TRANSCRIPTIONAL NETWORK REGULATED BY *CAENORHABDITIS ELEGANS* TRANSCRIPTION FACTORS CEH-23 AND CEP-1 MODULATES LIFESPAN THROUGH AMPK SIGNALINGS WHEN MITOCHODNRIAL ELECTRON TRANSPORT CHAIN FUNCTION IS IMPAIRED

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Abstract

TRANSCRIPTIONAL NETWORK REGULATED BY *CAENORHABDITIS ELEGANS* TRANSCRIPTION FACTORS CEH-23 AND CEP-1, MODULATES LIFESPAN THROUGH AMPK SIGNALINGS WHEN MITOCHODNRIAL ELECTRON TRANSPORT CHAIN FUNCTION IS IMPAIRED

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The global population is aging rapidly due to advances in technology and improvements in public health. Furthering knowledge about the aging process will allow discoveries of interventions and treatment to promote healthy aging that will hopefully, in turn, grant individuals access to a better quality of life during their later years. Several longevity modulators have been identified in various model organisms, and many of them seem to be conserved. One example of such a longevity modulator is the mitochondrial electron transport chain (ETC) function.

Mitochondrial ETC function has long been associated with aging and lifespan determination, where moderately reduced mitochondrial ETC function extends the lifespan of various organisms, suggesting that the pro-longevity effects of ETC function are well-conserved. The detailed mechanism of how mitochondrial function affects organismal lifespan remains unknown. Several transcription factors have been shown to promote lifespan when mitochondrial ETC function is impaired, suggesting that ETC dysfunction may induce a transcriptional network reprogramming response. Whether these transcription factors collaborate to modulate lifespan has not been fully explored. My thesis work shows that transcription factors CEH-23 and

p53/CEP-1 likely collaborate to modulate lifespan under ETC stress as these transcription factors shows an epistatic relationship in their longevity effects. Consistently, microarray analyses revealed that CEH-23 and CEP-1 share a significant overlap in their transcriptional outputs, which are enriched in kinases and phosphatases. Intriguingly, the majority of CEH-23 and CEP-1 common targets are also regulated by AMP-activated protein kinase, suggesting that these two transcription factors engage AMPK signaling to modulate the lifespan of animals with impaired ETC. This study links the transcriptional response to the cell signaling response in organisms with impaired mitochondrial ETC, which provides important insights into how mitochondria function to modulate organismal lifespan.

BIOGRAPHICAL SKETCH

Hsin –Wen (Ella) Chang grew up in Tainan city, which is the ancient capital of the Taiwan. Growing up in a loving family with parents who encourage their children to explore different interests. Ella's interests toward biological science sprout in her earlier childhood, as she was hanging out at the histology lab in a hospital where her mother worked. She was fascinated by how one could freeze the human tissue and sliced it into thin layers, which allow the tissues to be visualized under the microscope and help the doctors to diagnose for diseases. Ella moved to the Seattle, WA when her mother decided to accept a job there. During this time, she developed her passion in science and coffee. During her undergraduate years at University of Washington, Ella took the opportunity to conduct the undergraduate research at Elton Ted Young's lab, at which she was studying transcription regulation in budding yeast. The working environment of the Young lab is very friendly and encouraging, which sparked Ella's interests in doing research as her career. Entered college as a pre-med student, the experience at the Young lab really blew her mind so she decided to pursue a career in research instead. In 2007, Ella graduated from the University of Washington with a BS in Biochemistry and a BS in Microbiology. After staying in the Young working as a full time research technician, Ella found herself ready for the next challenge and came to Cornell University to study aging in Dr. Sylvia Lee's lab.

The time at Cornell wasn't easy at first, due to the freezing cold winter in the upstate New York. However, the stimulating academic environment, friendly colleagues and friends, and the beautiful Cornell campus quickly made Ella found Ithaca home. During her time at Cornell, while learning how to be a good scientist, Ella also developed several interests outside of the lab, such as cooking, baking and running. In her last year of graduate school, she ran her first half

marathon, which is very similar to her journey in the graduate school: both are very long, tough, but very rewarding toward the end. After studying aging for six years, Ella found her new passion in science, microbiota and its effects on host biology. The knowledge and skills that she acquired from her research experience in the past six year will definitely equipped her to continue her journey to become independent scientist.

DEDICATION

To my parents, Chia-Chun Chang and Shu-Er Lee

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This work won't be possible without helps and supports from many people. I would like to first express my gratitude to my mentor, Dr. Sylvia Lee. Sylvia is an outstanding mentor, who is extremely supportive and invested a lot of her time and efforts to help me develop into a more independent research scientist. I am extremely thankful that she has been very patient and understanding even in the days when the project did not go well. Sylvia has demonstrated what a good scientist should be: think critically, be collaborative and never oversell the data. Sylvia not only set up a good role model as a great scientist, she has also demonstrated that a female scientist can have a well-balanced life between her career and family.

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ABBREVIATIONS

ETC	Electron Transport Chain
CEH-23	*C. elegans* Homeobox
CEP-1	*C. elegans* P53-like protein
AAK-2	AMP-Activated Kinase (catalytic subunit)
AAK-2 ca	Constitutively active AAK-2
AMPK	AMP-activated protein kinases
ISP-1	Iron-Sulfur Protein
NUO-6	NADH Ubiquinone Oxidoreductase
CLK-1	Demethoxyubiquinone (DMQ) Hydroxylase
MEV-1	Succinate Dehydrogenase Cytochrome b560 Subunit
GAS-1	Mitochondrial complex I subunit
FTN-1	Ferritin
TTBK-2	Tau Tublin Kinase
GFP	Green Fluorescent Protein
PQ	Paraquat

CHAPTER 1

INTRODUCTION[1]

Mitochondria are essential for bioenergetics and metabolism and are central to cell viability and survival. A major function of mitochondria is oxidative phosphorylation and ATP production, which occurs through a series of electron transferring reactions via the electron transport chain (ETC) located in the mitochondrial inner membrane. Mitochondria are also major sites of several key processes, including beta-oxidation, the tricarboxylic acid cycle, and apoptosis regulation. As a result, mitochondrial function is essential for maintaining cellular homeostasis and survival.

Mitochondrial function has long been linked to aging, and mitochondrial oxidative phosphorylation declines with age in diverse organisms (Pulliam et al., 2012). During oxidative phosphorylation, electrons can leave the ETC and react with oxygen prematurely in the mitochondria to produce toxic reactive oxygen species (ROS). In 1972, Harman proposed the "mitochondrial theory of aging", which suggests that ROS produced from normal mitochondrial metabolism can cause minor cellular damage, and their accumulation over time drives physiological function decline with age (Beckman and Ames, 1998; Harman, 1972). The mitochondrial theory of aging has been well accepted until recent years when cumulative observations from various model organisms started to challenge this theory. For example, inactivation of one of the five *C. elegans* mitochondrial superoxide dismutases, *sod-2*, which normally acts to detoxify

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superoxide, actually prolongs lifespan (Van Raamsdonk and Hekimi, 2009). Moreover, mild mitochondrial dysfunction has been shown to promote longevity from yeast to mammals (Copeland et al., 2009; Dell'agnello et al., 2007; Dillin et al., 2002; Kirchman et al., 1999; Lee et al., 2003; Liu et al., 2005; Van Raamsdonk and Hekimi, 2009; Wong et al., 1994). Together, these observations suggest that mitochondria influence the longevity of an organism in a more complex way than just via the production of toxic ROS molecules.

The mitochondrial ETC consists of five complexes, which are located in the mitochondrial inner membrane, and their perturbation has been shown to have disparate effects on animal longevity. For example, specific point mutations in several mitochondrial ETC subunits extend lifespan (Feng et al., 2001; Yang and Hekimi, 2010a). Similarly, genome-wide RNAi screens in *C. elegans* uncovered many mitochondrial ETC subunits that extend lifespan when attenuated (Chen et al., 2007; Curran and Ruvkun, 2007; Dillin et al., 2002; Lee et al., 2003). However, *C. elegans* with mutations in some ETC subunits can also exhibit shorter lifespans (Hartman et al., 2001). A threshold model has been proposed to explain the differential longevity effects of different ETC mutations. The model suggests that reducing ETC function up to a certain threshold can be beneficial in some aspects and extend organismal lifespan. However, when ETC function is reduced below this threshold, it is detrimental and shortens the lifespan of an organism (Van Raamsdonk and Hekimi, 2009). This model is further supported by RNAi experiments where different levels of mitochondrial inhibition were achieved by exposing *C. elegans* to different concentrations of siRNA against

specific mitochondrial subunits; optimal longevity was caused by intermediate levels of mitochondrial inhibition, whereas a high level of RNAi-mediated inhibition shortened lifespan (Rea et al., 2007). It is interesting to note that in addition to lifespan changes, *C. elegans* with mutations in or RNAi inhibition of ETC subunits often exhibit other physiological defects, including slower development, slower feeding and defecation rates, and a reduced brood size. Therefore, lifespan extension via mitochondrial dysfunction in *C. elegans* has a physiological cost.

Similar findings in other organisms support a conserved role for mitochondrial ETC dysfunction in longevity modulation. RNAi inhibition of many different ETC subunits in D. melanogaster robustly extend lifespan with little pleiotropy (Copeland et al., 2009). Additionally, two mouse mutants with compromised mitochondrial ETC function live longer (Dell'agnello et al., 2007; Liu et al., 2005). Although the lifespan extension associated with mitochondrial ETC dysfunction is well conserved, the underlying mechanisms are not well understood. This review is primarily focusing on the work that has been performed in *C. elegans* to elucidate these mechanisms, as *C. elegans* has been a leading and robust model system for studying aging and mitochondrial dysfunction. One way cells cope with mitochondrial dysfunction is through a retrograde transcriptional reprograming response, which has been extensively studied in yeast (Jazwinski and Kriete, 2012; Sekito et al., 2000). Activation of retrograde signaling has been shown to extend replicative lifespan and delay senescence in yeast, and parallel mitochondria-to-nucleus signaling pathways also exist in *C. elegans* (Cristina et al., 2009; Kirchman et al., 1999). Various longevity phenotypes observed in the different *C.*

elegans ETC mutants has been thoroughly reviewed (Dancy et al., 2014; Munkácsy and Rea, 2014). The relationship between mitochondrial dysfunction and longevity and the roles of several key transcription factors participate in this retrograde signaling are outlined in the followings.

1.1 *C. elegans* mitochondrial ETC mutants exhibit altered lifespans

Several mutations in different ETC complexes have been isolated in *C. elegans*, and these mutants exhibit differential lifespans. Therefore, these mutants represent powerful tools for understanding how mitochondrial function regulates organismal lifespan. The *clk-1* mutant was the first long-lived mutant to be isolated. Besides a longer lifespan, *clk-1* mutants exhibit a slower developmental rate, longer defecation cycle, and reduced brood size (Felkai et al., 1999). The *clk-1* gene encodes a hydroxylase that is required for ubiquinone biosynthesis. During oxidative phosphorylation, ubiquinone transfers an electron from complexes I and II to complex III in the ETC. Therefore, *clk-1* mutation does not directly affect mitochondrial ETC complexes but rather the electron transfer efficiency during oxidative phosphorylation. Indeed, Felkai et al. found that electron transport was reduced in mitochondria isolated from *clk-1* animals. Consistently, *clk-1* overexpression increased mitochondrial activity and shortened lifespan (Felkai et al., 1999). Excitingly, the role of *clk-1* in longevity is conserved, as heterozygous *clk-1*/MCLK1 mutant mice also exhibit a longer lifespan and attenuated aging phenotypes (Lapointe et al., 2009; Liu et al., 2005).

Two independent genetic screens that aimed to uncover mutants with phenotypes similar to the *clk-1* mutant identified two additional long-lived ETC mutants, nuo-6 and *isp-1* (Feng et al., 2001; Yang and Hekimi, 2010a). The *isp-1* gene encodes the Rieske iron sulfur protein and is an ETC complex III subunit, and nuo-6 encodes a conserved subunit of mitochondrial ETC complex I. Both mutations confer a robustly long life in *C. elegans* when mutant (Feng et al., 2001). Similar to *clk-1* mutants, both *isp-1* and nuo-6 mutant animals develop slower and exhibit reduced reproductive capacity and other behavioral phenotypes. Since all three of these mutations affect mitochondrial ETC function, it is not surprising that these ETC mutants consume less oxygen. However, while *isp-1* mutants exhibit similar ATP levels to wildtype, both *clk-1* and nuo-6 mutants actually possess increased ATP levels (Braeckman et al., 1999; Yang and Hekimi, 2010a). Additionally, the mechanisms that mediate the long lifespan of these ETC mutants appear to be distinct. For example, both nuo-6 and *isp-1* mutants have slightly elevated mitochondrial superoxide, but *clk-1* mutants have wildtype levels. Moreover, the antioxidant N-acetyl-cysteine (NAC) can suppress the lifespan extension of both nuo-6 and *isp-1* mutants but not of *clk-1* mutants. Together these observation suggest that the long-lived phenotypes of nuo-6 and *isp-1* mutants depend on mitochondrial superoxide, whereas the longevity of *clk-1* mutants might be independent of mitochondrial superoxide (Yang and Hekimi, 2010b).

As briefly mentioned in the introduction, not all mitochondrial ETC mutations promote longevity. In *C. elegans*, two mutations in mitochondrial ETC components, *gas-1* and *mev-1*, shorten lifespan. The *gas-1* gene encodes a conserved iron protein

subunit of complex I of the ETC, and *mev-1* encodes succinate dehydrogenase subunit c, which is part of ETC complex II. Similar to the abovementioned long-lived ETC mutants, these short-lived mutants develop slowly and exhibit reproductive defects and slow-behavior phenotypes. The *gas-1* and *mev-1* mutations reduce complex I and complex II activity, respectively, but retain wildtype ATP levels, suggesting that *mev-1* mutants either consume less energy or exhibit increased complex I activity to compensate for the complex II defect (Kayser et al., 2004; Senoo-Matsuda et al., 2001). The short lifespan of *mev-1* mutants has been proposed to be caused by increased oxidative stress, as *mev-1* mutants possess higher mitochondrial superoxide (Kondo et al., 2005; Senoo-Matsuda et al., 2001), and superoxide can be produced by complex II of the ETC (Ishii et al., 2004; Senoo-Matsuda et al., 2001). These observations suggest that the *mev-1* mutation may directly increase mitochondrial superoxide. Consistently, as a consequence of increased mitochondrial superoxide, more oxidative damage to proteins has been observed in *gas-1* and *mev-1* mutants (Dingley et al., 2010; Kayser et al., 2004).

Despite the many phenotypic similarities between *gas-1* and *mev-1* mutants, each mutant possesses some distinct characteristics. For example, *gas-1* mutants exhibit a decreased mitochondrial membrane potential and reduced mitochondrial density, suggesting that the short life of these mutants may be due to severely reduced mitochondrial ETC function (Dingley et al., 2010). Indeed, Pujol et al. demonstrated that the *gas-1* mutation caused an over compensation of complex II, which destabilized complex I and thus limited *gas-1* mutant lifespan (Pujol et al., 2013). It is therefore

interesting to speculate that in *mev-1* mutants, as in *gas-1*, a compensatory upregulation of complex I or complex III activity might ensue, as RNAi knock down of the ETC complex III component cyc-1 partially restored the lifespan of these animals (Cristina et al., 2009). In addition to increased oxidative damage, developing *mev-1* mutant embryos possess more apoptotic cells and fail to upregulate the anti-apoptotic gene *ced-9* under hypoxia (Senoo-Matsuda et al., 2003). Dysregulation of apoptosis could be a cause of the short life of these mutants. Indeed, deleting the pro-apoptotic gene *ced-3* restores *mev-1* mutant longevity (Senoo-Matsuda et al., 2003). However, adult *mev-1* mutants do not have altered apoptosis (Baruah et al., 2014).

As elaborated above, the different *C. elegans* mitochondrial ETC mutants exhibit distinct lifespans. These mitochondrial ETC mutants share some similar characteristics but each has their own unique properties. To date, three genome-wide studies have surveyed the transcriptional changes in response to nuo-6, *isp-1*, *clk-1*, *gas-1*, and *mev-1* mutations (Cristina et al., 2009; Falk et al., 2008; Yee et al., 2014). These investigations revealed that a compensatory transcriptional response likely plays an important role in the longevity of these mutants. Interestingly, different transcriptional changes appear to respond to distinct ETC perturbations even when longevity outcomes are similar. For example, microarray analyses indicate that the long-lived *isp-1* and *clk-1* mutants share some similarities in gene expression patterns while there are also unique gene expression pattern associate with each ETC dysfunctions(Cristina et al., 2009). The genes fstr-1/2 (F57F4.3/F57F4.1) showed expression changes only in the *clk-1* mutant but not in *isp-1* mutant, and fstr-1/2 RNAi suppressed the long life of the *clk-1*

mutant but not of the *isp-1* mutant (Cristina et al., 2009). The comparison of gene expression profiles between *isp-1* and nuo-6 mutants revealed a significant overlap between these mutants suggesting a common mechanism regulates their lifespans. Consistent with the requirement of mitochondrial ROS signaling in mediating the lifespans of *isp-1* and nuo-6, many genes that display expression changes in these mutants have also been shown to respond to pro-longevity doses of ROS (Yee et al., 2014). On the contrary, the short-lived *mev-1* and *gas-1* mutants also exhibit differential gene expression patterns. These data indicate that different disruptive ETC mutations can induce distinct transcriptional responses with unique physiological consequences (Falk et al., 2008). Next, several transcription factors currently known to engage in compensatory transcriptional responses in the various mitochondrial ETC mutants are described in the following.

1.2 HIF-1: hypoxia inducing factor

C. elegans hif-1 encodes the mammalian HIF-1α ortholog, which is a subunit of the HIF-1 transcription factor complex that responds to reduced oxygen levels in the environment. It is important to note that wild *C. elegans* live in soil, which provides a lower oxygen environment than the atmosphere. Living in such hypoxic conditions has likely driven *C. elegans* to become tolerant of a wide range of oxygen levels, from 0% to 60% (normoxia is 10-21% oxygen). Although *C. elegans* can survive in a wide oxygen range, oxygen levels can modulate lifespan, as worms grown in hypoxia live longer, and worms grown in high oxygen exhibit shortened lifespans (Honda et al., 1993). Interestingly, hif-1 mutant animals live longer when grown at 25°C. However, hif-1

mutants exhibit a normal lifespan when grown at lower temperatures suggesting that hif-1 modulates longevity in response to specific environmental cues (Leiser et al., 2011; Mehta et al., 2009).

It is possible that a *C. elegans*-specific pathway evolved to allow these animals to adapt to a lower oxygen environment. ETC dysfunction might create a stress similar to hypoxia; therefore, dissecting the physiological and metabolic consequences of hypoxia and ETC dysfunction may further our understanding of how the mitochondrial ETC mediates longevity. The link between hypoxia signaling and mitochondrial ETC mutant longevity has been extensively explored in *C. elegans*. Several mitochondrial ETC mutants are more resistant to chronic oxygen deprivation (Butler et al., 2010), suggesting that a hypoxia signaling response is activated in the ETC mutants. HIF-1 activity is upregulated in *isp-1* and *clk-1* mutants, and hif-1 is required for their lifespan extension. Moreover, knocking down several mitochondrial ETC components using RNAi activated the HIF-1 target, nhr-57, indicative of HIF-1 activity (Lee et al., 2010). Additionally, stabilizing HIF-1 by inhibiting its negative regulators vhl-1 and egl-9 using RNAi extended the lifespan of wildtype worms but not of the ETC mutants. Therefore, mitochondrial dysfunction-induced activation of HIF-1 activity contributes to the long life of ETC mutants (Lee et al., 2010).

Under hypoxia, where oxygen is lacking, HIF-1α cannot be hydroxylated by EGL-9, which blocks the subsequent ubiquitination by VHL-1 and therefore remains stable. Consistent with a mitochondrial dysfunction-induced role for HIF-1, it can also be activated and stabilized by ROS in *C. elegans* (Lee et al., 2010). The link between ROS

and HIF-1 is conserved in mammals, as human cells exhibit increased ROS levels in response to hypoxia (Chandel et al., 1998; Vanden Hoek, 1998; Waypa, 2002). Some ETC mutants, such as *isp-1* and nuo-6, possess higher mitochondrial superoxide, and this increase is required for their lifespan extension (Yang and Hekimi, 2010b). Increased ROS in the ETC mutants is proposed to stabilize HIF-1 under normoxia. This ROS-mediated HIF-1 stabilization might be explained by changes in the redox state of free iron in the cell. Increased cellular ROS oxidizes Fe2+ to Fe3+ in mammalian cells, which deactivates HIF prolyl hydroxylase activity and thus blocks the degradation of HIF-1α (Gerald et al., 2004). A recent study also suggested that HIF-1 has a direct role in enforcing ROS production in the ETC mutants, which is necessary for promoting their lifespan (Hwang et al., 2014).

