression and anti-aging effects (Matheu *et al.* 2007).

p53-activating stressors can lead to transient cell-cycle arrest or apoptosis. While we can not rule out the possibility that apoptosis via CEP-1 independent pathways play a role in the appearance of Mit mutant phenotypes, our results with *egl-1* and *atm-1* knock out strains, suggest that apoptosis is not the main mechanism through which CEP-1 is extending longevity in the Mit mutants. On the other hand, deregulated induction of a CEP-1 dependent apoptotic pathway may explain the short lifespan induced by severe mitochondrial stress (this study and (Senoo-Matsuda *et al.* 2003). Independent of its pro-apoptotic activity, EGL-1 is known to also induce mitochondrial fragmentation (Delivani *et*

al. 2006), a process that may contribute to its lifespan shortening effect (Chan & Mattson 1999).

In worms the pro-apoptotic and anti-proliferative effects of *cep-1* can follow different pathways (Derry *et al.* 2007). Moreover, in the *gld-1* mutant, the two long-lived Mit mutants, *isp-1 (qm150)* and *clk-1(qm30)*, reduce germ cell hyperproliferation without inducing germ cell apoptosis (Pinkston *et al.* 2006). Furthermore, *aak-2* is an AMP kinase recently shown to be required for *isp-1* and *clk-1* Mit mutant longevity (Curtis *et al.* 2006). This same kinase is also necessary for the longevity increase induced by mild treatment of *atp-3* RNAi (our unpublished observation). Two papers have shown that, in response to mitochondrial dysfunction and energy deprivation, p53 induces cell-cycle arrest via AMP kinase-dependent phosphorylation (Jones *et al.* 2005; Mandal *et al.* 2005). These observations suggest that an AMPK/CEP-1 metabolic checkpoint may play an integral role in specifying Mit mutant longevity.

Another role of p53, which is Janus-faced and therefore potentially relevant to mitochondrial stress, is the control of autophagy (Maiuri *et al.* 2007; Levine & Abrams 2008). Autophagy is a form of organelle and cellular digestion that can be either necessary or detrimental for tissue homeostasis and organismal survival. It has been associated both with prevention and causation of diseases in human, including neurodegenerative disorders (Vellai *et al.* 2007; Levine & Kroemer 2008). Autophagic genes are required during normal development in *C. elegans* and also for the increased lifespan observed in several mutant backgrounds, including a p53/*cep-1* knock out strain (Melendez *et al.* 2003; Tavernarakis *et al.* 2008; Toth *et al.* 2008). Moreover, gain-of function mutation of the sole *C. elegans* BH3-only protein EGL-1, induces autophagy, while deletion of EGL-1 compromises starvation-induced autophagy (Maiuri *et al.* 2007). Based on our results it will be interesting to determine whether autophagy is associated and/or necessary to specify longevity resulting from different degrees of mitochondrial dysfunction.

A developmental checkpoint role for *cep-1*: the whole p53 family in one gene?

In our studies we found that the absence of *cep-1* partially rescued the developmental arrest induced by severe mitochondrial damage. Importantly, we showed that *cep-1* mutants also prevented the developmental arrest induced by treatment with EtBr, ruling out the possibility that lack of *cep-1* is simply modulating RNAi potency rather than the response to mitochondrial stress. A developmental role of *cep-1* in response to stress is consistent with a *cep-1*-dependent metabolic checkpoint acting during *C. elegans* development (Rea *et al.* 2007) and/or by *cep-1* functions reminiscent of other p53 family members, such as those of p63 and p73 in differentiation and development (D'Erchia *et al.* 2006; Ou *et al.* 2007). Under normal, well-fed conditions, *cep-1* appears to be dispensable for *C. elegans* development. Only under conditions of increased energy and nucleotide demand, when its function may be required for mitochondrial biogenesis, we observed a requirement for *cep-1*. These conditions may exist when cells are rapidly proliferating, such as during embryonic *C. elegans* development or at the transition between L3–L4 stages, the point of gonad formation. The regulation of animal lifespan through a mitochondrial checkpoint in proliferating cells during animal development is an interesting possibility.