As HIF-1 is a transcription factor, its effect on longevity is likely mediated through its transcriptional targets. Shen et al. identified HIF-1 hypoxia-responsive targets by global gene expression profiling using microarrays (Shen et al., 2005). Sixty-three of these targets are predicted to participate in signal transduction, metabolism, transport, and extracellular matrix remodeling. These data support the idea that HIF-1 helps the cell to survive under hypoxia by altering the metabolism and other crucial cellular processes. ETC dysfunction caused by *isp-1* or *clk-1* mutations and hypoxia were shown to induce not completely overlapping HIF-1 dependent transcriptional responses in *C. elegans* (Lee et al., 2010). Consistent to this notion, genome wide study revealed that HIF-1 regulated a distinct transcriptional response in the ETC mutants compared to that induced under hypoxia stress(Hwang et al., 2014). In the *isp-1* mutant, HIF-1 might

amplify the ROS signal by suppressing the expression of the free iron chelator, *ftn-1* and activating the free iron transporter, *smf-3* (Hwang et al., 2014). A more thorough comparison of HIF-1 targets under hypoxia and ETC dysfunction will provide important insights into how HIF-1 modulates lifespan under these stresses.

1.3 SKN-1: *C. elegans* Nrf

C. elegans ortholog NF-E2-related factor (Nrf), *skn-1*, has also been implicated as a longevity regulator, as it is required to maintain the normal lifespan for *C. elegans* (An and Blackwell, 2003). Similar to HIF-1, SKN-1 can be activated by ROS and is necessary for the lifespan extension in response to transient increased ROS levels. For example, a low-dose arsenite extends lifespan due to transient induction of ROS, and Schmeisser et al. demonstrated that SKN-1 is required for this extended lifespan (Schmeisser et al., 2013a). Since the mitochondrial ETC is one of the major sites for ROS production, ETC dysfunctions often perturb ROS production. For example, both long-lived ETC mutants *isp-1* and nuo-6 have increased mitochondrial ROS that serve as an important signal to promote organismal longevity (Yang and Hekimi, 2010b). Likewise, inhibiting the ETC complex I in *C. elegans* using chemicals also yields a ROS-dependent lifespan extension (Schmeisser et al., 2013b). Schmeisser et al. further demonstrated that this lifespan extension also requires active neuronal SKN-1. Together, these data suggest that SKN-1 likely regulates lifespan in response to reduced mitochondrial function. The role of SKN-1 in longevity in response to specific defects in ETC is less defined due to a collection of inconsistent data across different studies. Both Rea et al. and Tullet et al. provided evidence that SKN-1 is not required for the lifespan

extension observed when ETC components are knocked down with RNAi (Rea et al., 2007; Tullet et al., 2008). However, Park et al. argued that knocking down *skn-1* by RNAi partially suppressed the extended lifespan of *clk-1* mutants, suggesting an inhibitory role for SKN-1 in longevity (Park et al., 2010) This discrepancy further highlights the hypothesis that defects in different parts of the mitochondrial ETC could be distinct.

SKN-1 is a well conserved transcription factor and participates in many different biological processes, including embryonic development, stress responses, and normal lifespan (An and Blackwell, 2003; Maduro et al., 2007; Paek et al., 2012; Tullet et al., 2008). SKN-1 activity in response to the environmental and physiological cues is tightly regulated. In mammals, Nrf activity is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1), which sequesters Nrf in the cytoplasm and prepares it for subsequent degradation. Although a clear Keap ortholog in *C. elegans* has not been identified, *C. elegans* WDR-23 shares a similar function with Keap, as it also negatively regulates SKN-1(Choe et al., 2009). Interestingly, Paek and colleagues discovered that a pool of SKN-1 protein associates with the mitochondrial outer membrane and represents another mechanism that sequesters SKN-1 from the nucleus. When this interaction between SKN-1 and the mitochondrial outer membrane is disrupted, SKN-1 is constitutively active (Paek et al., 2012). This finding provides a link between mitochondria and SKN-1 regulation and hints at a possibility that mitochondrial dysfunction might affect SKN-1 activity. Moreover, SKN-1 has been demonstrated to be the downstream target of two MAPKs, PMK-1/P38 and MPK-1/ERK (Inoue et al., 2005;

Okuyama et al., 2010). As the activation of the MAPK pathway relies on ATP availability, reduced mitochondrial ETC function likely affects SKN-1 activation through altering the phosphate metabolism balance. Together, current data suggest that mitochondrial function likely regulates the activity of SKN-1. However, further investigation is needed to strengthen the regulatory link between mitochondrial ETC function and SKN-1 activity. As mentioned above, SKN-1 is a transcription factor with many complex roles. The transcriptional targets of SKN-1 in response to oxidative stress, reduced insulin-like pathway signaling, and during development have already been identified (Celniker et al., 2009; Oliveira et al., 2009; Tullet et al., 2008). Identifying SKN-1 targets in response to ETC dysfunction will enhance our understanding of how this transcription factor helps the organism cope with mitochondrial dysfunction.

1.4 CEP-1: *C. elegans* p53

The sole p53 homolog in *C. elegans*, *cep-1*, has also been demonstrated to mediate the lifespans of ETC mutants. p53 is a major tumor suppressor with a variety of conserved roles, including its well-characterized pro-apoptotic function. Abrogation of *cep-1* on its own has been shown to slightly increase worm lifespan (Arum and Johnson, 2007), and *cep-1* transcripts actually decrease over time in wildtype aging animals, (McGee et al., 2012), so the relationship between the absence of *cep-1* and longevity is unclear. Ventura et al. first observed that *cep-1* was required for the different lifespan outcomes of animals that exhibited varying degrees of mitochondrial dysfunction. A mild RNAi inhibition of several mitochondrial components, *atp-3*, *cco-1*, and *isp-1*, prolonged the lifespan of wildtype worms, which was abrogated upon *cep-1* mutation. Conversely,

cep-1 mutation increased the lifespan of animals that lived shorter lives when these same components were more severely knocked down using more concentrated RNAi (Ventura et al., 2010). Recently, it has been demonstrated that *cep-1* mutation decreased the lifespan of the long-lived ETC mutants *isp-1* and nuo-6 but increased the lifespan of the short-lived ETC mutants *mev-1* and *gas-1* (Baruah et al., 2014); detailed in Chapter 2 of this thesis. These results harken back to the observations by Ventura et al. (2010) that suggested that *cep-1* responds to different mitochondrial stress levels in disparate ways. To identify mediators of these distinct responses, the CEP-1-regulated transcriptional profiles of *isp-1* and *mev-1* mutants were compared, which surprisingly yielded a large overlap (Baruah et al., 2014). However, a closer analysis revealed that a small subset of genes were, in fact, differentially regulated between these two mutant backgrounds. As this group was enriched for the "aging" Gene Ontology term, suggesting that this analysis successfully identified genes with functions important for longevity. We confirmed that one of these candidates, the iron transporter ferritin (*ftn-1*), indeed exhibited different CEP-1-mediated regulation between the long-lived and short-lived mutants. Importantly, RNAi-mediated knockdown of *ftn-1* & ftn-2 (a close homolog of *ftn-1*) only partially attenuated the lifespan of *isp-1* mutants but had no effect on *mev-1* lifespan, indicating that *cep-1* mediates *isp-1* lifespan uniquely via *ftn-1* regulation and likely additional pathway (Baruah et al., 2014).

Iron homeostasis may represent an important CEP-1-mediated process that potentiates longevity. Neither knockdown or overexpression of *ftn-1* in wildtype animals alters lifespan (Baruah et al., 2014; Valentini et al., 2012), so its partial suppression of

the long lifespan of *isp-1* animals may be due to the already iron-sensitized *isp-1* mutant background, as *ISP-1* is a Rieske iron sulfur protein. Interestingly, RNAi-mediated knockdown of the nuclear-encoded mitochondrial protein frataxin (frh-1) alone extends the lifespan of wildtype animals (Ventura et al., 2005). Frataxin mediates iron–sulfur cluster formation as well as mitochondrial iron bioavailability and defective frataxin function is the main cause of Friedreich's ataxia in humans. Notably, *cep-1* mutation can partially suppress the longer lifespan of animals treated with frh-1 RNAi (Ventura et al., 2010), which may indicate that CEP-1 responds to iron homeostasis distress and that this response promotes longevity in an iron-stressed environment. This hypothesis is consistent with the observation that *cep-1* is required for the induction of *ftn-1* expression upon iron stress (i.e., in *isp-1* mutants) but *cep-1* mutants do not affect *ftn-1* expression in the absence of iron stress (i.e., in *mev-1* mutants) (Baruah et al., 2014). Analysis of genes regulating processes other than iron homeostasis that were differentially changed between the CEP-1-regulated transcriptional profiles of *isp-1* and *mev-1* mutants should uncover aging-related functions for those genes.

In addition to studying CEP-1 targets and their impact on longevity, understanding what occurs upstream of CEP-1 could also uncover how CEP-1 potentiates distinct lifespan outcomes in response to mitochondrial dysfunction. Genes that encode proteins that participate in ROS-generating or ROS-responsive mechanisms represent particularly good candidates for further investigation, as ROS are well-known activators of p53 (Liu et al., 2008). ROS can activate p53 indirectly via oxygen radical-induced DNA damage that in turn unleashes p53-mediated pro-

apoptosis or pro-antioxidant responses. ROS can also regulate p53 activity directly by interacting with p53's redox-sensitive Cysteine residues. These residues reside in p53's DNA-binding domain and affect the ability of p53 to specifically recognize the consensus sequence in its targets (Liu et al., 2008). Importantly, the residues that govern DNA binding are conserved between p53 and CEP-1 (Huyen et al., 2004). While several mitochondrial ETC mutants are known producers of ROS, and therefore p53/CEP-1's role in mediating their lifespans may not be surprising, how CEP-1 responds to other ETC mutants to yield disparate lifespan outcomes remains to be clarified.

1.5 CEH-23: *C. elegans* homeobox protein

Previous works from our lab identified *ceh-23* through an RNAi screen for suppressors of the long lifespan of *isp-1*. Inactivating *ceh-23* by mutation or RNAi partially suppresses the long-lived phenotype of *isp-1* mutant animals but does not shorten the lifespan of wildtype worms or other mutants that live longer due to perturbations distinct from the mitochondrial ETC. Therefore, *ceh-23* mediates part of the lifespan extension in ETC mutants, but its absence does not affect the general health of the organism. More interestingly, inactivating *ceh-23* exclusively shortened the lifespan of ETC mutants without affecting their developmental or reproductive phenotypes (Walter et al., 2011). Thus, *ceh-23* activity uncouples longevity from the other pleiotropic phenotypes associated with mitochondrial ETC inhibition. This observation provides hope for identifying mechanisms specific to the longevity role of the mitochondrial ETC that are distinct from its essential energetic role. Consistent with

the role of CEH-23 in longevity, overexpression of *ceh-23* is sufficient to prolong lifespan in wildtype worms (Walter et al., 2011).

The CEH-23 protein contains a highly conserved homeobox domain. Homeobox proteins have been found to bind DNA and act as transcription factors (Hawkins and McGhee, 1990). Consistent with a transcription function, CEH-23 is expressed in the nucleus of neuronal and intestinal cells (Walter et al., 2011). Unlike HIF-1 and CEP-1, little is known about how CEH-23 is regulated or the identity of its downstream targets that are important for longevity. The only known role of *ceh-23* in *C. elegans* is a possible function in AIY neuron differentiation. *ceh-23* expression is induced by TTX-3 in the AIY neuron, and elevated *ceh-23* expression is required to maintain one of the AIY differentiation markers, *sra-11* (Altun-Gultekin et al., 2001). However, no functional defects in the AIY neurons have been detected in *ceh-23* null mutants. Interestingly, ablation of the AIY interneurons shortened the lifespan of wildtype and long-lived daf-2 mutant worms, which positions the AIY neuron as an important determinant of longevity. However, the CEH-23-mediated regulation of SRA-11 is unlikely to be important for the longevity of ETC mutants, as *sra-11* mutants do not exhibit a longer lifespan (Shen et al., 2010). Higher *ceh-23* expression is observed in the ETC mutants using both a transcriptional reporter and RT-qPCR (Walter et al., 2011), indicating that *ceh-23* might respond to mitochondrial dysfunction. However, it is unclear whether the regulatory circuit observed during AIY neuron differentiation is also responsible for the up-regulation of *ceh-23* in ETC mutants or if another signaling pathway activates *ceh-23* expression in the ETC mutants. Nevertheless, the neuronal and intestinal expression of

CEH-23 is particularly interesting in the context of data indicating that mitochondrial ETC inhibition in either of these tissues alone is sufficient to alter the lifespan of the entire organism (Durieux et al., 2011). Further elaboration of CEH-23 functions will likely illuminate how neuronal or intestinal CEH-23 might respond to mitochondrial dysfunction to modulate overall longevity.

1.6 UBL-5, DEV-1, ATFS-1: key regulators of mitochondrial unfolded protein response

The lifespan of several ETC mutants is also mediated by the transcription factors UBL-5, DVE-1, and ATFS-1, which govern the mitochondrial unfolded protein response (UPR^{mt}), a mechanism that monitors protein homeostasis and maintains proper mitochondrial protein function (Durieux et al., 2011; Yoneda et al., 2004). Upon mitochondrial dysfunction, UBL-5, a ubiquitin-like protein, and the transcription factor DVE-1 are translocated from the cytoplasm into intestinal nuclei and form a complex (Benedetti et al., 2006; Haynes et al., 2007). Reduced *ubl-5* and *dve-1* levels via RNAi abrogated the longer lifespans of the ETC mutants *isp-1* and *clk-1* (albeit dve-1 RNAi also reduced the lifespan of wildtype animals) (Durieux et al., 2011). These long-lived ETC mutants also rely on the cytoplasmic bZip transcription factor ATFS-1 to mitigate their mitochondrial stress. These mutants do not tolerate the absence of atfs-1 and cannot develop when grown on atfs-1 RNAi (Nargund et al., 2012a). Given that ATFS-1 harbors both a nuclear and mitochondrial localization signal, it is predicted to be a critical toggle between a stressed and unstressed state, where it is shuttled to either the mitochondria or the nucleus depending on the absence or presence of the UPR^{mt}, respectively (Nargund et al., 2012a). A perturbed UPR^{mt} is also deleterious for the short-lived ETC mutant *mev-1*, where RNAi-mediated knock down of *dve-1* and *ubl-5* even further reduced the lifespan of these animals

(Fitzenberger et al., 2013). Notably, the UPR^{mt} in *C. elegans* can be visualized by the induction of hsp-6::gfp and hsp-60::gfp—two mitochondrial chaperones, and all three of these transcription factors are required for this induction (Yoneda et al., 2004).

Although UPR^{mt} components clearly contribute to the lifespan-defining mechanisms of ETC mutants, it remains unclear how an activated UPR^{mt} can result in distinct lifespan outcomes in response to different ETC perturbations. For example, while short-lived *gas-1* mutants display increased hsp-6::gfp expression, so do long-lived nuo-6 mutants, albeit at lower levels compared to *gas-1* (Pujol et al., 2013). Interestingly, a recent study demonstrated that the degree of hsp-6::gfp induction actually correlated with the extent of lifespan increase in worms inhibited for various mitochondrial ribosomal proteins (mrps) (Houtkooper et al., 2013). In this study, RNAi of mrps-5 extended the lifespan of *C. elegans mev-1* mutants but not of *cco-1* mutants. Unlike *cco-1* mutant, *mev-1* mutants did not display a stoichiometric imbalance between the nuclear and mitochondrial-encoded oxidative phosphorylation subunits, which has been shown to trigger UPR^{mt}. Consistently, *mev-1* RNAi did not induce UPR^{mt}. The authors proposed that mrps knockdown extended the lifespan of *mev-1* mutants by inducing a UPR^{mt} specifically via perturbing the nuclear- to mitochondrial-encoded protein ratio of the mitochondrial protein complexes (Houtkooper et al., 2013). However, other studies suggested that *mev-1* mutants did exhibit increased hsp-6::gfp expression (Durieux et al. 2011; Runkel et al., 2013). Thus, the importance of mitochondrial protein imbalance in activating the UPR^{mt} and its effects on lifespan merits further investigation.

Several proteins mediate the signaling between unfolded/misfolded mitochondrial proteins and nuclear-encoded gene expression characteristic of the UPR^{mt}. Upon mitochondrial protein stress, the ClpP protease, in concert with its binding partner ClpX, degrade the perturbed

mitochondrial proteins (Haynes et al., 2007, 2010). The transporter HAF-1 then transports these degraded peptides outside of the mitochondrial matrix (Haynes et al., 2010). This initiates a yet-to-be thoroughly defined signaling cascade that promotes the relocalization of UBL-5 (Benedetti et al., 2006), DVE-1, and ATFS-1 (Haynes et al., 2007, 2010). In parallel to the UPR^{mt} pathway, the eIF2α kinase GCN-2 has also been demonstrated to be required for the longevity of *clk-1* mutant animals (Baker et al., 2012). Interestingly, instead of abolishing hsp-60::gfp expression, which would be consistent with the requirement of GCN-2 in *clk-1* mutant lifespan, gcn-2 deletion in *clk-1* mutants actually further increased hsp-60::gfp expression. Even more curious, knocking down the eIF2α phosphatase gsp-1, which acts in antithesis to the gcn-2 kinase, also attenuated the lifespan of *clk-1* mutants as well as hsp-60::gfp expression. Therefore, at least in this context, hsp-60::gfp induction (i.e., UPR^{mt} induction) does not simply correlate with a positive outcome (lifespan extension) in the presence of ETC stress.

Additional factors have been documented to induce the UPR^{mt}. For example, paraquat induced both hsp-60::gfp and sod-3::gfp (a marker of ROS accumulation) expression in worms (Runkel et al., 2013; Yoneda et al., 2004). A recent investigation by Runkel et al. into the paraquat-induced UPR^{mt} revealed that ATFS-1 was required for this hsp-60::gfp induction but HAF-1 was not. Further, treating animals with the ROS scavenger NAC reduced the paraquat-induced expression of hsp-60::gfp by 75%, suggesting that, whether directly or indirectly, ROS induces a UPR^{mt} (Runkel et al., 2013). Interestingly, when animals were treated with acrylamide, a compound that induces cytoplasmic but not mitochondrial ROS, hsp-6::gfp expression is barely increased further clarifying that ROS generated in the mitochondria are likely necessary to activate UPR^{mt} (Runkel et al., 2013). Conversely, hsp-60::gfp expression was actually induced, and not repressed, when *clk-1* and *isp-1* mutants were treated with the ROS scavenger ascorbate

in another study (Baker et al., 2012). Moreover, NAC treatment did not attenuate the UPR^{mt} and the longevity of mrps-5 mutants, also suggesting that these mechanisms are ROS-independent. Therefore, while it appears that ROS and mitochondrial protein stress share similar stress-response mechanisms, their interactions are complex and can even be uncoupled.

The components of the UPR^{mt} summarized above largely work cell-autonomously. Intriguingly, UPR^{mt} can also be induced via non-cell-autonomous signaling that may be distinct from cell-autonomous signals. Knock down of the nuclear encoded cytochrome c oxidase-1 *cco-1* in the neurons alone induced hsp-60::gfp expression in the intestine and increased organismal lifespan (Durieux et al., 2011). A "mitokine" signal was proposed to be induced upon mitochondrial dysfunction in neurons and then transmitted to other cells in the animal, including the intestine, to coordinate a systemic compensatory response. Although *ubl-5* is required for the long life and UPR^{mt} induction of *cco-1* mutants, these phenotypes were not affected when *ubl-5* was knocked down in intestinal cells in animals that were also neuronally depleted for *cco-1*. While a neuronal signal can induce intestinal UPR^{mt} with pro-longevity effects, this signal does not rely on UBL-5, which appears to function exclusively in a cell-autonomous manner in the intestine (Durieux et al., 2011). Further elaboration of the signaling components mediating this non-cell-autonomous stress signal will be essential for a comprehensive understanding of how an organism copes with mitochondrial stress.

1.7 Mitochondrial ETC dysfunction induces complex transcriptional networks

Although the activities of several transcription factors were discussed separately, it is likely that at least some of these factors coordinate and act in a transcriptional network to respond to mitochondrial ETC dysfunction. For example, HIF-1 can inhibit DNA damage-induced apoptosis and CEP-1 activity in the germline (Sendoel et al.,

2010). Whether HIF-1, CEP-1, CEH-23, the factors that mitigate the UPR^{mt}, and others collaborate in response to mitochondrial dysfunction awaits further investigation.

Consistent with a complex transcriptional network, the link between mitochondrial dysfunction and longevity is highly dependent on temporal and spatial regulation. Partial inhibition of mitochondrial ETC function either by RNAi knockdown or pharmacological intervention must occur prior to the fourth larval stage of development to confer a longer lifespan in worms (Dillin et al., 2002; Rea et al., 2007). And while ETC inhibition in adulthood effectively reduced respiration and ATP production, it had no effect on longevity (Dillin et al., 2002). Furthermore, using a genetic trick to limit the RNAi-mediated inhibition of the ETC only during development, but not after reaching adulthood, also extended lifespan robustly (Dillin et al., 2002). Collectively, these data suggest that inactivation of ETC components during development is crucial for longevity determination, and the signal initiated by the ETC stress maintains its effect later in life even if the original stressor no longer exists. It is important to note that the ETC inhibition timing requirement coincides with massive mitochondrial biosynthesis that accompanies germline development, suggesting that ETC dysfunction at this stage might exert the most potent effect. Looking forward, it will be important to determine whether and how the transcription factors and pathways we have discussed here respond to mitochondrial dysfunction in a temporal-specific manner.

Tissue-specific mitochondrial function is also important for longevity determination. Neuronal and intestinal tissues are key for the lifespan extension in ETC mutants (Durieux et al., 2011). For example, inducing mitochondrial ETC dysfunction

only in neurons or the intestine was sufficient to promote the lifespan of the entire organism, whereas the same ETC defect in the body wall muscle did not significantly alter lifespan. Similar findings also hold true in D. melanogaster (Owusu-Ansah et al., 2013). In *C. elegans*, some of the transcription factors that we have discussed exhibit distinct expression patterns. For example, CEH-23 is expressed in the neurons and intestine (Walter et al., 2011), CEP-1 is expressed in pharyngeal cells and the germline (Jaramillo-Lambert et al., 2010), and HIF-1 is ubiquitously expressed in all somatic cells (Jiang et al., 2001). To clearly delineate the cell-autonomous and non-cell-autonomous compensatory responses of mitochondrial ETC dysfunction, the cell types/tissues where the various factors we have discussed, as well as others, act in to modulate longevity must be investigated in the future. Given that ETC dysfunction can trigger both cell-autonomous and non-cell-autonomous compensatory responses with relatively strict temporal and spatial requirements (Durieux et al., 2011), the existence of a complex and collaborative transcriptional network is likely.

While I have focused on the transcription factors HIF-1, CEP-1, CEH-23 and the UPR^{mt} inducers, emerging research suggests that many more transcriptional regulators might respond to mitochondrial dysfunction and affect lifespan. Indeed, two independent RNAi screens identified a total of nine additional transcription factors that likely participate in longevity outcomes in response to ETC perturbation (Khan et al., 2013; Walter et al., 2011). Therefore, further investigation of how these promising new candidates act singly or cooperatively with known transcription factors will likely lead to

fruitful insights into the link between mitochondrial dysfunction and longevity in *C. elegans*.

1.8 Dietary restriction likely modulates lifespan through a mechanism distinct from that in ETC mutants

Dietary restriction (DR) has been shown to extend the lifespan of various organisms (Fontana et al., 2010). Despite the conflicting findings of its effects on the longevity of rhesus monkeys, the health benefits of DR have consistently been observed (Bodkin et al., 2003; Colman et al., 2009; Mattison et al., 2012). In mammals, the effects of DR on mitochondrial function have been studied extensively. DR has been shown to attenuate mitochondrial ROS emission from ETC complex I (Gredilla et al., 2001; Hagopian et al., 2005, 2011; López-Torres et al., 2002), a poignant finding given that increased ROS levels are one of the hallmarks of aging. Although there have been conflicting data regarding how DR impacts mitochondrial proton leak (Asami et al., 2008; Bevilacqua et al., 2004; Hagopian et al., 2005; Lal et al., 2001), recent evidence suggest that DR might in fact promote the efficiency of mitochondrial respiration(Desai et al., 1996; Hempenstall et al., 2012; Hepple et al., 2005; Lanza et al., 2012). Lastly, DR has been shown to reduce the sensitivity of mitochondria to apoptosis stress, where less apoptosis is observed in DR animals (Hafner et al., 2010; Hofer et al., 2009) Together, these mammalian studies suggest that the longevity effects of DR might depend on altering mitochondrial functions. Consistent with this notion, the mitochondrial protein deacetylase SIRT3 can be activated by an increased NAD+/NADH ratio under DR conditions in mammals (Sauve, 2010; Shi et al., 2005; Yang et al., 2010a). Furthermore,

deacetylation of several factors has been demonstrated to mediate the beneficial effects of DR. For example, deacetylation of SOD2 is thought to reduce overall ROS in DR animals (Qiu et al., 2010), and deacetylation of the mitochondrial protein cyclophilin D in DR animals lowers their sensitivity to apoptosis by delaying the opening of the mitochondrial permeability transition pore (mPTP), which is a key step towards inducing apoptosis (Hafner et al., 2010).

In *C. elegans*, dietary restriction can be implemented using various regimens, including dilution of bacterial food on agar plates or in liquid culture, or complete bacterial deprivation post reproduction, all of which extend the organismal lifespan robustly but are thought to act through partially overlapping pathways (Greer and Brunet, 2009). Similar to the mammalian models, several DR interventions in *C. elegans* have been shown to increase mitochondrial respiration (Bishop and Guarente, 2007; Houthoofd et al., 2002; Schulz et al., 2007), suggesting a conserved role for DR in mitochondrial function. Contrary to the effects of DR on mitochondria, the ETC mutants described in this review exhibit moderately reduced respiration and mitochondrial function. Several genetic studies suggest that although both DR and moderate ETC dysfunction prolong lifespan, the longevity phenotypes are likely mediated through distinct molecular players. For example, the eat-2 mutant is commonly used to study DR in *C. elegans*. eat-2 encodes a subunit of a ligand-gated channel of the pharyngeal muscle, thus eat-2 mutants exhibit a slower pharyngeal pumping rate that reduces food intake (Lakowski and Hekimi, 1998). Consistent with a role for DR in longevity, eat-2 mutants live longer. Notably, the eat-2 mutant lifespan is further increased with the

addition of the ETC mutation nuo-6(qm200), suggesting that eat-2-mediated DR can act additively with mitochondrial dysfunction to prolong lifespan (Yang and Hekimi, 2010a). Moreover, the extended lifespan of eat-2 mutants requires the transcription factor pha-4, but the long-lived phenotype of *isp-1* mutants does not (Panowski et al., 2007). However, the relationship between mitochondrial dysfunction and DR in mediating lifespan is complicated by the epistatic relationship between *clk-1* and eat-2 mutations. Unlike the *isp-1* and nuo-6 mutants, the *clk-1* mutation seems to activate a similar pathway to the eat-2 mutation, as the *clk-1*(e2519) mutation does not further lengthen the lifespan of eat-2 mutants (Lakowski and Hekimi, 1998). As discussed in the previous section, *clk-1* mutants have phenotypes that are quite distinct from the other ETC mutants. The epistatic relationship between *clk-1* and eat-2 further supports the idea that *clk-1* mutations trigger a longevity mechanism that is distinct from other ETC mutations.

While the mitochondrial ETC longevity and DR longevity pathways appear to be distinct, they both promote the activity of the transcription factor skn-1 in *C. elegans*. SKN-1 is required for the extended lifespan of DR worms when they are cultured in liquid media with diluted bacteria as the food source (Bishop and Guarente, 2007). Moreover, one of the skn-1 targets identified by Park et al., nlp-7, is required for the prolonged lifespan of the eat-2 mutant (Park et al., 2010), which reinforces the pivotal role of skn-1 in DR-mediated longevity. As previously discussed, while the roles of SKN-1 in the longevity of ETC mutants are still controversial, the activity of SKN-1 is likely to be induced by mitochondrial ROS in the ASI neurons under ETC stress (Schmeisser et al., 2013b). Interestingly, under conditions of glucose deprivation, increased

mitochondrial respiration yields increases in ROS production, which is essential for the extended lifespan associated with this type of glucose deprivation-mediated DR (Schulz et al., 2007). Notably, SKN-1 activation and its requirement for prolonged longevity is only observed in some DR regimens but not all, suggesting that food availability plays a more complex role in lifespan than originally anticipated. How skn-1 affects longevity in response to ETC dysfunction is equally complicated as discussed in the previous section. A comparison of the activities of skn-1 under both ETC stress and DR stress may provide a more thorough look at the contribution of skn-1 in modulating longevity in response to these perturbations.

1.9 Mammalian mitochondrial dysfunction and aging

Mitochondrial dysfunction in mammals has largely yielded detrimental effects. In humans, inherited mitochondrial respiratory chain disorders encompass a large spectrum of clinical symptoms, including muscle weakness, neurological disorders, and lactic acidosis (McInnes, 2013). These disorders occur approximately 1 in 5,000 live births (Menezes et al., 2014). Furthermore, mitochondrial DNA itself accumulates mutations over time, and this has been proposed to be a cause and not an effect of aging using mouse models deficient for mtDNA polymerase proofreading activity. These mice not only lived shorter lives but also displayed premature aging-related pathologies in multiple tissues (Trifunovic et al., 2004). Mitochondrial dysfunction has also been observed in a variety of age-related human diseases, including neurodegeneration, type II diabetes, and cancer, where defects in nuclear-encoded proteins are suspected to contribute to mitochondrial dysfunction and thus some of the observed disease symptoms (Kwong et al., 2006). Given all these, how, if at all, could findings in *C. elegans* inform adaptive responses upon mitochondrial dysfunction in mammals?

Since, as discussed earlier, the age-dependent decline of mitochondrial ETC function is evolutionarily conserved, the presence of mitochondrial dysfunction-related aging pathologies in

mammals is consistent with observations in *C. elegans*. Furthermore, several mouse models of ETC dysfunction support the idea that mitochondrial ETC function also modulates lifespan in mammals. Similar to what has been observed in *C. elegans*, reduced function of the ubiquinone biosynthesis enzyme CLK-1/MCLK1 in mice causes reduced mitochondrial ETC function and extends lifespan (Lapointe and Hekimi, 2008; Lapointe et al., 2009; Liu et al., 2005). While heterozygous loss of MCLK1 induces long life, homozygous loss of MCLK1 is lethal, reminiscent of the threshold model observed in *C. elegans* ETC mutants. In another model, Hughes et al. constructed RISP (+/P224S) mice to mimic the *C. elegans isp-1*(qm150) mutation (Hughes and Hekimi, 2011). Similar to the MCLK1 model, the homozygous RISP mutant mice are not viable. Decreased function of RISP in mice reduces mitochondrial respiration in a substrate-dependent manner, where only the electron transfer from complex II to III to IV is affected. Interestingly, RISP (+/P224S) mice have a gender-specific longevity phenotype: only RISP (+/P224S) males are short-lived. Even more curious, RISP (+/P224S) females that survive past the median wild-type lifespan live slightly longer than wild-type mice. This suggests that RISP heterozygosity is mildly protective for female mice later in life. The beneficial effect of RISP (+/P224S) is reminiscent of the pro-longevity effect of the *isp-1* mutation in *C. elegans*. Lifespan data for *isp-1* mutants, and for most lifespan experiments, were collected using hermaphrodites, so a possible gender-specific effect would likely be overlooked in *C. elegans*.

In addition to MCLK1 and RISP mutant mice, mice with dysfunctional SURF1 are also used to study aging. One type of SURF1 mouse model harbors a prematurely truncated and highly unstable SURF1 protein, $SURF1^{loxP}$ (Dell'agnello et al., 2007). As SURF1 encodes an assembly protein that is important for cytochrome c oxidase (complex IV) formation, $SURF1^{loxP}$ mice have reduced cytochrome oxidase activity and a long lifespan. In humans, SURF1 has been

implicated in Leigh syndrome, which is typically caused by mutations that disrupt mitochondrial function. Moreover, patients with a cytochrome c oxidase deficiency usually have mutations in SURF1. While $SURF1^{loxP}$ mice exhibit lifespan extension in response to a mild reduction in ETC function, another SURF1 knockout mouse model exhibits the opposite longevity phenotype: $SURF1^{neo}$ mice, which carry an allele of SURF1 with a neomycin-resistant cassette (Agostino, Alessandro; Invernizzi, Federica; Tiveron, Cecilia; Fagiolari, Gigliola; Zeviani, 2003), are actually short-lived. Curiously, these mice exhibit reduced cytochrome c oxidase activity similar to the $SURF1^{loxP}$ mice. Besides the short-lived phenotype, $SURF1^{neo}$ mice also exhibit high embryonic lethality, which is not observed in $SURF1^{loxp}$ mice. These inconsistencies may be due to an artifact of using a NEO cassette, as explained in Dell'Agnello et al., who pointed out that the $SURF1^{loxP\text{-}NEO\text{-}loxP}$ mice also have high embryonic lethality (Dell'agnello et al., 2007). Taken together, several mouse models with ETC dysfunction described here demonstrate that reduced mitochondrial ETC function can also lead to lifespan extension in mammals, suggesting a possible conserved underlying mechanism between worms and mice.

In addition to the effects on lifespan, the broad spectrum of pathological manifestation associate with mitochondrial functions echoes the pleiotropy of *C. elegans* mitochondrial ETC mutants. In humans, mitochondrial DNA heteroplasmy, or a mixture of mutated and normal DNA within one cell, is one phenomenon that can account for these differences (Lagouge and Larsson, 2013). In these cases, penetrance depends on whether the presence of mutated mtDNA reaches a certain threshold in one or more tissues to manifest in a disease phenotype. Such a threshold phenomenon is reminiscent of the observations in *C. elegans* with dysfunctional mitochondria, where some ETC mutants live shorter than normal, whereas others live longer than normal. Furthermore, it has been observed that dietary intake and genetic background of

individuals influence final phenotypic outcomes of human patients and mammalian models of mitochondrial dysfunction. Findings from *C. elegans* therefore could illuminate the cellular and organismal compensatory responses that determine the phenotypic manifestations (Chandel et al., 1998; Runkel et al., 2013; Yang and Hekimi, 2010b).

The emerging data reviewed here indicate that cells and organisms have a large capacity to respond to mitochondrial stress, and different degrees of mitochondrial dysfunction likely induce compensatory responses that lead to divergent phenotypic outcomes. While *C. elegans* and mammals have very different physiologies, they likely share common cellular and organismal adaptive signaling responses to dysfunctional mitochondria. Insights gained from further analysis of the mechanistic basis of these responses in *C. elegans* and other model systems could be harnessed to provide therapeutic opportunities aimed at improving diverse mitochondrial disorders and possibly age-related diseases.

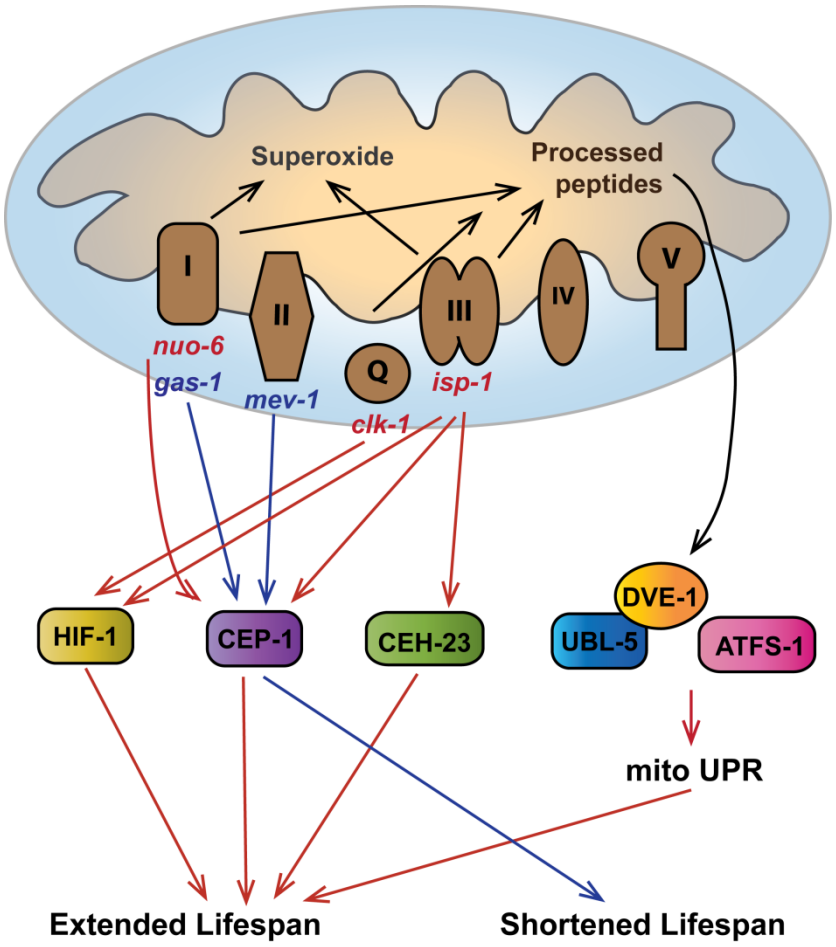


Figure 1.1 Mitochondrial ETC dysfunction affects life span in C. elegans by inducing a transcriptional network.
Mitochondrial mutations trigger differential transcriptional responses by activating distinct transcription factors. Red arrows indicate transcriptional pathways that are activated in long-lived ETC mutants and are important for their life-span extension. Blue arrows indicate the transcriptional responses that are activated in the short-lived ETC mutants and are important for limiting the life span of these animals.

CHAPTER 2

CEP-1, the *Caenorhabditis elegans* p53 Homolog, Mediates Opposing Longevity Outcomes in Mitochondrial Electron Transport Chain Mutants[2]

2.1 Abstract

Caenorhabditis elegans CEP-1 and its mammalian homolog p53 are critical for responding to diverse stress signals. In this study, we found that *cep-1* inactivation suppressed the prolonged lifespan of electron transport chain (ETC) mutants, such as *isp-1* and *nuo-6*, but rescued the shortened lifespan of other ETC mutants, such as *mev-1* and *gas-1*. We compared the CEP-1-regulated transcriptional profiles of the long-lived *isp-1* and the short-lived *mev-1* mutants and, to our surprise, found that CEP-1 regulated largely similar sets of target genes in the two mutants despite exerting opposing effects on their longevity. Further analyses identified a small subset of CEP-1-regulated genes that displayed distinct expression changes between the *isp-1* and *mev-1* mutants. Interestingly, this small group of differentially regulated genes are enriched for the "aging" Gene Ontology term, consistent with the hypothesis that they might be particularly important for mediating the distinct longevity effects of CEP-1 in *isp-1* and *mev-1* mutants. We further focused on one of these differentially regulated genes, *ftn-1*, which encodes ferritin in *C. elegans*, and demonstrated that it specifically contributed to the extended lifespan of

2 Chapter 2 is modified from Baruah, A.; Chang, H.-W.; Hall, M.; Yuan, J.; Gordon, S.; Johnson, E.; Shtessel, L. L.; Yee, C.; Hekimi, S.; Derry, W. B.; Lee, S. S. PLoS Genet. 10: e1004097; 2014. My role in this paper is to perform some of the key experiments including investigating the requirement of *cep-1* and its transcriptional target, *ftn-1*, in various ETC mutants lifespan (Figure 2.5 A-C, Figure 2.6 A, B). I also showed that *ftn-1* is differentially expressed in various ETC mutant, which correlate to its longevity effect (Figure 2.6 C). Last I showed that some of the CEP-1 targets identified in the *isp-1* and *mev-1* background are universal CEP-1 targets in other ETC mutants (Figure 2.7 and 2.8). The manuscript was mainly written by AB and revised by LS, SSL and me. The sections I contribute are highlighted in Helvetica.

isp-1 mutant worms but did not affect the *mev-1* mutant lifespan. We propose that CEP-1 responds to different mitochondrial ETC stress by mounting distinct compensatory responses accordingly to modulate animal physiology and longevity. Our findings provide insights into how mammalian p53 might respond to distinct mitochondrial stressors to influence cellular and organismal responses.

2.2 Introduction

Mitochondria are major sites of numerous metabolic processes, in particular electron transport and ATP production, and are essential for life. Not surprisingly, mitochondria also play central roles in aging and disease (Wallace, 2005). In model organisms such as worms, flies, and mice, specific point mutations or RNAi knockdowns directly affecting the electron transport chain (ETC) result in varying effects on development and longevity, ranging from developmental arrest and shortened survival to extended lifespan. The extended lifespan associated with moderate mitochondrial ETC dysfunction was surprising and further highlights the complex relationship between mitochondrial function and aging. An emerging model posits that a moderate reduction in mitochondrial ETC function can lead to compensatory responses that lengthen lifespan (Copeland et al., 2009; Lapointe and Hekimi, 2008; Van Raamsdonk and Hekimi, 2009; Rea et al., 2007), whereas a more severe reduction in mitochondrial ETC function, beyond an innate threshold, will lead to developmental arrest and/or early death (Ishii et al., 1998; Walker et al., 2006). How different degrees of mitochondrial dysfunction result in opposing effects on longevity remains largely unknown.