Concluding Remarks

Our data suggest a model in which mild disruption of ETC function in *C. elegans* induces transient cellular damage (perhaps genotoxic stress, proliferation errors and/or organelle dysfunction), which is readily repaired or eliminated by sub-lethal activation of *cep-1*. A temporary halt of the cell-cycle would allow for metabolic adaptation to mild mitochondrial stress and to elicit proper defenses against oxidative and other stressors; *cep-1* activation

also mediates increased animal lifespan. When mitochondrial dysfunction surpasses a critical threshold, *cep-1* functionality is altered in an attempt to eliminate irreparably damaged cell leading to the detrimental phenotypes observed in the Mit mutants (Fig. 5). Our current findings suggest that p53 has a previously unrecognized role in modulating animal lifespan and development in response to mitochondrial stress. p53 activity is now a prime target for investigations into the pathogenesis and treatment of human mitochondrial disorders.

EXPERIMENTAL PROCEDURES

Nematode Strains and Maintenance

Standard nematode culturing techniques were employed (Wood 1988). The following strains were utilized: N2 (wild-type), SJ4100 [*hsp-6::gfp(zcIs13)*], TJ375 [*hsp-16.2::gfp(gplIs1)*], CL2166 [*dvIs19[pAF15(gst-4::gfp::NLS)] III*], CL691 [*dvIs19[pAF15(gst-4::gfp::NLS) III]*]; *skn-1(zu67) IV/nT1[unc-?(n754);let-?(IV;V)]*, EU1 [*skn-1(zu67)IV/nT1[unc-?(n754)let-?(IV;V)]*, EU31 [*skn-1(zu135)IV/nT1[unc-?(n754)let-?(IV;V)]*], GR1307 [*daf-16(mgDf50)*], TJ1052 [*age-1(hx546)*], VC199 [*sir-2.1(ok434)*], RB654 [*sir-2.3(ok444)*], VC172 [*cep-1(gk138)*], TJ1 [*cep-1(gk138)*], XY1054 [*cep-1(lg12501)*], VC381 [*atm-1(gk186)*], MT1082 [*egl-1(n487)*], MD166 [*egl-1::gfp(bcIs1)*].

Feeding RNAi

RNAi feeding techniques were previously described (Ventura *et al.* 2005; Rea *et al.* 2007). The following RNAi feeding constructs were derived from the Ahringer *C. elegans* RNAi library (Kamath & Ahringer 2003): *atp-3* (F27C1.7), *cco-1* (F26E4.9), *nuo-2* (T10E9.7) (Kamath & Ahringer 2003) while feeding RNAi constructs against *frh-1* and *isp-1* have been previously described (Ventura *et al.* 2005; Rea *et al.* 2007).

Lifespan and Statistical Analysis

Survival analyses began from hatching and were recorded at 20°C using synchronous populations of 60–100 animals per strain. We calculated mean and standard deviation of the mean from pooled population of animals and used the log-rank test to assess differences in survival curves between pooled populations as previously described (Rea *et al.* 2007).

Developmental Analysis

Development was followed at 20°C on populations of synchronous animals from the time of hatching after a 4 hour synchronized egg lay, which was carried out directly on RNAi plates or EtBr plates. Images were collected 4 and 5 days after the time of egg lay for the parental and first generation animals, respectively. Live worm images, to record development, were captured with a digital camera connected to a dissecting microscope.

Microscopy

For fluorescence images, individual worms were randomly picked using a regular dissecting scope from a population of 200 worms for each condition. Remaining worms were then collected for western blotting or followed for lifespan analysis. Worms were arranged for image analysis on 2% agarose pads and Nomarski and fluorescence images were captured via a Zeiss Axioskop running SlideMaker 4.0 software retrofitted with deconvolution capabilities and a digital fluorescent camera containing a Sedat (Quad) filter set. GFP fluorescence was collected using a FITC 490/528nm absorption/emission filter pair (peak transmission with a 20/38 nm bandpass). All images were collected using identical exposure settings.

Quantitative RT-PCR

Quantitation of *egl-1* and *atp-3* mRNA in *frh-1* and *atp-3* RNAi-treated animals relative to vector-only treated animals was undertaken using primers specific for *egl-1* (Schumacher *et al.* 2005) and *atp-3* (Rea *et al.* 2007). Data was normalized to a non-variable control gene (*F23B2.13*). mRNA was extracted from approximately 5000 synchronized worms per sample, using a Total RNA and Protein Isolation Kit (Macherey-Nagel). Oligo(dT) primed cDNA was synthesized using a SuperScript cDNA Synthesis Kit (Invitrogen). Quantitative PCR (qPCR) was undertaken on an ABI Prism® 7000 instrument with SYBR Green PCR Master Mix kit (Applied Biosystems).

Western Blot Analysis

Western Blot analysis of whole-worm protein extracts were prepared from ~200 adult worms. Animals were boiled in 5% SDS, 0.02% β -mercaptoethanol and 1mM protease inhibitor cocktail (SIGMA P2714). Following protein quantitation (BCA, Pierce), protein levels were measured by western analysis, quantified using densitometry, and normalized against actin. Antibodies employed were anti-GFP (MSA02, MitoSciences, Oregon, 1:1000 dilution) and anti-Actin (AC-15, Sigma, 1:5000).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CEP	<i>C. elegans</i> p53
ETC	Electron Transport Chain
FRDA	Friedreich's Ataxia
Mit mutants	Mitochondrial mutants
NDUFS3	NADH Dehydrogenase [Ubiquinone] iron (Fe)-Sulfur protein 3
HMAD	Human Mitochondrial-Associated Disease
FRH	frataxin
<i>gst</i>	Gluthathione-S-Transferase
<i>hsp</i>	heat shock protein
SKN	Skinhead
Nrf	Nuclear factor erythroid-2 Related Factor
ARE	Antioxidant Responsive Element
<i>Daf</i>	Abnormal Dauer Formation
SIRT	sirtuins

FOXO	Forkhead boX
ATM	Ataxia Telangectasia Mutated
EGL	EGg Laying defective
ATP	adenosine triphosphate
CCO	Cytochrome C Oxidase
ISP	Iron-Sulfur Protein
EtBr	ethidium bromide
GLD	defective in Germ Line Development
AMPK	adenosine triphosphate (AMP)-activated protein kinase.

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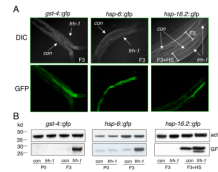


Figure 1. Frataxin suppression induces stress response genes

(A) Nomarski (DIC, top panels) and fluorescence (GFP, bottom panels) images of *gst-4*, *hsp-6* and *hsp-16.2* GFP reporter strains fed for three consecutive generations (F3) on vector control (con) or frataxin RNAi (*frh-1*). GFP expression reveals the induction of GST-4 and HSP-6 in the parental generation (P0) compare to the third generation (F3). For HSP16.2, GFP expression has been analyzed in animals cultured for three generations on *frh-1* RNAi (untreated F3) or after 12 hours recovery from a 1.5h heatshock (35°C) (F3+HS). (B) GFP induction of animals treated as in A was quantified by western blotting and normalized against actin. Shown are results from one experiment out of three performed with similar results.