Caenorhabditis elegans represents a powerful model to study the genetic basis of cellular and organismal changes in response to mitochondrial dysfunction. Previous findings in *C. elegans* have revealed a number of long-lived and short-lived ETC mutants. The nuo-6(qm200)

mutant, which harbors a point mutation in the NADH-ubiquinone oxidoreductase of complex I, the *isp-1*(qm150) mutant, which harbors a point mutation in the rieske iron sulphur subunit of complex III, and the *clk-1*(e2519) mutant, with a point mutation in a coenzyme Q biosynthesis enzyme, exhibit substantial lifespan extension (Feng et al., 2001; Walker et al., 2006). In contrast, the *mev-1*(kn-1) mutant, with a point mutation in the succinate dehydrogenase subunit c of complex II, and the *gas-1*(fc21) mutant, with a point mutation in the NADH:ubiquinone oxidoreductase NDUFS2 subunit of complex I, live significantly shorter than wild-type worms (Kondo et al., 2005). Furthermore, large-scale RNAi screens have revealed that RNAi-mediated inactivation of many of the ETC subunits result in prolonged or shortened lifespan (Lee et al., 2003).

Studies using genetic mutants and RNAi-mediated knockdown of ETC components in worms have begun to reveal the mechanistic basis of the longevity outcomes associated with mitochondrial dysfunction. Reactive oxygen species (ROS) have emerged as an important signaling intermediate in the ETC mutants. Specifically, nuo-6, *isp-1*, and *mev-1* mutants have been shown to exhibit elevated levels of mitochondrial superoxide, and antioxidant treatment of these worms was able to revert their longevity phenotype(Ishii et al., 2004; Yang and Hekimi, 2010b). Interestingly, the long-lived *clk-1* mutant was not found to exhibit a higher level of mitochondrial superoxide, and antioxidant treatment had no impact on its lifespan, suggesting that the *clk-1* mutation influences lifespan independent of ROS. In addition to increased ROS levels, the ETC mutants also exhibit an altered metabolism. The long-lived nuo-6, *isp-1*, and *clk-1* mutants share similar metabolic profiles that are distinct from that of the *mev-1* short-lived mutant (Yang et al., 2010b; Yee et al., 2014). An elevated production of metabolites, such as α-ketoacids and α-hydroxyacids, has been proposed to act as a pro-longevity signal in the long-

lived ETC mutants (Butler et al., 2010, 2013). Furthermore, studies that have largely employed RNAi-mediated knockdown of various ETC subunits demonstrated that an imbalanced stoichiometry of the ETC protein subunits triggered a strong mitochondrial unfolded protein response (mtUPR). In this scenario, processed peptides in the mitochondria are thought to serve as the signal that activates several transcriptional regulators, including UBL-5 and ATFS-1, to induce transcriptional responses necessary to restore proteostasis in the mitochondria, which contributes to longevity determination (Nargund et al., 2012b). Lastly, several RNAi and candidate screens have identified additional transcription factors, such as CEP-1 (Torgovnick et al., 2010), CEH-23 (Walter et al., 2011), and TAF-4 (Khan et al., 2013) that mediate the lifespan of various ETC mutants.

The transcription factor p53 has recently emerged as a key regulator of metabolic balance (Jones et al., 2005; Mandal et al., 2005; Matoba et al., 2006). Despite its importance, how p53 senses metabolic stress and accordingly regulates molecular changes that determine the physiological outcomes of an organism remains poorly understood. *C. elegans* CEP-1, the sole homolog of the mammalian p53 family (Bensaad and Vousden, 2007) (p53, p63 and p73), is known to mediate the lifespan changes in worms with mitochondrial dysfunction. Inactivation of *cep-1* has been shown to partially suppress the extended longevity of *isp-1* mutant worms (Derry et al., 2001). Furthermore, using different concentrations of RNAi to cause different degrees of knockdown of several ETC components demonstrated that CEP-1 is required for the increased longevity under mild mitochondrial disruption as well as the shortened lifespan when mitochondrial damage is more severe (Ventura et al., 2010). Therefore, CEP-1 exerts opposite effects on lifespan that likely depend on the levels of mitochondrial stress experienced. The underlying mechanism governing this intriguing duality of CEP-1 function is not known.

In this study, we sought to further characterize the role of CEP-1 in the longevity of several mitochondrial ETC mutants. Our results indicate that CEP-1 is a critical mediator of the lifespan of several mitochondrial mutants, suggesting that CEP-1 plays a central role in sensing mitochondrial distress and coordinating physiological outcomes accordingly. We also evaluated the CEP-1-regulated transcriptomes in the long-lived *isp-1* and the short-lived *mev-1* mitochondrial ETC mutants. Despite the opposing roles that CEP-1 appears to play in determining the lifespan of these mutants, the CEP-1-regulated transcriptional profiles were largely similar in these mutants. Nevertheless, the expression of a small group of genes was differentially regulated by CEP-1 between the long-lived *isp-1* and the short-lived *mev-1* mutants. Interestingly, this small group of genes is enriched for the Gene Ontology functional group "aging", indicating they are over-represented by genes previously known to have a role in aging in worms. We functionally validated one of these differentially regulated genes, *ftn-1*, which encodes ferritin in *C. elegans*, by demonstrating that its RNAi-mediated depletion significantly impacted the lifespan of the *isp-1* but not *mev-1* mutant. This result supports our hypothesis that CEP-1 can differentially regulate a small subset of target genes to achieve distinct longevity outcomes in response to mutations in different ETC components.

2.3 Results

2.3.1 CEP-1 exerts opposing effects on longevity and development in the long-lived *isp-1* and short-lived *mev-1* mutants

Previous results suggested that *cep-1* is required for the lifespan extension associated with mild mitochondrial dysfunction and the shortened lifespan associated with severe mitochondrial dysfunction (Ventura et al., 2010). We confirmed these results and demonstrated that inactivation of *cep-1* partially but consistently suppressed the extended lifespan of *isp-1*

mutant animals (Figure 2.1A, Table 2.1). We further showed that inactivation of *cep-1* largely restored the lifespan of *mev-1* mutants to wild-type (Figure 2.1B, Table 2.1). Our data are therefore consistent with previous findings suggesting that CEP-1 can respond to different degrees of mitochondrial dysfunction and modulate lifespan accordingly.

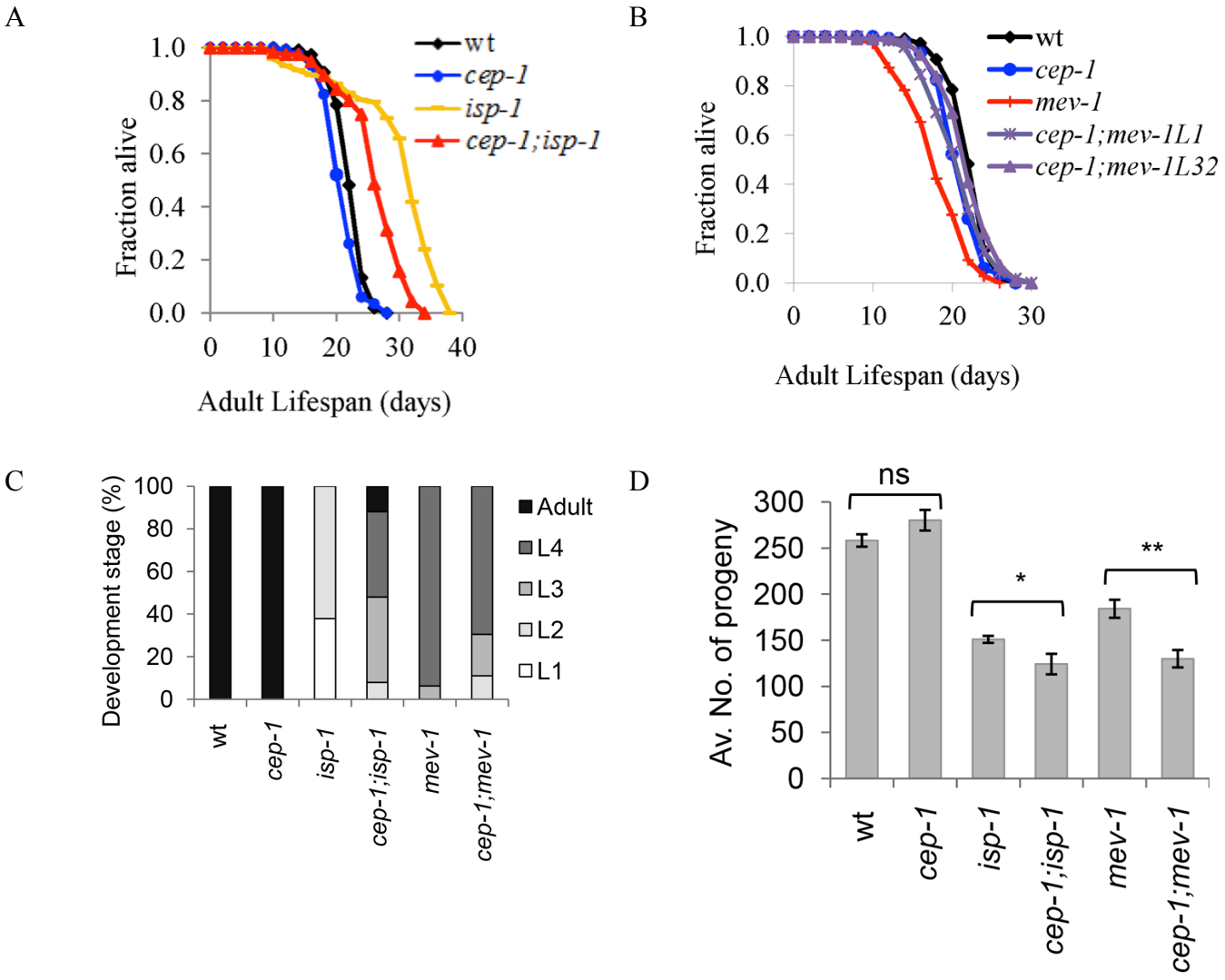


Figure 2.1 CEP-1 mediates the longevity and development of two mitochondrial mutants in *C. elegans*.

(A) *cep-1* mutation partially suppresses *isp-1* mutant longevity as the *cep-1*;*isp-1* double mutant lifespan is shorter than that of the *isp-1* single mutant. (B) *cep-1* mutation restores the *mev-1* mutant lifespan as the lifespans of two *cep-1*;*mev-1* isolates (L1, L32) are similar to that of wt. (C) The percentage of worms at each developmental stage was quantified for wt, *cep-1*, *isp-1*, *cep-1*;*isp-1*, *mev-1*, and *cep-1*;*mev-1* mutant worms after 60 hr of growth from the embryonic stage at 20°C. (D) The average number of progeny production for each line was calculated from 5 to 10 worms. The *isp-1* and *mev-1* mutants produce significantly less progeny than wt. The *cep-1*;*isp-1* and *cep-1*;*mev-1* double mutants display significantly lower brood sizes than their respective single mutant controls (*p,0.05, **p,0.0005). The error bars represent standard errors. Statistical analysis was performed using a two-tailed t-test.

Table 2.1: Adult lifespan of all individual experiments.

A. *isp-1, cep-1;isp-1* lifespan

A.1

Strain	Total N	mean ± Std. Error	median ± Std. Error	*P*-value *vs* wt	*P*-value *vs* *isp-1*	% mean lifespan difference to wt
wt	112	22.56 ± 0.25	22 ± 0.30			
cep-1	123	21.20 ± 0.26	22 ± 0.29	.000		-6.42
isp-1	117	30.29 ± 0.66	32 ± 0.38	.000		25.52
cep-1;isp-1	116	26.33 ± 0.47	26 ± 0.43	.000	.000	14.32

A.2

Strain	Total N	mean ± Std. Error	median ± Std. Error	*P*-value *vs* wt	*P*-value *vs* *isp-1*	% mean lifespan difference to wt
wt	108	21.24 ± 0.32	22 ± 0.23			
cep-1	104	20.78 ± 0.26	20 ± 0.38	.119		-2.21
isp-1	29	25.24 ± 1.44	26 ± 1.33	.000		15.85
cep-1;isp-1	109	23.83 ± 0.51	26 ± 0.72	.000	.010	10.87

B. *clk-1, cep-1; clk-1* lifespan

B.1

Strain	Total N	mean ± Std. Error	median ± Std. Error	*P*-value *vs* wt	*P*-value *vs* *clk-1*	% mean lifespan difference to wt
wt	114	22.44 ± 0.31	22 ± 0.32			
cep-1	115	21.36 ± 0.32	22 ± 0.29	.015		-5.06
clk-1	107	26.1 ± 0.43	26 ± 0.54	.000		14.02
cep-1;clk-1 L4	115	25.88 ± 0.50	26 ± 0.77	.000	.794	13.29
cep-1;clk-1 L6	113	26.52 ± 0.49	26 ± 0.65	.000	.240	15.38

B.2

Strain	Total N	mean ± Std. Error	median ± Std. Error	*P*-value *vs* wt	*P*-value *vs* *clk-1*	% mean lifespan difference to wt
wt	112	21.89 ± 0.28	22 ± 0.29			
cep-1	65	19.81 ± 0.32	21 ± 0.23	.000		-10.50
clk-1	107	25.09 ± 0.34	26 ± 0.39	.000		12.75
cep-1;clk-1 LN4	94	23.81 ± 0.38	24 ± 0.69	.000	.032	8.06
cep-1;clk-1	101	25.77 ± 0.52	26 ± 0.57	.000	.041	15.06

LN6						

Table 2.1 continued.

C. *mev-1, cep-1;mev-1* lifespan

C.1

Strain	Total N	mean ± Std. Error	median ± Std. Error	P-value vs wt	P-value vs *mev-1*	% difference of mean lifespan to wt
wt	112	22.56±0.25	22±0.30			
cep-1	123	21.2±0.26	22±0.29	.000		-6.42
mev-1	111	18.18±0.37	18±0.41	.000		-24.09
cep-1;mev-1L1	117	20.97±0.35	22±0.43	.013	.000	-7.58
cep-1;mev-1L2	119	19.51±0.38	20±0.48	.000	.011	-15.63
cep-1;mev-1 L32	112	22.2±0.33	22± 0.33	.930	.000	-1.62
cep-1;mev-1 L9	97	20.47±0.41	20±.37	.009	.000	-10.21

C.2

Strain	Total N	mean ± Std. Error	median ± Std. Error	P-value vs wt	P-value vs *mev-1*	% difference of mean lifespan to wt
wt	108	21.24±0.32	22±0.23			
cep-1	104	20.78±0.26	20±0.38	.119		-2.21
mev-1	99	18.87±0.47	20±0.43	.001		-12.56
cep-1,mev-1 L1	132	22.12±0.42	22±0.54	.004	.000	3.98
cep-1;mev-1 L2	125	21.95±0.43	22±0.39	.005	.000	3.23
cep-1;mev-1 L32	124	23.87±0.40	24±0.50	.000	.000	11.02

C.3

Strain	Total N	mean ± Std. Error	median ± Std. Error	P-value vs N2	P-value vs *mev-1*	% difference of mean lifespan to wt
wt	120	19.7 ±0.33	20 ±0.39			
cep-1	121	19.75 ±0.28	20 ±0.29	.456		0.25
mev-1	107	16.64 ±0.42	16 ±0.31	.000		-18.39
cep-1;mev-1 L9	126	20.91 ±0.40	20 ±0.58	.002	.000	5.79

Table 2.1 continued.

D. *gas-1, cep-1;gas-1* lifespan

D.1

Strain	Total N	mean ± Std. Error	median ± Std. Error	P-value vs wt	P-value vs *gas-1*	% difference of mean lifespan to wt
wt	103	19.42 ±0.26	20 ±0.19			
cep-1	101	19.64 ±0.20	20 ±0.13	.837		1.12
mev-1	83	13.49 ±0.45	14 ±0.40	.000		-43.96
gas-1	69	13.97 ±0.59	12 ±0.54	.000		-39.01
gas-1;cep-1 L22	106	16.06 ±0.58	18 ±0.71	.000	.018	-20.92
gas-1;cep-1 L34	86	16.56 ±0.93	14 ±1.31	.441	.014	-17.27

D.2

Strain	Total N	mean ± Std. Error	median ± Std. Error	P-value vs wt	P-value vs *gas-1*	% difference of mean lifespan to wt
wt	106	13.87 ±0.20	14 ±0.22			
cep-1	108	14.30 ±0.22	14 ±0.31	.075		3.01
mev-1	89	11.96 ±0.31	12 ±0.38	.000		-15.97
gas-1	103	10.72 ±0.26	10 ±0.25	.000		-29.38
cep-1;gas-1 L22	142	16.95 ±0.30	18 ±0.49	.000	.000	18.17
cep-1;gas-1 L34	169	16.35 ±0.33	16 ±0.63	.000	.000	15.17

Table 2.1 continued.
E.wt and *cep-1, isp-1, cep-1;isp-1, mev-1, cep-1;mev-1* mutants lifespan after *ftn-1*&2 RNAi
E.1

strain	Total N	mean ± Std. Error	median ± Std. Error	P-value vs wt	% difference of mean lifespan to wt
wt_control	114	17.15 ± 0.19	18 ± 0.09		
wt_*ftn-1*&2 RNAi	115	18.35 ± 0.22	18 ± 0.23	.000	6.54
*cep-1*_control	111	18.56 ± 0.24	18 ± 0.24	.000	7.60
cep-1_ftn-1&2RNAi	118	18.92 ± 0.20	18 ± 0.26	.000	9.36
*isp-1*_control	103	27.46 ± 0.78	30 ± 0.67	.000	37.55
isp-1_ftn-1&2RNAi	77	25.40 ± 0.76	26 ± 1.31	.000	32.48
cep-1;*isp-1*_control	124	22.55 ± 0.35	24 ± 0.41	.000	23.95
cep-1;*isp-1_ftn-1*&2RNAi	114	23.16 ± 0.46	24 ± 0.44	.000	25.95
*mev-1*_control	103	13.73 ± 0.26	14 ± 0.32	.000	-24.91
mev-1_ftn-1&2RNAi	110	14.11 ± 0.31	14 ± 0.40	.000	-21.55
cep-1;*mev-1*_control	118	15.78 ± 0.38	16 ± 0.47	.308	-8.68
cep-1;*mev-1_ftn-1*&2 RNAi	117	15.16 ± 0.45	16 ± 0.79	.105	-13.13

Table 2.1 continued
E.2

strain	Total N	mean ± Std. Error	median ± Std. Error	P-value vs N2_control	% difference of mean lifespan to wt control
wt_control	108	19.23 ± 0.30	20 ± 0.31		
wt_*ftn-1*&2 RNAi	116	20.23 ± 0.22	20 ± 0.21	.049	1.49
*cep-1*_control	111	19.52 ± 0.27	20 ± 0.33	.653	1.49
cep-1_ftn-1&2RNAi	115	19.51 ± 0.22	20 ± 0.25	.978	1.44
*isp-1*_control	144	28.25 ± 0.64	30 ± 0.94	.000	31.93
isp-1_ftn-1&2RNAi	118	22.30 ± 0.78	24 ± 0.89	.000	13.77
cep-1;*isp-1*_control	119	21.08 ± 0.54	22 ± 0.41	.000	8.78
cep-1;*isp-1_ftn-1*&2RNAi	108	20.78 ± 0.49	22 ± 0.35	.000	7.46
*mev-1*_control	115	12.99 ± 0.36	14 ± 0.36	.000	-48.04
mev-1_ftn-1&2RNAi	112	13.61 ± 0.38	14 ± 0.41	.000	-41.29
cep-1;*mev-1*_control	110	16.10 ± 0.48	16 ± 0.54	.000	-19.44
cep-1;*mev-1_ftn-1*&2RNAi	112	16.11 ± 0.44	16 ± 61	.000	-19.37