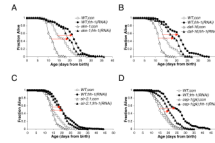


Figure 2. Loss of *cep-1*, but not *skn-1*, *daf-16* or *sir-2.1* KO, significantly shortened the increase in lifespan induced by *frh-1* RNAi

Survival analysis of different knock-out strains, *skn-1(zu67)*, *daf-16(mgDf50)*, *sir2.1(ok434)* and *cep-1(gk138)* carried out, along with their wild-type (WT) control strains, after feeding for three consecutive generations with vector alone (con) or RNAi targeting frataxin (*frh-1*). *frh-1* RNAi increased lifespan on wild-type strains (closed vs open circles) as well as on *skn-1(zu67)* (A), *daf-16(mgDf50)* (B) and *sir2.1(ok434)* (C) (closed vs open triangles). (D) *cep-1(gk138)* significantly shortened the increase in lifespan induced by *frh-1* RNAi (closed vs open triangles). Compare red arrows. Shown are cumulative data from at least 2 experiments.

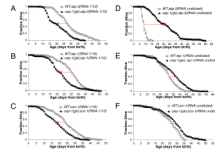


Figure 3. *cep-1* modulates lifespan in opposite directions depending on the level of mitochondrial protein suppression

Survival curves of wild-type (WT) and *cep-1(gk138)* animals fed bacteria containing RNAi against *atp-3* (A), *isp-1* (B) or *cco-1* (C) diluted 1/10 with empty vector-containing bacteria (*RNAi* 1/10); or RNAi against *atp-3* (D), *isp-1* (E) or *cco-1* (F) left undiluted (*RNAi* undiluted). Shown are cumulative data from at least 2 experiments.

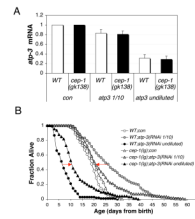


Figure 4. *cep-1(gk138)* effects on Mit mutant longevity are not due RNAi efficacy reduction, and phenocopy in *cep-1(lg12501)*

(A) Quantitation of *atp-3* mRNA levels in wild-type (WT) and *cep-1(gk138)* animals after growth on 1/10 diluted *atp-3* RNAi (*atp-3* 1/10) or undiluted *atp-3* RNAi (*atp-3* undiluted) relative to vector only treated animals (con). Samples have been normalized to a housekeeper gene (F23B12.3). Shown are the mean and SEM of three independent experiments. (B) Survival curves of wild-type (WT) and *cep-1(lg12501)* animals fed *atp-3* RNAi diluted 1/10 with vector –only bacteria, or left undiluted, and compared to vector-only fed animals (con). Shown are cumulative data from at least 2 experiments.

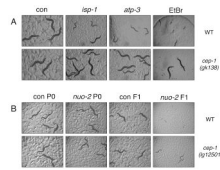


Figure 5. *cep-1* regulates animal development in response to severe mitochondrial disruption
(A) Digital images of four day old wild-type (WT) and *cep-1(gk138)* animals fed from the time of hatching with either empty vector (con), undiluted *isp-1* or *atp-3* RNAi, or EtBr (75 μ g/ml). **(B)** Digital images of five day old animals fed from the time of hatching with empty vector (con) or undiluted *nuo-2* RNAi ('P0' indicates starting generation animals, 'F1' specifies progeny animals derived from P0 hermaphrodites and maintained under the same feeding RNAi conditions).

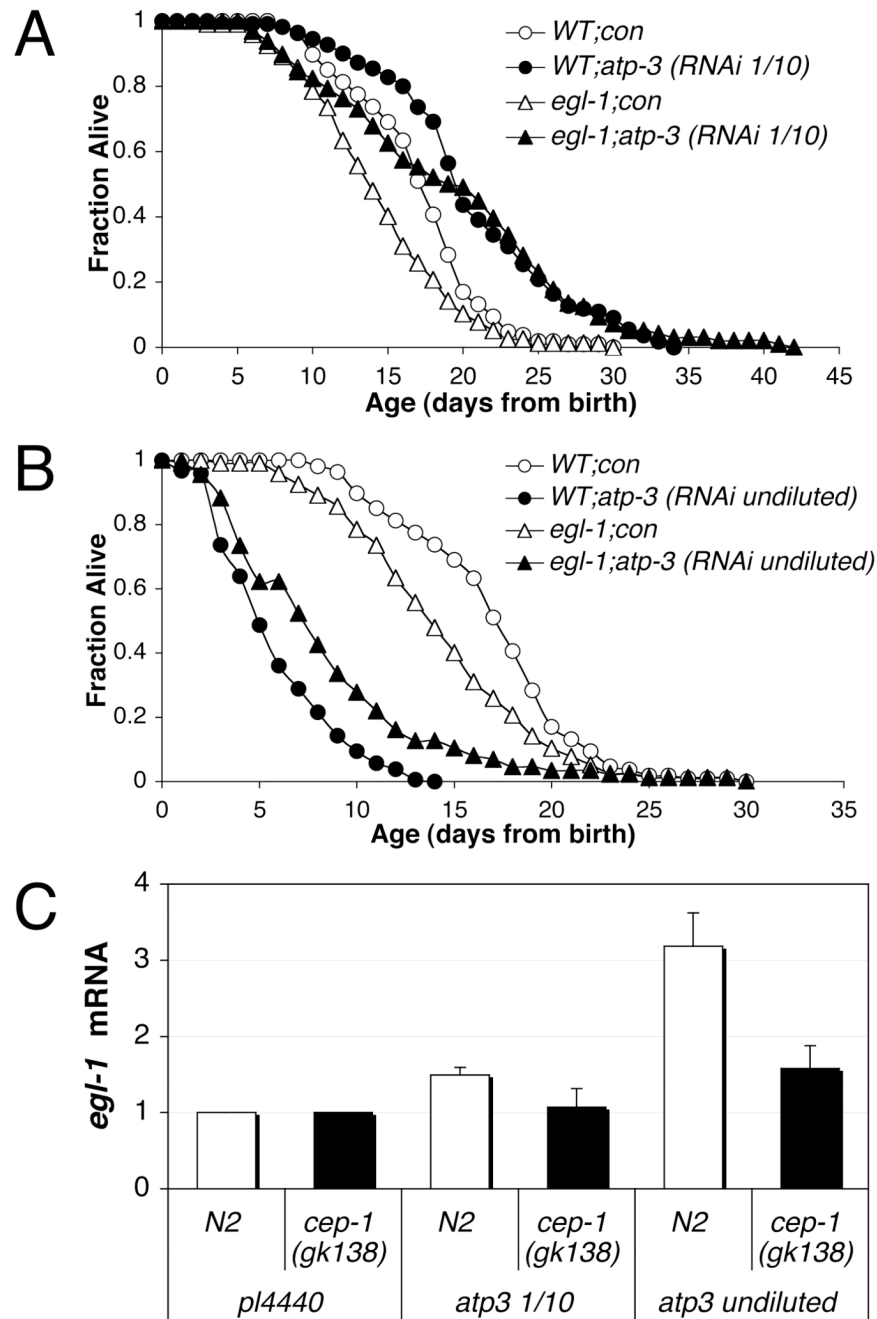


Figure 6. *egl-1* is a downstream target of *cep-1* that contributes to lifespan modulation after severe *atp-3* disruption

(A, B) Survival analysis of wild-type (WT) and *egl-1(n487)* animals fed *atp-3* RNAi diluted 1/10 with vector –only bacteria, or left undiluted, and compared to vector-only fed animals (con). Shown are cumulative data from 2 experiments. (C) Quantitative measurement of *egl-1* mRNA in wild-type (WT) and *cep-1 (gk138)* animals following growth on 1/10 diluted *atp-3* RNAi (*atp-3 1/10*) or *atp-3* RNAi left undiluted (*atp-3 undiluted*). Data is shown relative to vector-control fed animals (con) and is normalized to a housekeeping gene (F23B12.3). Shown are mean and SEM of three different experiments.