Table 2.1 continued.
E3. WT, *mev-1*, *gas-1*, nuo-6, and *isp-1* lifespan after *ftn-1* & 2 RNAi

strain	Total N	mean ± Std. Error	median ± Std. Error	P-value vs N2_E.V. control	% difference of mean lifespan to wt control
wt_control	97	22.41+ /-0.28	22+ /-0.33		
wt_*ftn-1*&2 RNAi	99	22+ /-0.29	22+ /-0.4	.412	-1.84
*gas-1*_control	100	15.28+ /-0.37	14+ /-0.42	.000	-31.82
*gas-1*_*ftn-1*&2RNAi	103	14.85+ /-0.39	14+ /-0.47	.000	-33.71
*mev-1*_control	99	13.11+ /-0.35	12+ /-0.47	.000	-41.50
*mev-1*_*ftn-1*&2RNAi	98	13.38+ /-0.4	12+ /-0.54	.000	-40.27
nuo-6_control	181	25.36+ /-0.2	26+ /-0.23	.000	13.18
nuo-6_*ftn-1*&2RNAi	122	20.57+ /-0.3	20+ /-0.32	.001	-8.21
*isp-1*_control	123	24.4+ /-0.3	24+ /-0.47	.000	8.90
*isp-1*_*ftn-1*&2RNAi	112	21.72+ /-0.27	22+ /-0.33	.164	-3.07

F. effect of *cep-1 on various ETC mutant longevity*

strain	Total N	mean ± Std. Error	median ± Std. Error	p-vlaue vs. WT	p-value vs. ETC mutants	% difference of mean lifespan to wt
mev-1	108	14.00+/-0.35	14+/-0.27	<0.001		-17.485
cep-1;mev-1	91	19.83+/-0.49	18+/-0.95	<0.001	0.000	16.925
gas-1	100	13.06+/-0.27	14+/-0.27	<0.001		-22.992
cep-1;gas-1	54	16.53+/-0.70	16+/-0.91	0.751	0.000	-2.587
wt	102	17.00+/-0.34	16+/-0.38			
clk-1(e2519)	103	21.00+/-0.38	20+/-0.53	<0.001		23.715
cep-1;clk-1	109	14.44+/-0.50	14+/-0.56	0.019	0.000	-14.888
nuo-6	114	22.85+/-0.33	24+/-0.49	<0.001		34.660
nuo-6; cep-1	231	17.57+/-0.33	18+/-0.49	<0.001	0.000	3.353

In addition to lifespan changes, mitochondrial ETC mutants also develop slowly and display reduced brood sizes. To assess whether CEP-1 participates in the development of mitochondrial ETC mutants, the development time of *isp-1* and *mev-1* mutants with or without *cep-1* was compared to that of wild-type (wt) worms. Synchronized embryos of the various strains were allowed to develop at 20°C for 60 hours, and the number of adults and larvae were counted (Figure 2.1C, Table 2.2A). The data showed that wt and *cep-1* mutant worms developed at similar rates, and 100% of the populations had reached adulthood by 60 hr. As expected, the *isp-1* mutant worms grew slowly, and the majority were in the L1 (37%) and L2 (62%) stages after 60 hr. However, *cep-1*;*isp-1* double mutants developed noticeably faster, and the majority were L3s (40%), L4s (40%), and adults (12%) after 60 hr. These data suggest that *cep-1* inactivation partially recues the slow development of the *isp-1* mutant. Interestingly, *cep-1* inactivation exerted an opposite effect on *mev-1* mutant development. The *mev-1* mutants were slightly developmentally delayed, where the majority of *mev-1* mutant worms were in the L3 (6%) and L4 (93%) stages at 60 hr. The development rate of the *cep-1*;*mev-1* double mutant worms was heterogeneous and further delayed (L2 (11%), L3 (19%) and L4 (69%) at 60 hr) compared to *mev-1* single mutant worms.

To examine a possible role for CEP-1 in the reproduction of mitochondrial ETC mutants, the average brood size of *isp-1* and *mev-1* mutants with or without *cep-1* was compared to that of wt animals. The brood size of the *cep-1* mutant did not significantly differ from wt ($p = 0.1$), and, as expected, the mitochondrial mutants (*isp-1*, *mev-1*) displayed significantly lower brood sizes compared to wt ($p<0.0001$). The double mutants *cep-1*;*isp-1* and *cep-1*;*mev-1* exhibited a further brood size reduction compared to *isp-1* and *mev-1* single mutants, respectively ($p\leq0.05$) (Figure

2.1D, Table 2.2B). These results suggest that *cep-1* inactivation further exacerbates the reproductive defect associated with mitochondrial dysfunction.

Table 2.2: Rate of Development and brood size

A1. Rate of Develoment (exp.1) :

Stage	wt (av.% ± Std. Error)	*cep-1* (av.% ± Std. Error)	*isp-1* (av.% ± Std. Error)	*cep-1;isp-1* (av.% ± Std. Error)	*mev-1* (av.% ± Std. Error)	*cep-1;mev-1* (av.% ± Std. Error)
L1	0.00	0.00	37.93± 0.71	0.00	0.00	0.00
L2	0.00	0.00	62.07± 1.41	8± 1.41	0.00	11.11± 1.41
L3	0.00	0.00	0.00	40± 0	6.25± 1.41	19.44± 0.71
L4	0.00	0.00	0.00	40± 0	93.75± 2.83	69.44± 0.71
Adult	100.00	100.00	0.00	12± 0.71	0.00	0.00

A2. Rate of Development (exp.2):

Stage	wt(%)	*cep-1* (%)	*gas-1* (%)	*cep-1*;*gas-1*(%)
L1	0.0	0.0	0.0	0.0
L2	0.0	0.0	0.0	0.0
L3	0.0	0.0	0.0	0.0
L4	0.9	0.0	24.1	40.7
YA	0.9	0.9	75.9	59.3
GA	98.2	99.1	0.0	0.0

A3. Rate of Development (exp.3):

Stage	wildtype(%)	*cep-1* (%)	nuo-6 (%)	*cep-1*;nuo-6 (%)	*gas-1* (%)	*cep-1*;*gas-1*(%)
L1	0.0	0.0	12.7	3.6	0.0	0.0
L2	0.0	0.0	85.9	92.9	0.0	0.0
L3	0.0	0.0	1.4	3.6	0.0	0.0
L4	0.0	0.0	0.0	0.0	3.9	6.6
YA	10.2	3.4	0.0	0.0	96.1	93.4
GA	89.8	96.6	0.0	0.0	0.0	0.0

A4. Rate of Development (exp.4):

	wildtype (%)	nuo-6 (%)	*cep-1*;nuo-6(%)
L1	0.0	25.0	2.0
L2	0.0	73.1	81.6
L3	0.0	25.0	14.3
L4	0.0	0.0	2.0
YA	0.0	0.0	0.0
GA	100.0	0.0	0.0
N		52.0	49.0

Table 2.2 continued.
B. Brood size:

	average number± sd	*p* value to wt	*p* value to single mutants
wt	258.33± 20.12		
cep-1	280.29± 29.6	0.1	
isp-1	151± 8.49	<0.0001	
cep-1;isp-1	124.2± 24.89		0.05
mev-1	184.21± 36.59	<0.0001	
cep-1;mev-1	130± 34.13		0.0005

Taken together, *cep-1* appears to participate in multiple physiological outcomes of ETC mutants. *cep-1* activity promotes, at least partially, both the slower development and the longer lifespan of the *isp-1* mutant but is required to prevent further reproductive deterioration. In contrast, *cep-1* activity promotes a shortened *mev-1* mutant lifespan but is required for a relatively normal developmental rate. Similar to its role in *isp-1* reproduction, *cep-1* prevents reproductive decline in *mev-1* mutants. Since *cep-1* loss similarly impacts the reproductive success of *isp-1* and *mev-1* mutants, but its inactivation has opposing effects on the lifespan and developmental rate of these mutants, the role of *cep-1* in reproduction might be independent of its role in lifespan and development in ETC mutants.

2.3.2 CEP-1 mediates reduced physiological germline apoptosis in the long-lived *isp-1* mutant

CEP-1 is a well-established key regulator of stress-induced apoptosis (Derry et al., 2001). Since CEP-1 is a crucial mediator of the longevity outcomes of *isp-1* and *mev-1* mutants, we asked whether CEP-1 does so by modulating apoptosis in these mutants. While apoptosis occurs throughout embryonic and larval development in *C. elegans*, we reasoned that monitoring apoptosis in adults would be more relevant in investigating adult lifespan. In *C. elegans* adults, physiological and stress-induced apoptosis occurs in the germline. We monitored physiological apoptosis in the germline of wild-type, *isp-1* and *mev-1* mutants with or without *cep-1* inactivation. CEP-1 is best characterized for its role in stress-induced apoptosis, and our data indicated that loss of *cep-1* alone only mildly affected physiological apoptosis in the germline. The short-lived *mev-1* mutant and *mev-1*;*cep-1* double mutant exhibited wt levels of physiological germline apoptosis (Figure 2.2). Interestingly, the long-lived *isp-1* mutant displayed a significantly lower level of physiological germline apoptosis, which was completely

rescued in the *cep-1*;*isp-1* double mutant (Figure 2.2). These data suggest that CEP-1 protects against physiological germline apoptosis in the *isp-1* mutant. As CEP-1 has largely been demonstrated to promote apoptosis in *C. elegans*, this new role of CEP-1 in protecting against apoptosis merits further investigation.

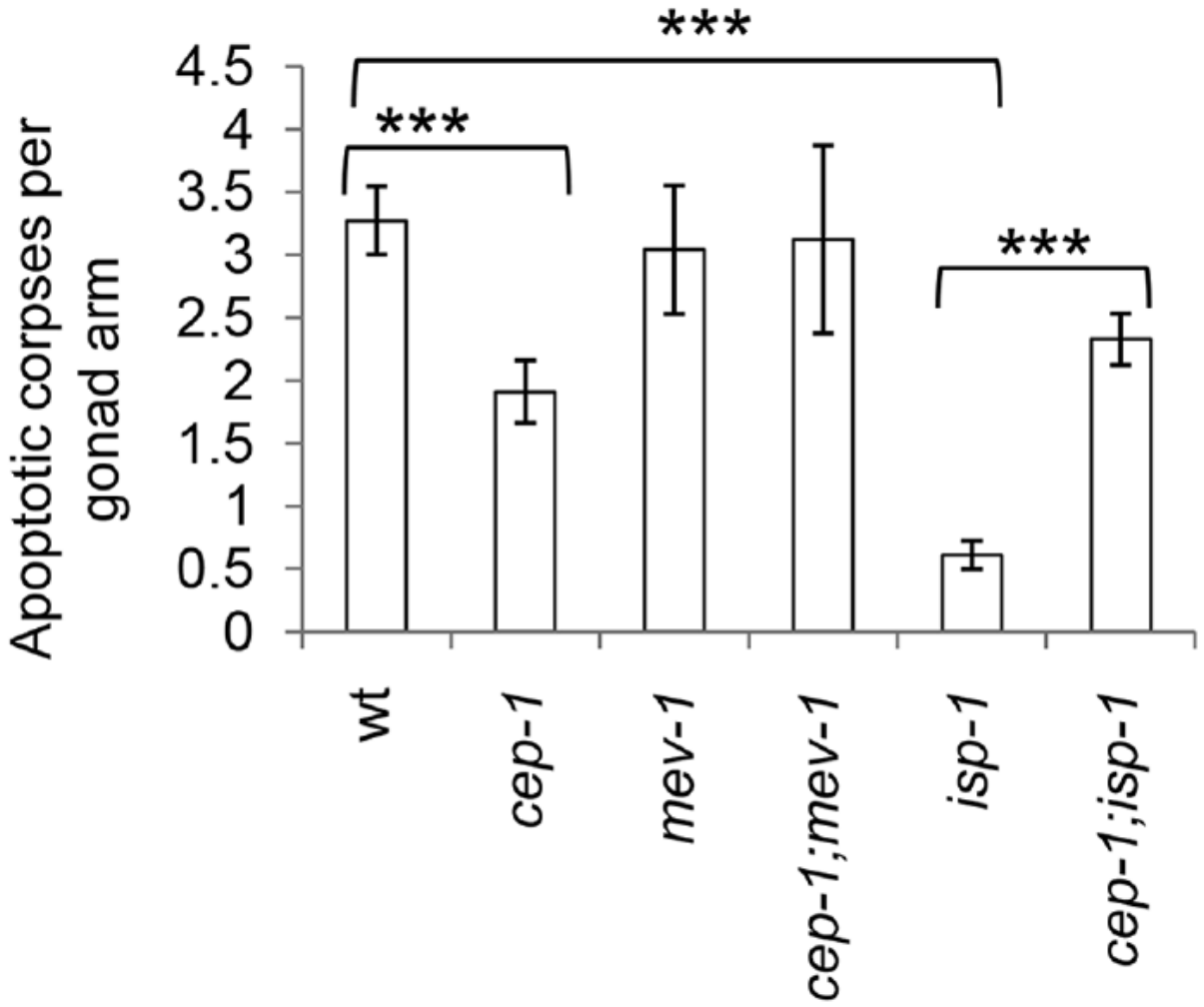


Figure 2.2 CEP-1 mediates reduced physiological germline apoptosis in the *isp-1* mutant. Physiological levels of apoptosis were quantified by counting the number of apoptotic corpses per gonad arm in various *C. elegans* strains. The corpses were counted using DIC microscopy at 636magnification 48 hr post L4. The data represent the average of at least 3 independent experimental replicates (n≥15 gonad arms for each) ±standard error. Statistical analysis was done using the Mann-Whitney U-test. ***p,0.001.

2.3.3 CEP-1-mediated transcriptional profiles in long-lived *isp-1* and short-lived *mev-1* mutants

CEP-1 appears to dually mediate the lifespan of mitochondrial mutants. We hypothesized that moderate mitochondrial dysfunction initiates a CEP-1-dependent defensive response that promotes longevity in long-lived mitochondrial mutants. Conversely, decreasing mitochondrial function beyond a threshold in short-lived mitochondrial mutants likely engages CEP-1 in a different way that results in a shortened lifespan. Since mammalian p53 is a well-established transcription factor, we sought to investigate the transcriptional response induced by CEP-1 in both a long-lived, *isp-1*, and a short-lived, *mev-1*, mitochondrial mutant. We hypothesized that the genes differentially regulated by CEP-1 between *isp-1* and *mev-1* mutants may mediate the distinct CEP-1 lifespans of the *isp-1* and *mev-1* mutants (see below). We compared the transcriptional profiles of synchronized *isp-1(qm150)* and *cep-1(gk138);isp-1(qm150)* young adults, as well as *mev-1(kn1)* and *cep-1(gk138);mev-1(kn1)*, using the Agilent 4×44K oligonucleotide microarray.

Although CEP-1 exerts opposing effects on the lifespans of *isp-1* and *mev-1* mutants, hierarchical gene cluster analysis revealed that the CEP-1-regulated transcriptional profiles in long-lived *isp-1* and short-lived *mev-1* mutants were largely similar (correlation coefficient of 0.58) (Figure 2.3A). Only a small number of genes exhibited *cep-1*-dependent differential regulation. Data analysis using the statistical tool SAM (Significance Analysis of Microarray) with a FDR (false discovery rate) of 0.5% revealed that CEP-1 regulated the expression of 3,404 genes in a similar manner in the long-lived *isp-1*(qm150) and short-lived *mev-1*(kn1) mutants (Figure 2.3B). SAM analysis with a FDR of 1% followed by a gene list comparison also revealed 71 genes (Table 2.3, see Materials & Methods for details on gene list filtering) that were

differentially regulated by CEP-1 in the *isp-1*(qm150) and *mev-1*(kn1) mutants (Figure 2.3B). The expression of these genes largely differed quantitatively rather than qualitatively, i.e., they generally showed a greater CEP-1-mediated regulation in the *isp-1* or *mev-1* mutant backgrounds.

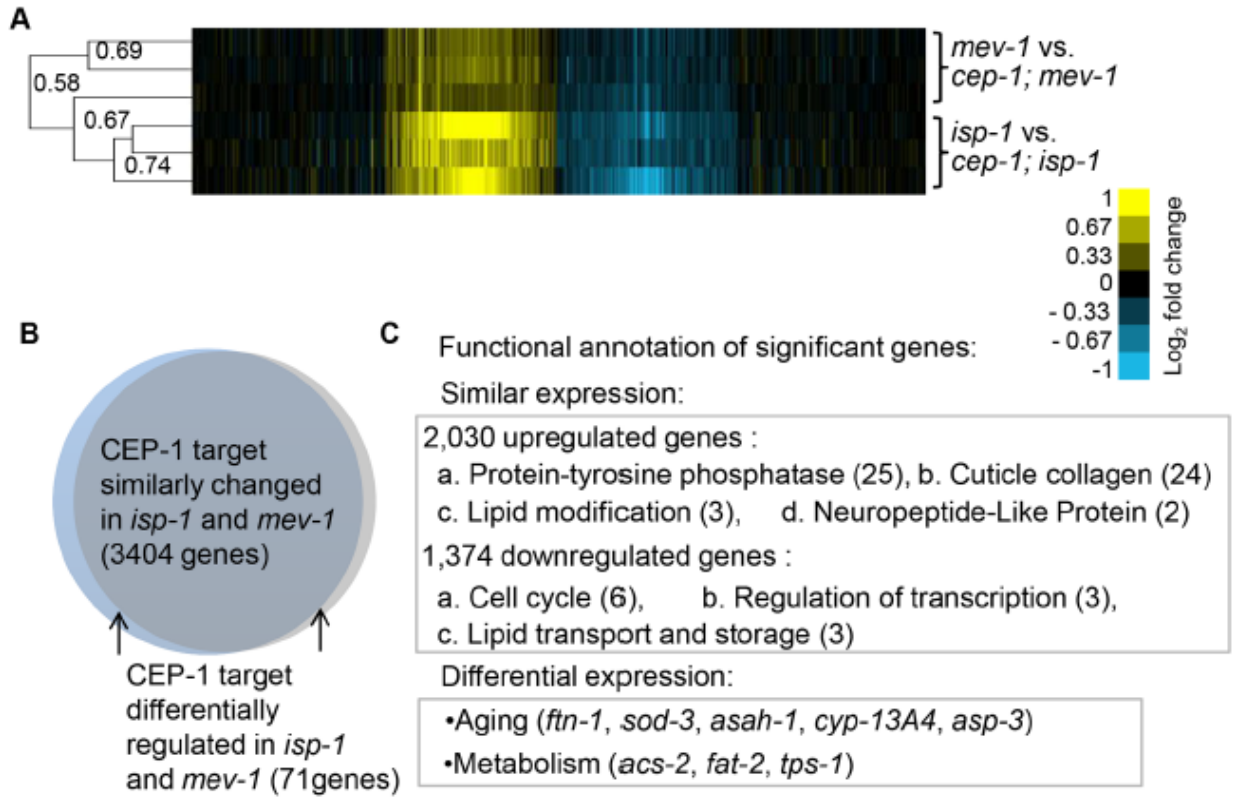


Figure 2.3 CEP-1-regulated transcriptomes in *isp-1* and *mev-1* mutants.
(A) CEP-1-regulated genes in *isp-1* and *mev-1* mutants are largely similar. Hierarchical single linkage gene clustering was performed, and the dendrogram shows the clustered relationship of individual arrays. The numbers on the dendrogram represent the correlation coefficients between arrays. Yellow: upregulated, Blue: downregulated, Black: no change. (B) The expression of CEP-1-regulated genes that were significantly changed in *isp-1* and *mev-1* mutants, identified by SAM analysis, are represented in the Venn diagram. (C) DAVID functional annotation of similarly and differentially expressed CEP-1-regulated genes in *isp-1* and *mev-1* mutants. The numbers represent the enrichment scores for each group (score.1.3 is considered as significant). Several examples of aging and metabolic genes are listed.

Table 2.3: The CEP-1 regulated genes that are differentially changed in the *isp-1* and *mev-1* mutants

GENE ID	gene name/ description
AH10.1	*acs-10 (fatty Acid CoA Synthetase family)*
C01B10.6	*protein coding*
C04E12.4	*protein coding*
C07G1.7	*protein coding*
C08A9.1	*sod-3 (SOD (superoxide dismutase))*
C08E3.1	*protein coding*
C12D8.14	*fipr-4 (FIP (Fungus-Induced Protein) Related)*
C14A6.10	*pseudogene*
C14C6.2	*protein coding*
C14C6.5	*protein coding*
C17D12.3	*protein coding*
C18H7.11	*protein coding*
C25A8.5	*protein tyrosine kinase*
C32A3.2	*mrps-31 (Mitochondrial Ribosomal Protein, Small)*
C44B7.10	*protein coding*
C45B2.2	*protein coding*
C52A10.2	*carboxylesterase*
C54F6.14	*ftn-1 (FerriTiN)*
D2096.3	*aagr-1 (Acid Alpha Glucosidase Relate)*
F09B12.3	*protein coding*
F09C8.1	*phospholipase precursor*
F10F2.4	*lron-5 (eLRR (extracellular Leucine-Rich Repeat) ONly)*
F19C7.2	*Lysosomal carboxypeptidase*
F19C7.4	*Lysosomal carboxypeptidase*
F26F12.1	*col-140 (COLlagen)*
F27C8.4	*spp-18 (SaPosin-like Protein family)*
F28B4.3	*EFG like repeat*
F28F8.2	*acs-2 (fatty Acid CoA Synthetase family), mitochondrial*
F42G8.8	*ser/ thr protein phosphatase*
F47B8.1	*protein coding*
F47G9.3	*cutl-18 (CUTiclin-Like)*
F48G7.5	*protein coding*
F53C3.12	*bcmo-2 (Beta-Carotene 15,15'-MonoOxygenase)*

Table 2.3 continued.