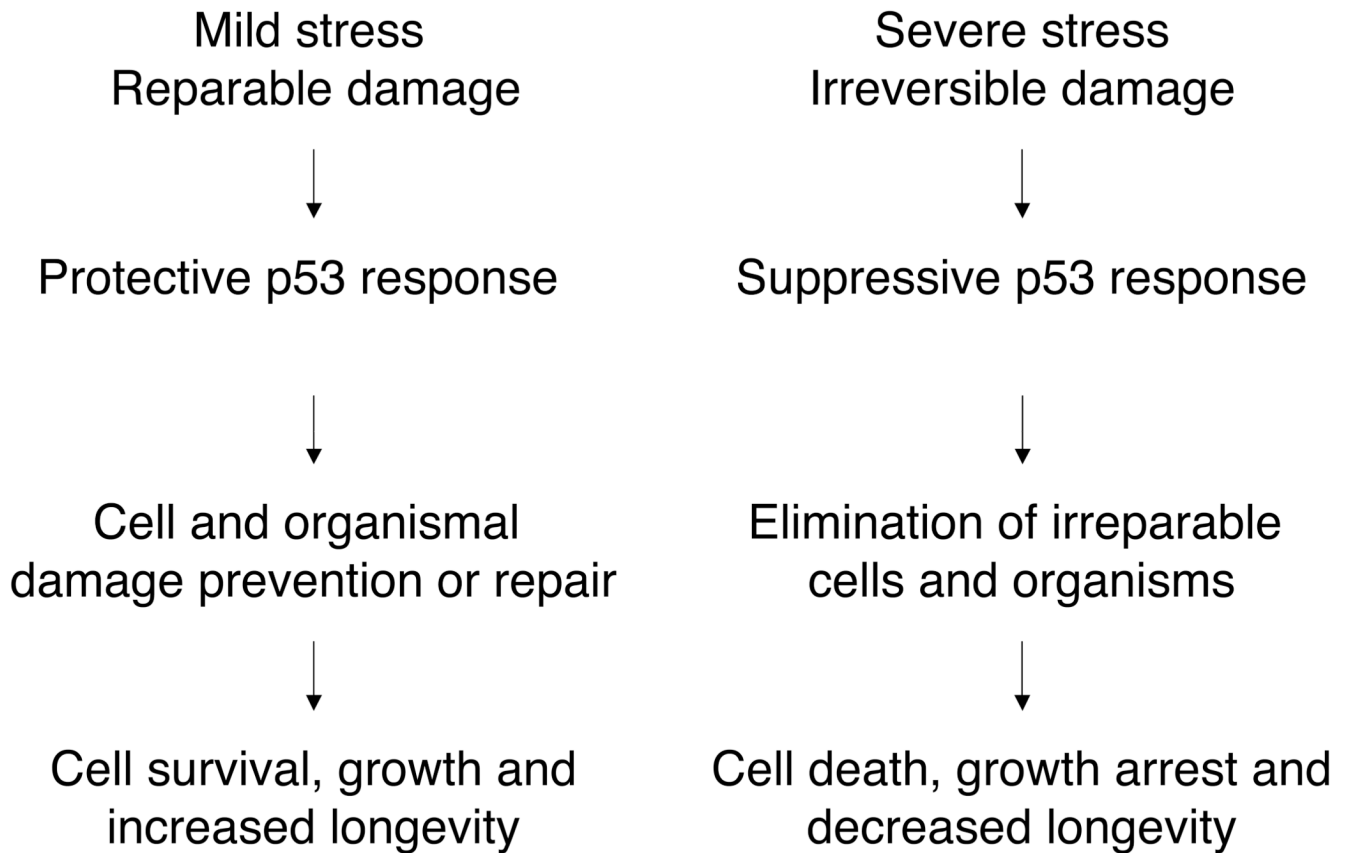


Figure 7. Evolutionary conserved functionality of Janus-faced p53/cep-1 in *C. elegans*
 Mild or transient cellular and organismal damage induces p53 protective pathways leading to damage repair, growth, survival and increased longevity. More severe or irreversible damage induces p53 suppressive responses that eliminate cells and irreversibly compromise the organism. Such drastic actions lead to growth arrest and/or decreased survival probability.