F53E10.5	*protein coding*
F53F4.5	*fmo-4 (Flavin-containing MonoOxygenase family)*
F55A11.11	*histidine phosphatase superfamily*
F56D6.15	*clec-69 (C-type LECtin)*
F56D6.2	*clec-67 (C-type LECtin)*
H22K11.1	*asp-3 (ASpartyl Protease)*
H34I24.2	*protein coding*
K04A8.3	*protein coding*
K07C6.5	*cyp-35A5 (CYtochrome P450 family)*
K08H10.1	*lea-1 (plant Late Embryo Abundant (LEA) related)*
K10C2.3	*aspertyl protease*
K10D3.2	*unc-14 (UNCoordinated)*
K11D12.3	*srr-4 (Serpentine Receptor, class R)*
K11D2.2	*asah-1 (AcylSphingosine AmidoHydrolase)*
K11G9.2	*esterase*
R05H10.7	*protein coding*
T05A1.2	*col-122 (COLlagen)*
T10B9.1	*cyp-13A4 (CYtochrome P450 family), dod-1*
T10B9.2	*cyp-13A5 (CYtochrome P450 family)*
T22B7.3	*protein coding*
T24D8.5	*nlp-2 (Neuropeptide-Like Protein)*
W02A2.1	*fat-2 (FATty acid desaturase)*
W09C3.2	*protein coding*
Y105C5A.12	*protein coding*
Y105C5B.15	*protein coding*
Y37A1B.5	*protein coding*
Y37B11A.2	*protein coding*
Y41C4A.16	*col-95 (COLlagen)*
Y41E3.2	*dpy-4 (DumPY: shorter than wild-type)*
Y47G6A.33	*protein coding*
Y49G5A.1	*trypsin inhibitor*
Y66A7A.5	*ceh-91 (C. elegans Homeobox)*
Y67D8B.5	*protein coding*
Y69A2AR.23	*protein coding*
Y75B8A.29	*zip-12 (bZIP transcription factor family)*

Table 2.3 continued.

ZC412.7	*nspa-3 (Nematode Specific Peptide family, group A)*
ZK54.2	*tps-1 (Trehalose 6-Phosphate Synthase)*
ZK945.7	*protein coding*

To validate our microarray findings, we selected six genes (F15E6.8/dct-7, C37A5.2/fipr-22, F28C6.1/AP-2 like TF, C52D10.6/skr-12, C08A9.1/sod-3 and F57B9.9/abu-13) based on their classification by SAM analysis and performed quantitative reverse transcription PCR (qRT-PCR) using an independent biological sample. The expression changes of all six genes echoed the microarray results (Figure 2.4).

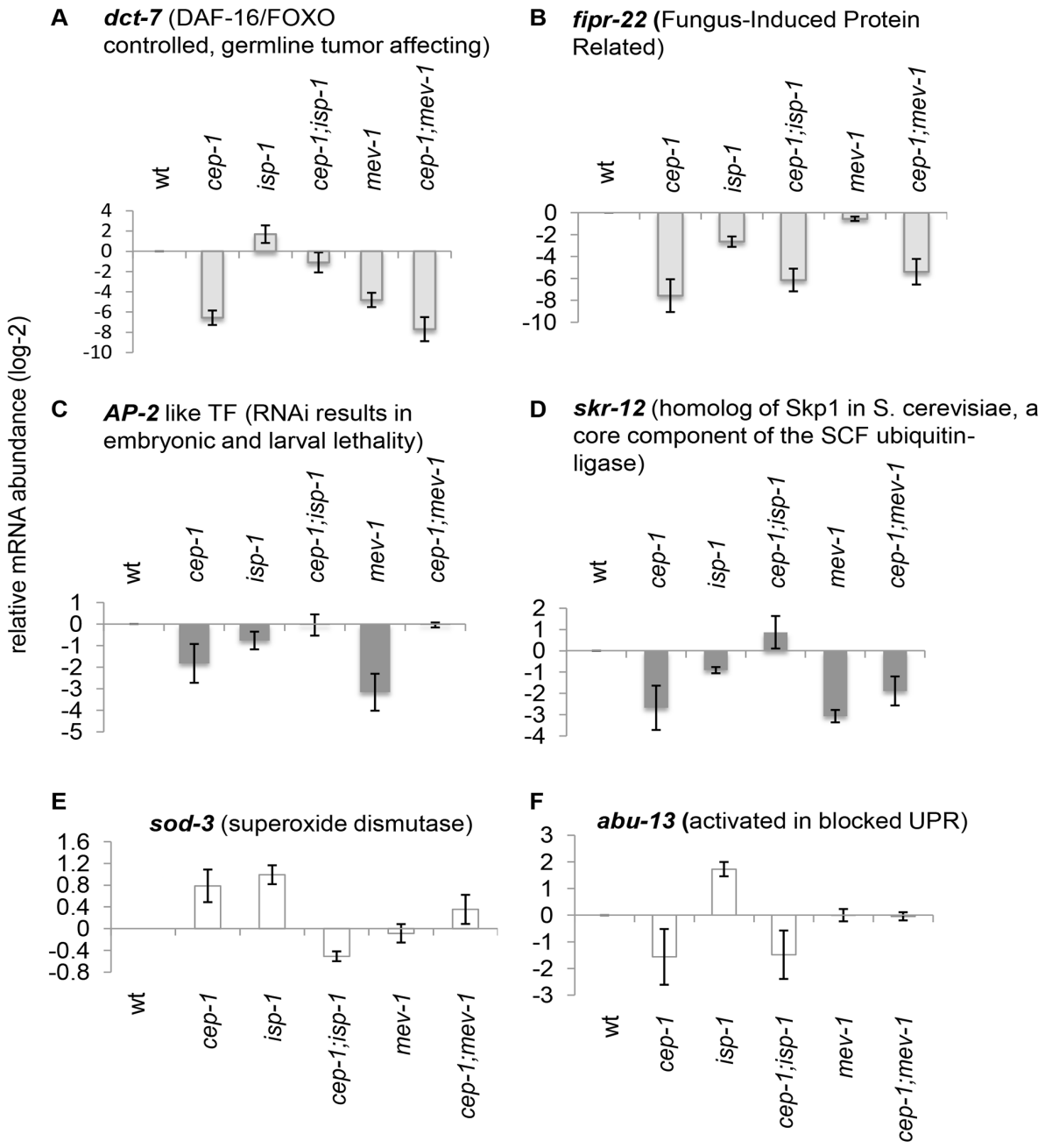


Figure 2.4 Validation of microarray results using qRT-PCR.

(A, B) *dct-7* and *fipr-22* represent genes positively regulated by CEP-1 in *isp-1* and *mev-1* mutants. (C, D) *AP-2* and *skr-12* represent genes negatively regulated by CEP-1 in *isp-1* and *mev-1* mutants. (E, F) *sod-3* and *abu-13* represent genes that are differentially regulated by CEP-1 in *isp-1* and *mev-1* mutants. The relative expression of each gene was normalized to *act-1*. The log2 ratios of the average expression for each gene compared to wt from three independent experiments are plotted. Error bars represent standard errors.

To examine the biological processes of the CEP-1-regulated genes identified by SAM, we performed Gene Ontology (GO) analysis using DAVID (Database for Annotation, Visualization, and Integrated Discovery). We focused on the GO term categories that were most significantly enriched in our dataset compared to the distribution in the *C. elegans* genome. Interestingly, the small group of CEP-1-regulated genes that differentially changed in *isp-1* and *mev-1* mutants were enriched for genes with known roles in aging and metabolism (Figure 2.3C). This finding supports our hypothesis that the genes differentially regulated by CEP-1 in *isp-1* and *mev-1* mutants are likely particularly important for the opposing effects on lifespan that CEP-1 exerts in these mutants. The genes that were similarly regulated by CEP-1 in *isp-1* and *mev-1* mutants likely account for the effects CEP-1 has on development, reproduction, and other physiological changes in these mutants and might also contribute to the final longevity (Figure 2.3C).

2.3.4 The CEP-1-regulated gene *ftn-1* contributes to the longer lifespan of the *isp-1* mutant

The microarray results helped narrow our analysis to a small number of CEP-1-regulated genes (Figure 2.3C) that might contribute to the dual effects CEP-1 exerts on the longevity of mitochondrial mutants with varying degrees of dysfunction. One of these genes, *ftn-1*, encodes the *C. elegans* homolog of the ferritin heavy chain. Using qRT-PCR, we observed that *ftn-1* expression was repressed ~2 fold in the *cep-1* mutant and induced ~1.5 fold in the *isp-1* mutant compared to wt. The *cep-1*;*isp-1* double mutant displayed a similar repressed level of *ftn-1* expression as observed for the *cep-1* single mutant (Figure 2.5A). While *ftn-1* expression was induced in the *mev-1* mutant, similar to the *isp-1* mutant, this induction was not changed in the *cep-1*;*mev-1* double mutant, consistent with the microarray results. Inspection of the *ftn-1*

upstream sequence did not reveal a known p53 binding motif, suggesting that CEP-1 might not directly regulate *ftn-1* transcription. *C. elegans* harbors another *ftn-1* homolog, ftn-2, but its expression was not changed in the ETC mutants compared to wt (Figure 2.5B). Lastly, we used the P*ftn-1*::gfp strain, where a GFP reporter is fused to the *ftn-1* promoter (Ackerman and Gems, 2012), to assess whether the microarray and qRT-PCR results translated into observably meaningful *ftn-1* expression changes. *Pftn-1::gfp* was substantially induced in *isp-1* mutants and was completely suppressed in *cep-1*;*isp-1* double mutants (Figure 2.6), consistent with our microarray and qRT-PCR results. Additionally, *Pftn-1::gfp* expression was slightly induced in *mev-1* mutants and unchanged in *cep-1*;*mev-1* double mutants. Taken together, our data indicate that CEP-1 is important for ferritin regulation in wild type and *isp-1* mutants but is dispensable in the *mev-1* mutant.

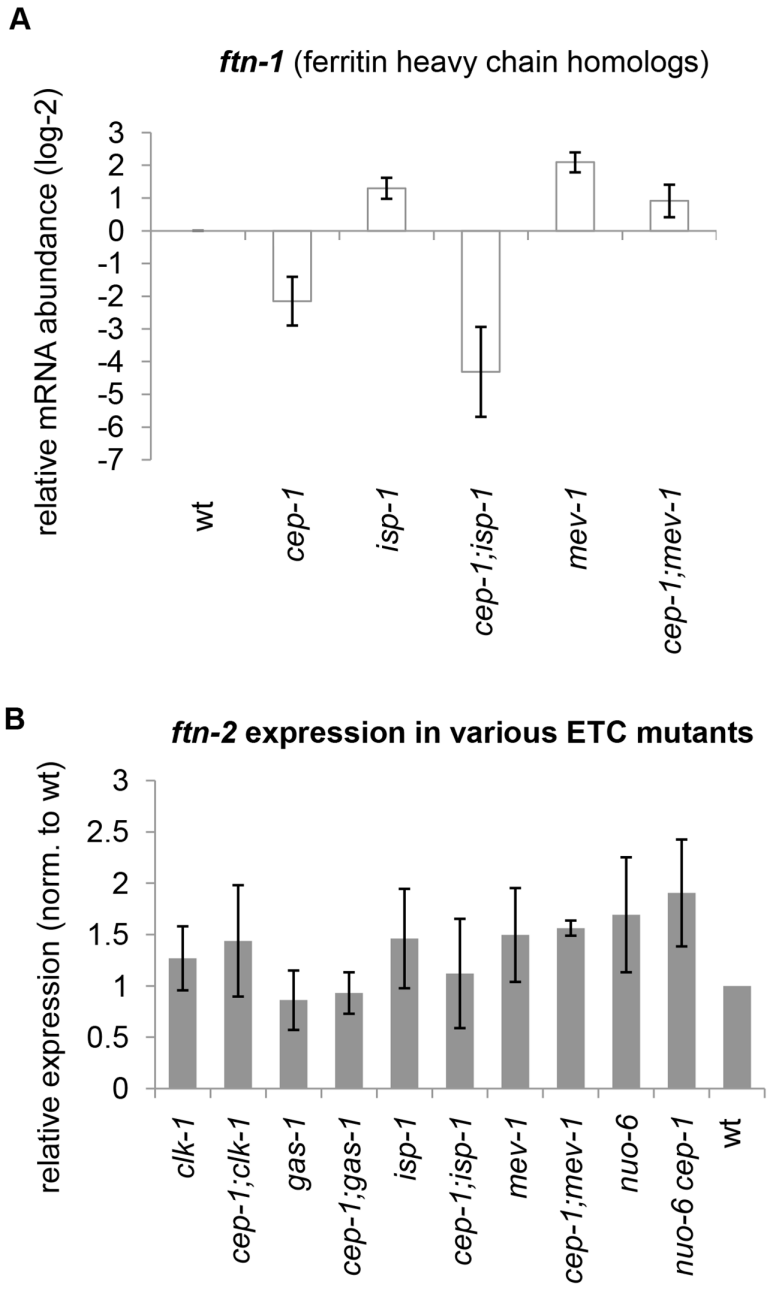


Figure 2.5 Expression analysis of *ftn-1* and *ftn-2* using qRT-PCR.
(A) *ftn-1* expression in each mutant strain was normalized to *act-1*. The log2 ratios of the average expression for *ftn-1* compared to wt from three independent experiments are plotted. Error bars represent standard errors. (B) Expression of *ftn-2* in various ETC mutants. The relative expression of each gene was normalized to *act-1*. The average expression ratios for each gene compared to wt from at least two independent experiments are plotted. Error bars represent standard errors.

Ferritin regulates the storage and release of iron. As iron homeostasis is known to play an important role during mitochondrial dysfunction, we wanted to investigate whether ferritin regulation was responsible for the dual roles of CEP-1 in mitochondrial ETC mutant longevity. To assess whether ferritin upregulation promoted *isp-1* mutant longevity, we examined the lifespan of *isp-1* and *cep-1*;*isp-1* mutants after *ftn-1* and ftn-2 knockdown by RNAi. We knocked down *ftn-1* and ftn-2 simultaneously to prevent possible functional redundancy, which may preclude observable phenotypes when either gene is knocked down alone. Double RNAi-mediated knockdown of *ftn-1* and ftn-2 significantly suppressed the extended lifespan of *isp-1* mutants, although not to the same degree as *cep-1* inactivation. Importantly, *ftn-1*/2 RNAi did not further suppress the lifespan of the *cep-1*;*isp-1* double mutant (Figure 2.6B), suggesting that *cep-1* and *ftn-1*/2 act in the same genetic pathway to mediate *isp-1* longevity, corroborating our model that *ftn-1*/2 are downstream targets of CEP-1. Consistent with our expression results suggesting that *ftn-1*/2 are not regulated by CEP-1 in the short-lived *mev-1* mutant, the *mev-1* mutant lifespan was not affected by *ftn-1*/2 RNAi (Figure 2.6C).

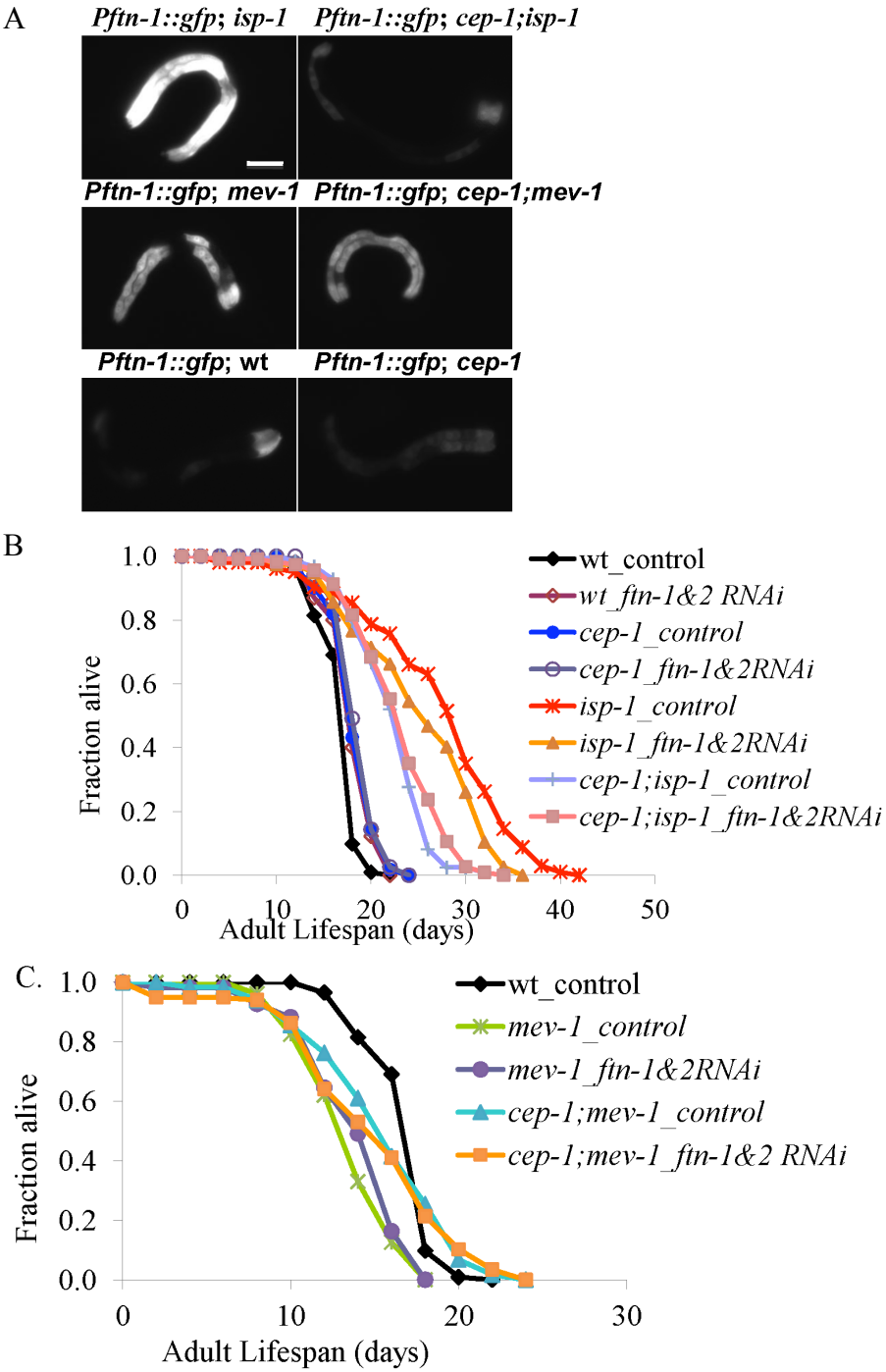


Figure 2.6 CEP-1-regulated ferritin inductions partially mediates the extended lifespan of *isp-1* mutants.

(A) *Pftn-1::gfp* expression in wildtype, *cep-1*, *isp-1*, *cep-1*;*isp-1*, *mev-1*, and *cep-1*;*mev-1* mutant worms. Scale bar = 100 mm. (B, C) The lifespans of wt, *cep-1*, *isp-1*, *cep-1*;*isp-1*, *mev-1* and *cep-1*;*mev-1* mutant worms treated with *ftn-1* and *ftn-2* double RNAi. L4440 is a treatment control.

2.3.5 CEP-1 is key to physiological changes in multiple ETC mutants

Given the important role CEP-1 plays in determining the lifespan of *isp-1* and *mev-1* mutants, we next tested whether *cep-1* is generally required for the longevity of additional ETC mutants that have been well characterized in *C. elegans*. We explored the long-lived nuo-6(qm200) mutant, which harbors a point mutation in the NADH-ubiquinone oxidoreductase of complex I, the long-lived *clk-1*(e2519) mutant, which harbors a point mutation in a coenzyme Q biosynthesis enzyme, and the short-lived *gas-1*(fc21) mutant, which has a point mutation in the NADH:ubiquinone oxidoreductase NDUFS2 subunit of complex I. The *cep-1* null mutation largely suppressed the long-lived phenotype of the nuo-6 mutant but did not affect the longevity of the *clk-1* mutant, as the *cep-1*;*clk-1* double mutant lived as long as the *clk-1* single mutant (Figure 2.7A, Figure 2.7B). Furthermore, *cep-1* deletion rescued the short-lived phenotype of the *gas-1* mutant, similar to its effect on the *mev-1* mutant lifespan (Figure 2.7C). Taken together, the data suggest that CEP-1 crucially participates in the longevity outcome of multiple ETC mutants.

The fact that *cep-1* abrogation did not affect the *clk-1* mutant lifespan was intriguing. All of the well-characterized mitochondrial ETC mutants in *C. elegans* (*isp-1*, nuo-6, *mev-1*, and *gas-1*), with the exception of *clk-1*, have been shown to harbor elevated levels of mitochondrial superoxide. In fact, antioxidant treatment of all of these mutants, again with the exception of *clk-1*, has been shown to suppress (the long-lived) *isp-1* and nuo-6 or rescue (the short-lived) *mev-1* and *gas-1* mutant lifespans. Therefore, the *cep-1*-mediated lifespans of these mitochondrial ETC mutants parallel the proposed

roles that elevated ROS is exerting in these mutants, i.e., CEP-1 mediates the lifespans of mutants with elevated ROS but not of mutants without. This observation suggests the interesting possibility that CEP-1 might somehow be linked to the ROS-mediated longevity increases observed in the long-lived mitochondrial ETC mutants. Future studies aimed at thoroughly investigating the relationship between CEP-1 and ROS-mediated longevity will likely yield fruitful insights.

As *cep-1* impacted the development rates of *isp-1* and *mev-1* mutants, we tested whether *cep-1* similarly affected the development of nuo-6 and *gas-1* mutants. Our data indicated that *cep-1* deletion had little impact on nuo-6 and *gas-1* mutant development (Figure 2.7D), unlike what we found for *isp-1* and *mev-1* mutants (Figure 2.1C). Therefore, whereas *cep-1* mediates the lifespans of all four ETC mutants tested here (*isp-1*, nuo-6, *mev-1*, and *gas-1*), its involvement in the development of each of the mutants is mixed, suggesting that the role of CEP-1 in development may be distinct from its role in longevity in these various mutants.

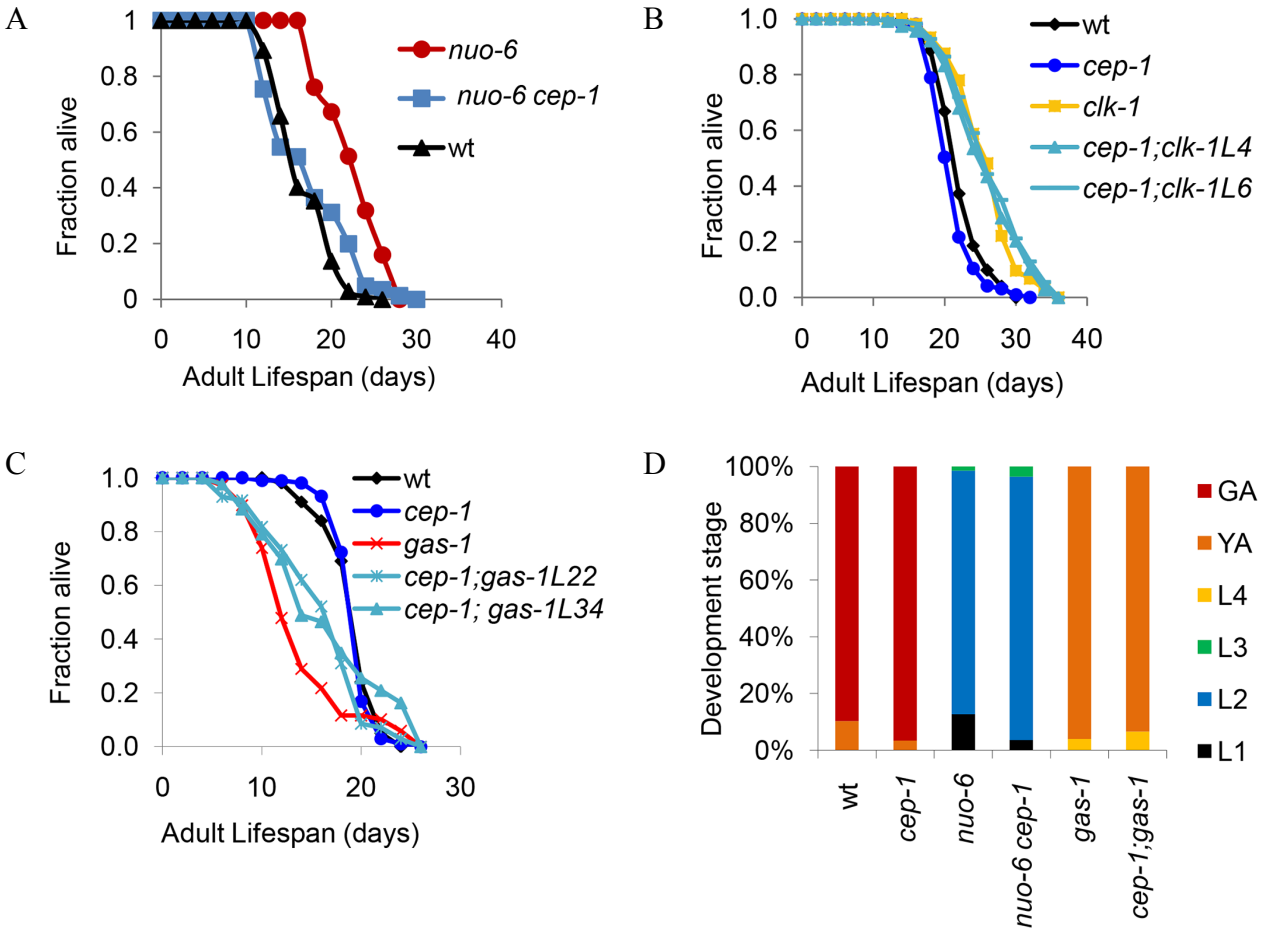


Figure 2.7 CEP-1 mediates the longevity and development of several mitochondrial mutants in *C. elegans*.
(A) *cep-1* mutation fully suppresses the long lifespan of the nuo-6 mutant. (B) *cep-1* mutation does not suppress *clk-1* mutant longevity as the lifespans of two *cep-1*;*clk-1* double mutant isolates (L4, L6) are similar to that of the *clk-1* single mutant. (C) *cep-1* mutation partially restores *gas-1* mutant lifespan as two isolates of *cep-1*;*gas-1* live longer than the *gas-1* single mutant. (D) The percentage of worms at each developmental stage was quantified as described in Figure 2.1C. *cep-1* deletion has little impact on nuo-6 and *gas-1* mutant development.

Given that we observed an important role for CEP-1 in *ftn-1* regulation and iron homeostasis in ETC mutant longevity, we further tested whether *ftn-1* & *ftn-2* are regulated by CEP-1 in *nuo-6* and *gas-1* mutants and whether *ftn-1/2* are required for their longevity. Strikingly, *ftn-1* was greatly induced in the *nuo-6* mutant, even more so than in the *isp-1* mutant (Figure 2.8C). Somewhat surprisingly, *cep-1* was not required for this induction, unlike in the *isp-1* mutant. We also demonstrated that RNAi-mediated depletion of *ftn-1/2* substantially suppressed the extended lifespan of the nuo-6 mutant, similar to their depletion in the *isp-1* mutant (Figure 2.8A). Although CEP-1 is essential for *ftn-1* induction in the *isp-1* mutant, it is dispensable in the nuo-6 mutant; however, both *cep-1* and *ftn-1*/2 are important for the longevity of nuo-6 and *isp-1* mutants. Therefore, while iron homeostasis and *cep-1* are both important for longevity determination in the long-lived *isp-1* and nuo-6 mutants, CEP-1 regulates *ftn-1* only in the *isp-1* mutant. We hypothesize that another transcription factor likely regulates *ftn-1* expression in the nuo-6 mutant. Interestingly, *ftn-1* was induced in the short-lived *gas-1* mutant independently of *cep-1*, similar to that in the short-lived *mev-1* mutant (Figure 2. 8C). Additionally, depleting *ftn-1*/2 did not rescue the shortened lifespan of the *gas-1* mutant, similar to that observed in the *mev-1* mutant (Figure 2.8B). Therefore, iron homeostasis might be a determinant of *isp-1* and nuo-6 longevity, but it does not appear to be important for mediating the lifespans of *gas-1* and *mev-1* mutants. Lastly, *ftn-1* was not induced in the *clk-1* mutant, another indication that *clk-1* mutant might engage a different mechanism to extend lifespan compared to the other mitochondrial ETC mutants studied here.

The distinct *ftn-1* results led us to consider more broadly whether *isp-1* & nuo-6 and *gas-1* & *mev-1* share some common molecular signatures, which could account for

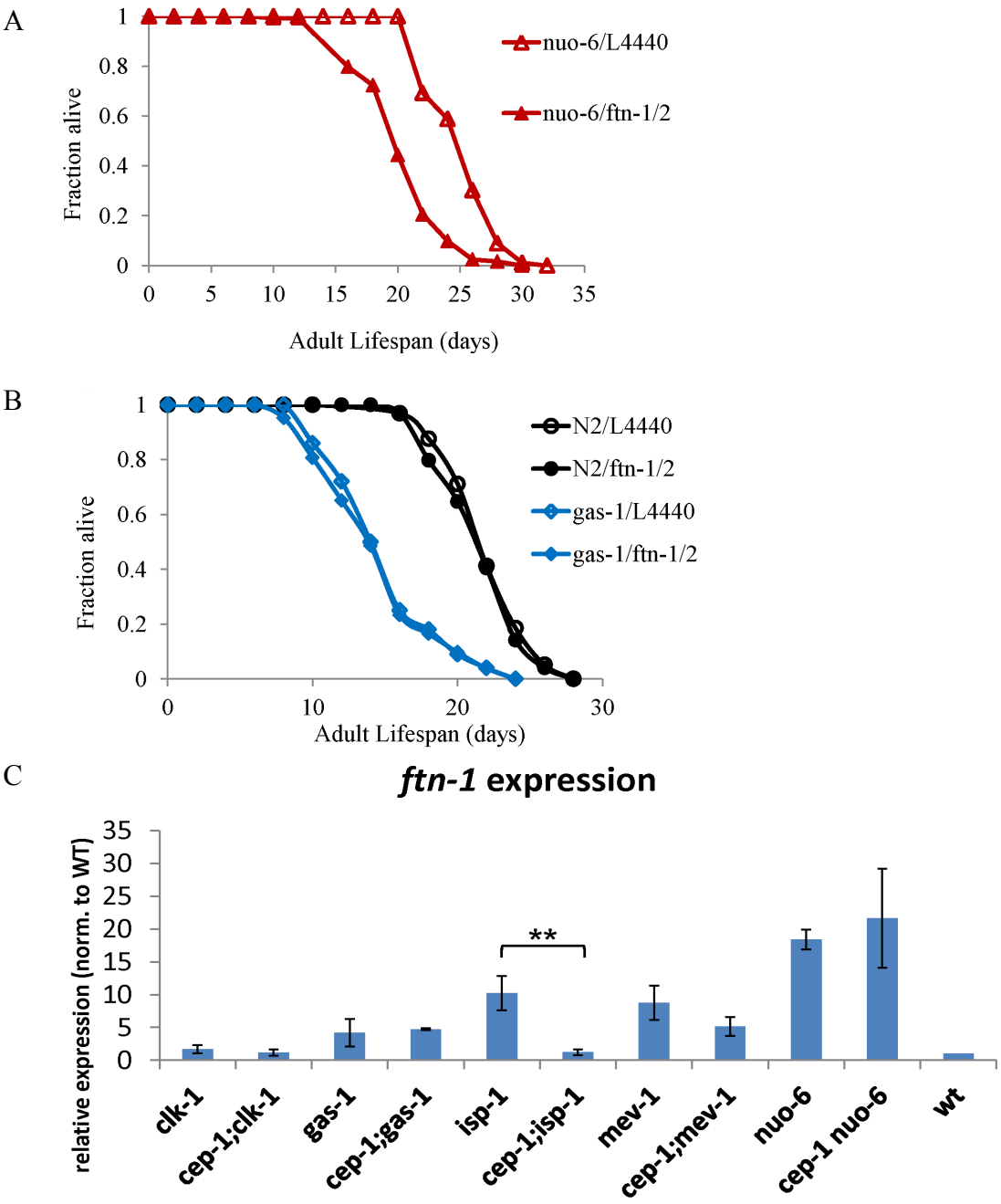


Figure 2.8 *ftn-1* is differentially expressed in various ETC mutants and mediates their lifespan outcomes.
(A, B) RNAi mediated knockdown of *ftn-1* attenuates the long life of the nuo-6 mutant but does not impact the lifespan of the short-lived *gas-1* mutant. (C) qRT-PCR results of *ftn-1* expression levels in various ETC mutants. The two-tailed student t-tests were performed to determine significant difference in *ftn-1* expression levels with and without CEP-1 in each ETC mutants.

their similar longevity (extended or shortened, respectively). We examined the expression of a handful of CEP-1-regulated genes that we identified earlier in *isp-1*/*mev-1* mutants. Using qRT-PCR, we first analyzed the expression of nine genes that were differentially regulated by CEP-1 in *isp-1* and *mev-1* mutants (Figure 2.9). Overall, we observed very different patterns for how these genes responded to the absence of *cep-1* in the two long-lived mutants (*isp-1* and nuo-6). The majority of genes that showed substantial *cep-1*-dependent induction in *isp-1* mutants barely changed when *cep-1* was depleted in nuo-6 mutants; only two genes (*cyp-35A5, F09C8.1*) showed consistent, but moderate, *cep-1*- dependent expression changes between *isp-1* and nuo-6. Interestingly, the response of these target genes to *cep-1* depletion was more similar between the two short-lived mutants (*mev-1* and *gas-1*). However, it is worth noting that the expression of the majority of these genes barely changed in *mev-1* and *gas-1* mutants in response to *cep-1* deletion (Figure 2.9).

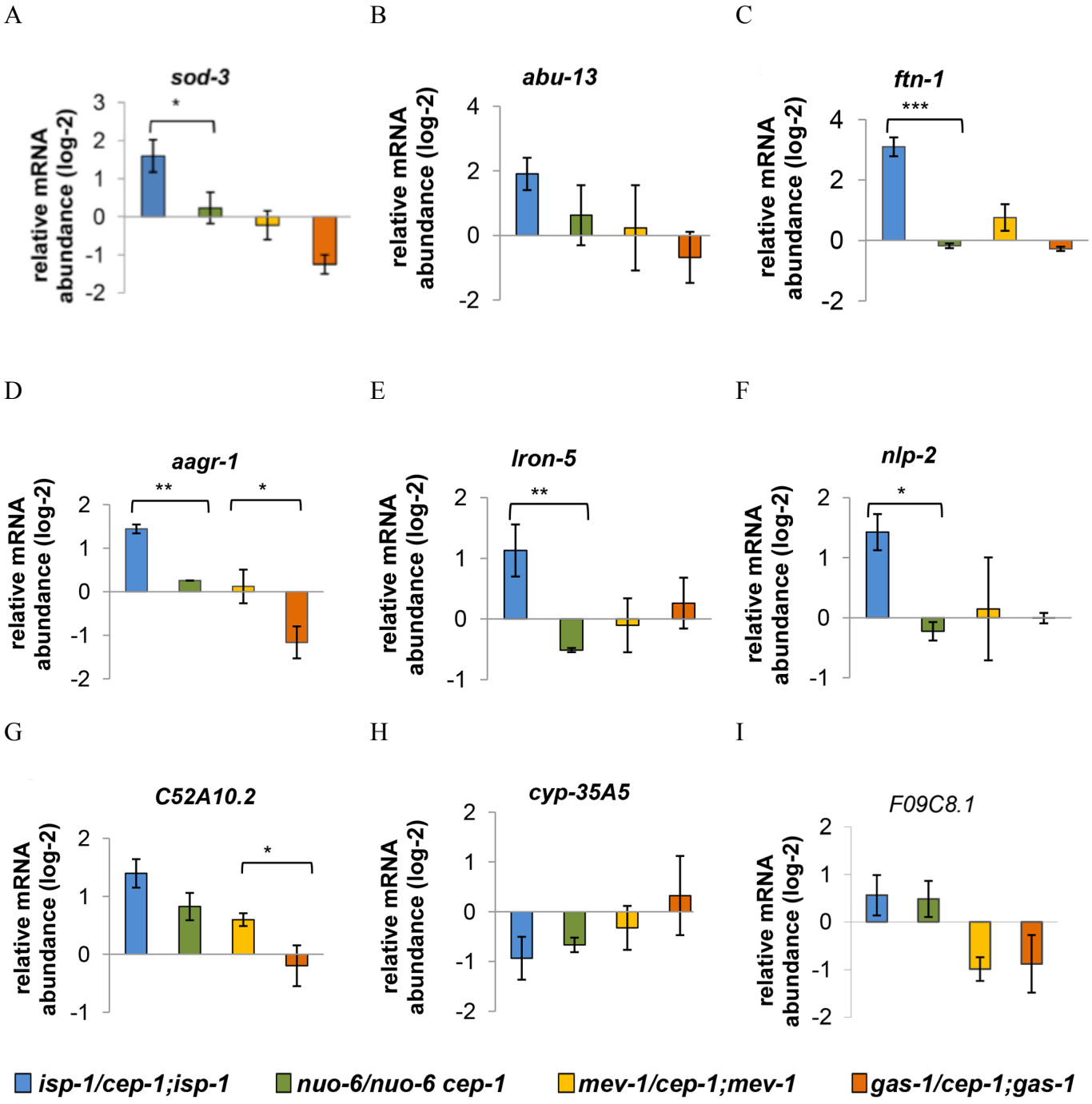


Figure 2.9 Expression of differentially regulated CEP-1 targets in other ETC mutants.
(A–I) These genes are differentially regulated by CEP-1 between *isp-1* and *mev-1* mutants. The relative expression of each gene was normalized to act-1 and wt. The average log2 ratio between ETC mutants with and without *cep-1* from three independent experiments are plotted. The error bars represent standard errors. Two-tailed t-tests were performed to determine significant differences of the expression of CEP-1 gene targets between the long-lived *isp-1*(qm150) and nuo-6(qm200) mutants and the short-lived *mev-1*(kn1) and *gas-1*(fc21) mutants. *p,0.05, ** p,0.01, *** p,0.001.

We also assayed the expression of genes that were similarly regulated by CEP-1 in *isp-1* and *mev-1* mutants and observed a similarly discordant pattern (Figure 2.10). The regulation of these genes by CEP-1 was strikingly different between *isp-1* and nuo-6 mutants but was much more similar between *mev-1* and *gas-1* mutants. In the absence of further genome-wide analysis, it is difficult to estimate the extent of common targets that are shared between (the long-lived) *isp-1* and nuo-6 and between (the short-lived) *mev-1* and *gas-1* mutants. However, based on the small number of genes tested here using qRT-PCR, it appears that CEP-1 regulates some common genes between *mev-1* and *gas-1* mutants, which might account for its ability to restore a normal lifespan in these short-lived mutants. For the long-lived mutants, the situation is more complex; CEP-1 appears to regulate largely distinct genes between *isp-1* and nuo-6 even though it is required for the extended lifespan of both mutants.