Table 1

Lifespan Analyses – Summary Statistics

Genotype	RNAi*	Mean LS	SEM	p vs con ^	p vs N2 ^	Sample size (censored)^^	Replicates (N)
Wild-type (N2)	con	15.9	0.2			393 (34)	5
<i>skr-1(zu67)</i>	<i>frrh-1</i>	20.4	0.3	4.4E-25		406 (44)	5
	con	12.3	0.3		8.4E-16	140 (20)	2
<i>gst-4::gfp</i>	<i>frrh-1</i>	20.2	0.5	2.5E-26	0.644	136 (18)	2
	con	18.1	0.4			165 (18)	2
<i>skr-1(zu67);</i>	con+O ₂	20.7	0.4	0.0034		181 (8)	2
	<i>frrh-1</i>	23.3	0.5	3.5E-11		125 (13)	2
<i>gst-4::gfp</i>	con	12.3	0.3			120 (20)	2
	con+O ₂	12.4	0.3	0.8		146 (18)	2
<i>age-1(hs546)</i>	<i>frrh-1</i>	20.1	0.5	9.3E-27		136 (18)	2
	<i>frrh-1</i> +O ₂ #	19.5	0.8	3.9E-14		76 (2)	1
<i>daf-16(mgD)50)</i>	con	11.3	0.2		3.39E-29	121 (2)	2
	<i>frrh-1</i>	16.8	0.8	4.9E-11	0.0002	65 (3)	1
<i>sirt2.1(ok434)</i>	con	22.2	1.1		5.5E-09	97 (10)	1
	<i>frrh-1</i>	31.3	0.9	1.0E-06	2.8E-26	96 (11)	1
<i>cep-1(gk138)BX10</i>	con	11.3	0.2		4.4E-21	121 (13)	2
	<i>frrh-1</i>	17.8	0.5	5.8E-19	0.003	124 (24)	2
<i>cep-1(lg12501)</i>	con	14.2	0.2		9.5E-13	178 (37)	2
	<i>frrh-1</i>	17.1	0.4	2.7E-07	0.005	191 (27)	2
<i>cep-1(gk138)BX10</i>	con	12.3	0.3		9.5E-09	81 (21)	1
	<i>frrh-1</i>	16.7	0.4	2.4E-11	0.25	80 (28)	1
<i>cep-1(lg12501)</i>	con	17.6	0.3		0.001	343 (130)	5
	<i>frrh-1</i>	19.1	0.3	2.2E-07	0.002	414 (45)	5
<i>cep-1(lg12501)</i>	con	17.8	0.4		5.5E-05	184 (27)	3

* Lifespan analyses were performed on 3rd generation worms cultured continuously on *frrh-1* RNAi. Unless specified, all other lifespan analyses were derived using worms cultured for a single generation on undiluted *isp-1*, *atp-3*, *cco-1* and *nuc-2* RNAi; O₂ is treatment with Hyperbaric Oxygen

^v *p*-values were calculated using the log-rank test

^w Pooled worms from N replicate experiments (censored individuals were included in the log rank analysis)

[#] *p*-values against *rhh-1* RNAi on the same strain is 0.9

^{BX10} indicates out-crossed ten times and refers to the *cep-1(gk138)* allele used in this work

Table II

Lifespan Analyses – Summary Statistics

Genotype	RNAi	Mean LS	SEM	p vs con [^]	p vs N2 [^]	Sample Size (censored) ^{^^}	Replicates (N)
Wild-type (N2)	con	15.9	0.2			393 (34)	5
	<i>atp-3</i> 1/10	28.5	0.8	2.8E-33		124 (14)	2
	<i>atp-3</i> 1/2	14.2	0.3	8.6E-05		157 (34)	2
	<i>atp-3</i>	8.4	0.2	4E-254		160 (55)	2
	<i>isp-1</i> 1/10	29.7	0.4	4.2E-91		375 (51)	5
	<i>isp-1</i>	25.3	0.4	2E-53		280 (29)	4
	<i>cco-1</i> 1/10	24.4	0.6	2.9E-30		181 (14)	3
	<i>cco-1</i>	24.9	0.7	2.5E-24		118 (23)	2
<i>cep-1(gk138)</i> ^{BX10}	con	17.6	0.3		0.0004	343 (130)	5
	<i>atp-3</i> 1/10	24.7	0.8	5.7E-13	0.008	118 (19)	2
	<i>atp-3</i> 1/2	20.3	0.8	6.8E-05	1.3E-09	160 (65)	2
	<i>atp-3</i>	25.7	0.9	8.6E-16	8E-256	160 (21)	2
	<i>isp-1</i> 1/10	22.7	0.5	6.4E-17	2.0E-14	339 (58)	5
	<i>isp-1</i>	29	0.6	1.1E-41	3.2E-10	282 (40)	4
	<i>cco-1</i> 1/10	18.3	0.6	0.005	2.3E-09	185 (23)	3
	<i>cco-1</i>	27	1	2.5E-18	0.009	119 (36)	2

[^] p -values were calculated using the log-rank test

^{^^} Pooled worms from N replicate experiments (censored individuals were included in the log rank analysis)

^{BX10} indicates out-crossed ten times and refer to the *cep-1(gk138)* allele used in this work

Table III

Lifespan Analyses – Summary Statistics

Genotype	RNAi	Mean LS	SEM	p vs con [^]	p vs N2 [^]	Sample Size (censored) ^{^^}	Replicates (N)
Wild-type (N2)	con	17	0.4			120 (10)	2
	<i>atp-3 1/10</i>	26.5	1	4.6E-14		121 (9)	2
	<i>atp-3</i>	7	0.4	4.2E-24		119 (9)	2
<i>cep-1(lg12501)</i>	con	19.1	0.5		0.006	124 (24)	2
	<i>atp-3 1/10</i>	22.9	0.9	0.02	0.02	124 (11)	2
	<i>atp-3</i>	12.3	0.7	4.0E-06	1.5E-08	117 (19)	2

[^] p-values were calculated using the log-rank test

^{^^} Pooled worms from N replicate experiments (censored individuals were included in the log rank analysis)

Table IV

Lifespan Analyses – Summary Statistics

Genotype	RNAi	Mean LS	SEM	p vs con [^]	p vs N2 [^]	Sample Size (censored) ^{^^}	Replicates (N)
Wild-type (N2)	con	16.3	0.3			241 (29)	4
	<i>fhh-1</i>	21.3	0.5	4.2E-15		234 (66)	4
<i>atp-3 1/10</i>	con	20.2	0.6	4.3E-09		119 (9)	2
	<i>atp-3</i>	5.3	0.3	1.2E-69		123 (12)	2
<i>atm-1(gk186)</i>	con	19.5	0.4		9.8E-12	237 (55)	4
	<i>fhh-1</i>	26.2	0.7	5.7E-13	2.6E-06	121 (25)	2
<i>atp-3 1/10</i>	con	25.5	0.9	2.5E-09	1.2E-06	121 (45)	2
	<i>atp-3</i>	5.1	0.3	4.4E-51	0.6	120 (28)	2
<i>egl-1(n487)</i>	con	14.6*	0.4		0.12	241 (74)	4
	<i>fhh-1</i>	25	0.9	1.1E-15	0.0007	120 (56)	2
<i>atp-3 1/10</i>	con	18.8	0.8	4.2E-06	0.9	115 (19)	2
	<i>atp-3</i>	7.7	0.5	1.1E-16	0.0002	115 (21)	2

[^] p-values were calculated using the log-rank test^{^^} Pooled worms from N replicate experiments (censored individuals were included in the log rank analysis)

* Mean LS is significantly shorter compared to N2, t-test = 0.002