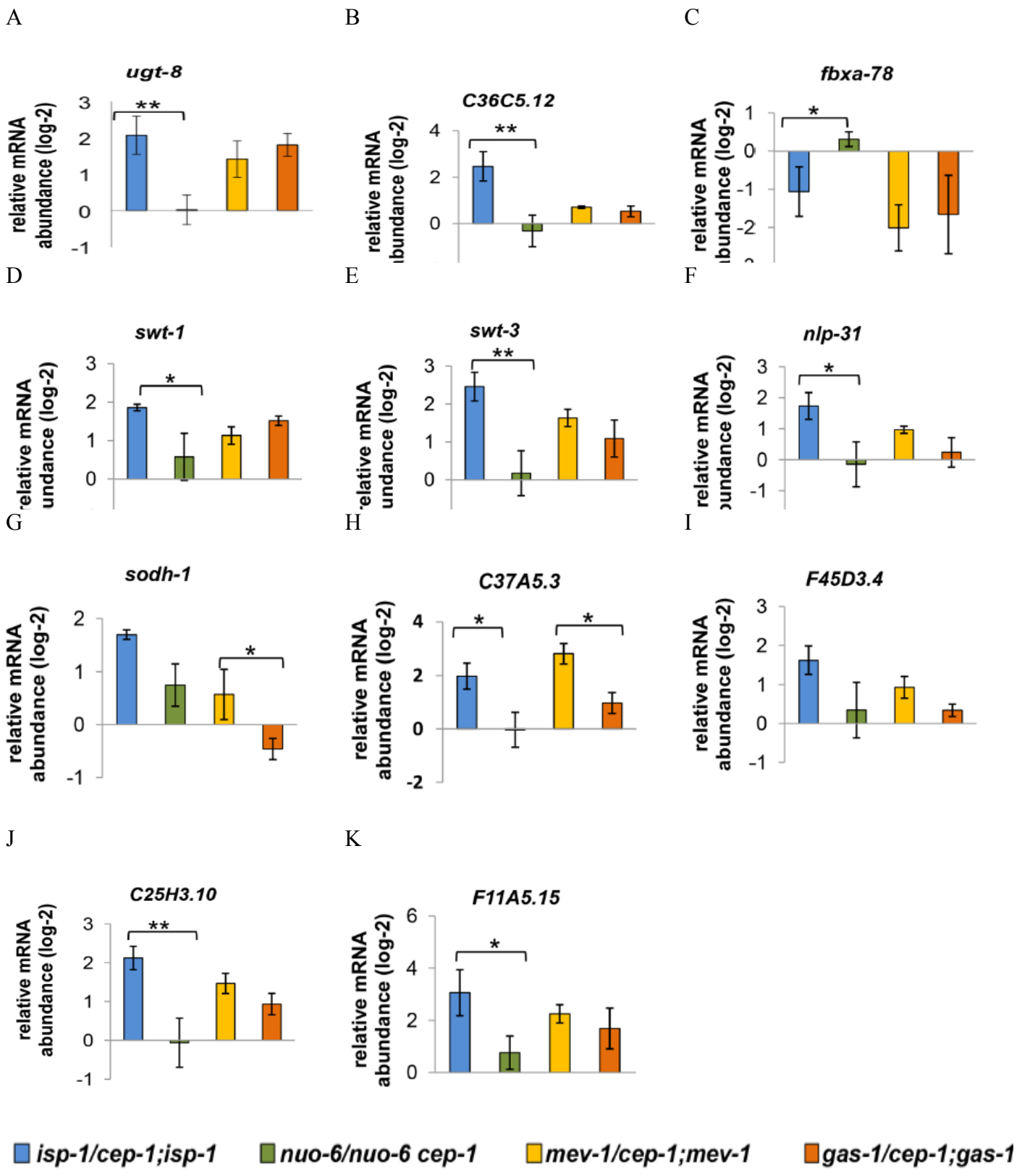

A
ugt-8
relative mRNA abundance (log-2)
B
C36C5.12
relative mRNA abundance (log-2)
C
fbxa-78
relative mRNA abundance (log-2)
D
swt-1
relative mRNA abundance (log-2)
E
swt-3
relative mRNA abundance (log-2)
F
nlp-31
relative mRNA abundance (log-2)
G
sodh-1
relative mRNA abundance (log-2)
H
C37A5.3
relative mRNA abundance (log-2)
I
F45D3.4
relative mRNA abundance (log-2)
J
C25H3.10
relative mRNA abundance (log-2)
K
F11A5.15
relative mRNA abundance (log-2)
isp-1/cep-1;isp-1
nuo-6/nuo-6 cep-1
mev-1/cep-1;mev-1
gas-1/cep-1;gas-1

Figure 2.10 Expression of similarly regulated CEP-1 targets in other ETC mutants.
(A–K) These genes are similarly regulated by CEP-1 between *isp-1* and *mev-1* mutants. The relative expression of each gene was normalized to *act-1* and wt. The average log2 ratio between ETC mutants with and without *cep-1* from three independent experiments are plotted. The error bars represent standard errors. Two-tailed t-tests were performed to determine significant differences of the expression of CEP-1 target genes between the long-lived *isp-1*(qm150) and nuo-6(qm200) mutants and the short-lived *mev-1*(kn1) and *gas-1*(fc21) mutants. * p,0.05, ** p,0.01, *** p,0.001.

2.3.6 The CEP-1-regulated transcriptional profiles of mitochondrial dysfunctional mutants are similar to those in worms exposed to UV irradiation

The best-characterized role of mammalian p53 is its ability to respond to DNA damage by inducing cell cycle arrest and repair or apoptosis. We wondered whether the CEP-1-regulated transcriptome changes in mitochondrial mutants would resemble changes observed in response to DNA damage. The global transcriptional profiles of wt or *cep-1* mutant worms treated with UV, gamma, or x-ray irradiation have previously been published (Derry et al., 2007; Greiss et al., 2008). Using clustering analysis, we compared the CEP-1-dependent global expression profiles in response to mitochondrial dysfunction (in *isp-1* or *mev-1* mutants) to profiles 4 hr after exposure to UV irradiation, 6 hr after gamma-ray, or 2 hr after X-ray (Figure 2.11). We observed a higher similarity between the CEP-1-regulated gene expression profiles in response to mitochondrial dysfunction and after UV treatment (correlation coefficient of 0.36) but less after gamma-ray treatment (correlation coefficient of 0.20). The published gene expression profiles after 2 hr of X-ray treatment did not display significant differences from the no treatment control and thus were not included in the downstream analysis.

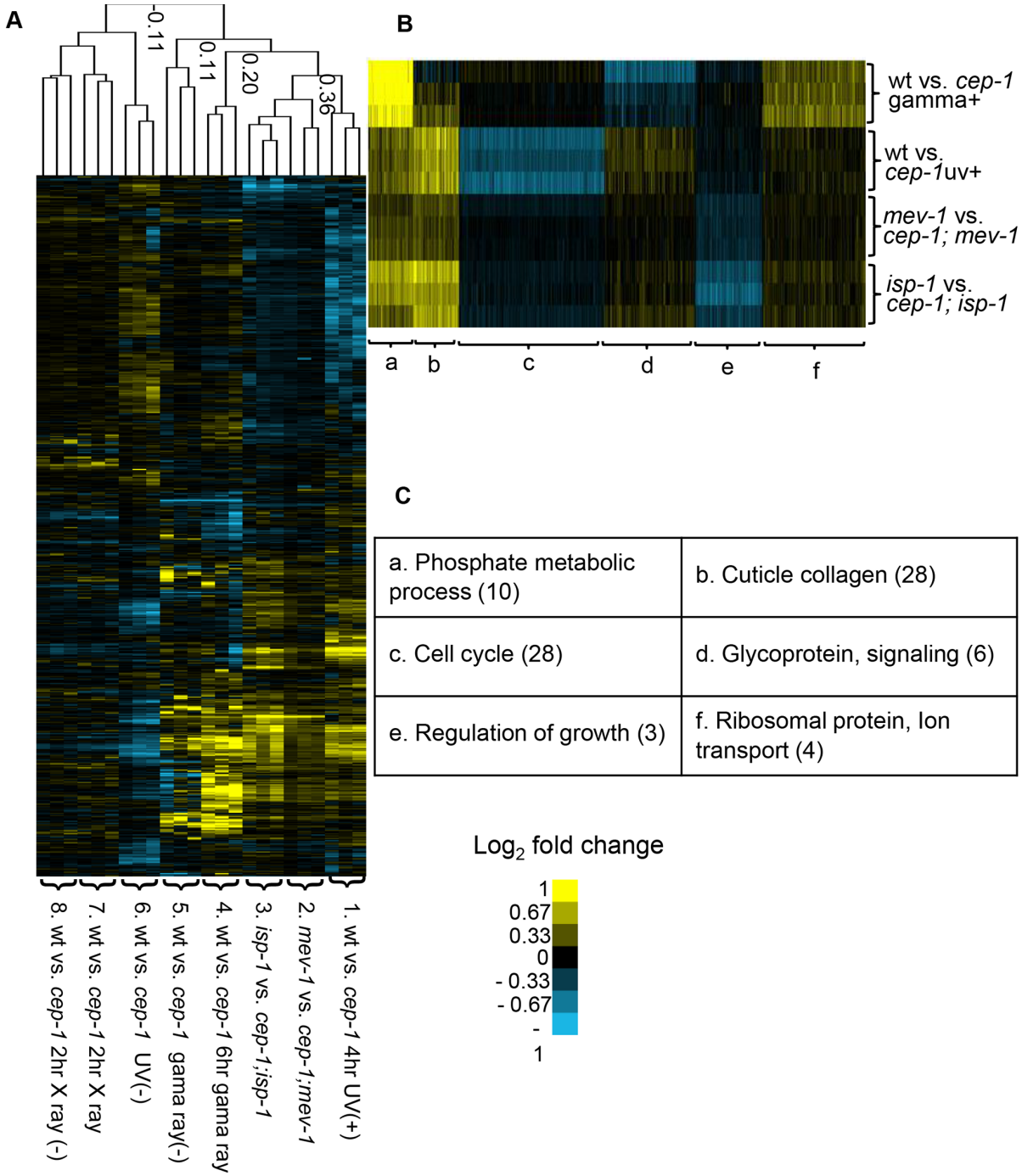


Figure 2.11 CEP-1 dependent transcriptional response under various stresses.
(A) Cluster analysis of the CEP-1-regulated transcriptomes in mitochondrial mutants, and in UV-, gamma- and X-ray-treated animals. Hierarchical single linkage gene cluster was performed and the dendrogram shows the clustered relationship of the arrays. The numbers represent the correlation coefficients of each condition. Each column represents a biological replicate and each row is a gene. (B) K-mean clustering (6 clusters) of CEP-1-regulated genes in *isp-1* and *mev-1* mutants and in UV- and gamma-irradiated animals. Genes that displayed a log2-fold change ≥0.5 in any two individual arrays were selected for clustering. (C) DAVID functional annotation of six K-mean clusters. The numbers represent the enrichment score for each group (score>1.3 is considered as significant).

We analyzed the CEP-1-regulated genes in response to mitochondrial dysfunction and UV and gamma irradiation. We focused on the gene sets that exhibited ≥1.4-fold changes under at least one of the stressed conditions (i.e., *isp-1* mutant, *mev-1* mutant, UV treatment, or gamma irradiation) and performed K-mean clustering to identify the gene sets that were either similarly regulated across all of the stressed conditions or differentially regulated under one or more conditions. The K-mean analysis revealed six distinct clusters (Fig. 2.11B). We employed DAVID to examine the GO terms generated from genes representing each cluster (Fig. 2.11C). Cluster-'a' (579 genes) represents genes that were upregulated by CEP-1 in response to all of the stressors. This group is particularly enriched for genes that function in phosphate metabolism, including kinases and phosphatases. Cluster-'f' (1,357 genes) represents genes that are also upregulated across all of the stressors but to a lesser extent than in cluster-'a'. Cluster-'f' is enriched for ribosomal and ion transport proteins. Cluster-'e' (901 genes) is enriched for growth regulation and represents genes that are repressed by CEP-1 in response to mitochondrial dysfunction and to UV and gamma irradiation. Clusters-'a', 'e' and 'f' together suggest that CEP-1 regulates common transcriptional programs in response to stress, which might in turn induce key signaling pathways to counter the stress and simultaneous suppression of growth. Clusters-'b', 'c', 'd' represent genes that are similarly regulated by CEP-1 in response to mitochondrial dysfunction and UV but are different after gamma irradiation. These gene groups are significantly enriched for nematode cuticle and collagen proteins, cell cycle regulators, glycoproteins and signaling proteins. In summary, both mitochondrial dysfunction and UV irradiation are known to induce ROS, which might reflect the common CEP-1-regulated transcriptional changes observed here.

2.4 Discussion

The major function of p53 is to integrate stress signals and to orchestrate appropriate cellular responses. Under normal, unstressed conditions, p53 is maintained at low levels via proteosome-mediated degradation. Upon stress, p53 levels stabilize and activate stress response programs that range from cell repair to cell death(Kruse and Gu, 2009). Our genetic data indicate that mutations in the *C. elegans* ETC subunits *isp-1* and nuo-6 engage CEP-1 in initiating a stress response that results in a longer lifespan. Conversely, mitochondrial defects, due to mutations in the ETC subunits *mev-1* and *gas-1*, act through CEP-1 to confer a shorter lifespan. Taken together, our data suggest the intriguing possibility that CEP-1 can sense distinct dysfunctional mitochondrial processes and modulate overall longevity accordingly.

How CEP-1 is able to sense mitochondrial dysfunction caused by different ETC mutations remains unclear. ROS have been proposed to be important regulators of p53(Liu et al., 2008). ROS can induce DNA damage, which leads to p53 activation via DNA damage checkpoint pathways. ROS are also known to engage p53 directly through modifying the redox-sensitive Cystein (Cys) residues on p53 (Hainaut and Mann, 2001). p53 contains several critical Cys residues located within the DNA-binding domain. Importantly, the Cys residues that are required to coordinate zinc and maintain the protein structure that enables interaction with the minor groove of target DNA are conserved between CEP-1 and human p53 (Huyen et al., 2004). As discussed earlier, many *C. elegans* ETC mutants have been shown to produce elevated levels of mitochondrial ROS. Altered ROS production in the mitochondrial ETC mutants might be coupled to CEP-1 via regulation of the conserved Cys residues. Upon activation, p53 is well-known to regulate the transcription of genes involved in ROS metabolism, including both

antioxidant and pro-oxidant genes (Sablina et al., 2005). Therefore, depending on upstream signaling, CEP-1/p53 can either alleviate ROS stress or promote further ROS accumulation.

Deficiencies in the ETC can also affect cellular energy homeostasis. The *isp-1* mutant has been shown to exhibit higher AMP:ATP ratios compared to wt worms, and *aak-2*, the AMPK α subunit of *C. elegans*, is partially necessary for the extended lifespan of *isp-1* mutants (Curtis et al., 2006). In mammals, the cellular energy sensor AMP kinase (AMPK) is known to directly phosphorylate p53 at Ser15, leading to stabilization and transcriptional activation of p53 (Imamura et al., 2001). Intriguingly, this Serine residue is conserved between CEP-1 and human p53, and thus, it is possible that an altered AMP:ATP ratio in ETC mutants engages AMPK to regulate CEP-1 function. Further experiments are necessary to definitively identify the signals generated by *C. elegans* ETC mutants that lead to CEP-1 activation.

Although CEP-1 plays opposite roles in *isp-1* and *mev-1* longevity, CEP-1-regulated genes in these two mutants are strikingly similar. This observation suggests that CEP-1 induces similar compensatory responses to restore cellular homeostasis regardless of the specific mitochondrial ETC defect. Our GO analyses suggested, upon mitochondrial dysfunction, that CEP-1 likely promotes a kinases/phosphatase- and/or neuropeptide-mediated signaling cascade, activates metabolic processes poised for defense and detoxification and represses the energy demanding cell cycle program. Although mtUPR has emerged as a key pathway that mediates the physiological outcomes of ETC mutants, we did not observe changes in major mtUPR response genes, such as atfs-1, hsp-6, or hsp-60. Our data suggest that CEP-1 likely does not act through mtUPR to affect the lifespan of ETC mutants.

We were excited to identify a small number of genes that were differentially regulated by CEP-1 in the *isp-1* and *mev-1* mutants, and this gene set was over-represented for genes previously known to modulate aging. We demonstrated that one of these genes, *ftn-1*, was indeed functionally important for the *cep-1*-mediated alterations to longevity in the *isp-1* mutant but not in the *mev-1* mutant, further underscoring CEP-1's functional duality. Ferritin functions to store iron in a non-toxic form, to deposit it in a safe form, and to transport it to areas where it is required. Mitochondria are the sites of iron-sulfur cluster synthesis, which are critical catalytic and structural components of many cellular proteins (Lill and Mühlenhoff, 2008). Conversely, the presence of iron in mitochondria must be tightly regulated, as free iron can react with ROS and further produce hydroxyl radicals through the Fenton reaction. As *isp-1* encodes an iron-sulphur cluster protein and can bind to iron, iron levels may accumulate in the *isp-1* mutant and thus upregulate *ftn-1* as a compensatory response to restore iron homeostasis. However, this hypothesis requires a thorough investigation of iron homeostasis in *isp-1* and the other ETC mutants to be validated. Additionally, further functional analysis of the small group of genes differentially regulated by CEP-1 in *isp-1* and *mev-1* mutants will likely reveal new genes important for longevity and illuminate how CEP-1 modulates the lifespans of animals with different ETC mutations.

Whereas CEP-1 is essential in mediating the extended lifespan of both nuo-6 and *isp-1* mutants, our results, albeit based on only a handful of target genes, suggest that CEP-1 regulates distinct genes in each case. This is quite surprising, especially considering that CEP-1 appears to regulate largely similar genes in *gas-1* and *mev-1* mutants. These differences might be due to the specific complex that is compromised and the precise point of electron transport that is defective in the various ETC mutants. During normal electron transport, electrons from complex I or

complex II can be passed onto complex III. In the nuo-6 mutant, where complex I is compromised, fuels can still enter the ETC via complex II, allowing for some degree of electron transport. The situation might be quite different in the *isp-1* mutant, which impairs complex III and would be expected to block electrons transferred from either complex I or complex II. In contrast, a defect in complex I, such as in *gas-1*, or in complex II, such as in *mev-1*, could have a similar consequence on electron transport if fuels are not limiting, as either likely partially compromises electron flow to complex III. Further experiments are necessary to elucidate whether and how different ETC mutations influence CEP-1 activity and gene regulation.

The best known function of CEP-1 in *C. elegans* is its ability to induce apoptosis upon stress. We, however, did not observe expression changes in egl-1, the key CEP-1 target for initiating apoptosis (Schumacher et al., 2005a). Intriguingly, our results suggest that CEP-1 may protect against physiological apoptosis specifically in the long-lived *isp-1* mutant. This is an aspect of CEP-1 function that remains poorly characterized. Interestingly, analysis of our microarray results revealed that *ced-8, ced-9, egl-38*, genes known to regulate apoptosis (Schumacher et al., 2005a), were specifically regulated in the *isp-1* mutant in a *cep-1*-dependent manner, but their expression was not changed in the *mev-1* mutant. Whether these genes might be central to the ability of CEP-1 to confer protection from physiological apoptosis will warrant further investigation. Future experiments to delineate whether repressed physiological apoptosis is required for the *isp-1* lifespan extension and which CEP-1 target genes might contribute to protection from physiological apoptosis will likely provide important new insights into the function and physiological role of CEP-1/p53.

DNA damage is a major p53-activating stressor, so we compared the CEP-1-mediated transcriptional profiles in mitochondrial mutants and worms treated with UV or gamma

irradiation. Our results demonstrated a considerably greater overlap between the CEP-1-regulated transcriptional response induced by ETC disruption and UV irradiation than by gamma irradiation. This might seem surprising given that UV irradiation and gamma irradiation are both genotoxic stressors that cause DNA damage, whereas mitochondrial ETC dysfunction might be considered a metabolic stress. However, UV irradiation is known to produce ROS, which might induce CEP-1 activation in a manner similar to mitochondrial ETC dysfunction, which is also known to induce ROS. Interestingly, the common set of genes that are upregulated by CEP-1 in mitochondrial mutants and in worms exposed to UV and gamma irradiation are highly enriched for kinases and phosphatases but not for DNA damage response genes. Therefore, it is likely that in response to mitochondrial dysfunction and different genotoxic stresses, CEP-1 can induce a core group of signal transduction molecules that initiate a downstream signaling cascade to mount a general stress response. In addition, CEP-1 appears to be able to sense specific damage and induce distinct responses associated with gamma irradiation and UV irradiation or mitochondrial ETC inhibition. Future dissection of the core and damage-specific responses of CEP-1/p53 will enhance our knowledge of the mechanisms that govern the function and regulation of p53, arguably one of the most important and ubiquitous tumor suppressors.

2.5 Materials and Methods

C. elegans strains

All strain stocks were kept at 16°C and grown under standard growth conditions. The following strains were used: Wild-type N2, *isp-1*(qm150), nuo-6(qm200), *mev-1*(kn-1), *gas-1*(fc21), *clk-1*(e2519), *cep-1*(gk138), and P*ftn-1*::gfp (GA641). Standard genetic methods were utilized to construct the following strains: *cep-1*(gk138);*isp-1*(qm150), *nuo-6(qm200) cep-1*(gk138), *cep-*

1(gk138);clk-1(e2519), *cep-1(gk138);mev-1(kn1)*, *cep-1(gk138);gas-1(fc21)*, *Pftn-1::gfp;isp-1(qm150),* and *Pftn-1::gfp;mev-1(kn-1).*

Lifespan analysis

All lifespan assays were performed at 20°C on Nematode Growth Media (NGM) plates seeded with E. coli OP50 or RNAi bacteria. A detailed experimental procedure is described in the Supplementary Materials and Methods (Text S1). The survival function of each worm population was estimated using the Kaplan-Meier method, and statistical analysis was performed using a log-rank test (SPSS software). $P \leq 0.001$ was considered as significantly different from the control population. The independent trials were analyzed separately, and representative experiments are shown in the figures. All of the data from all the trials are shown in Table 2.1.

Apoptosis assay

Worms of each strain were synchronized by picking, and the numbers of apoptotic corpses were counted 48 hours post L4. The corpses were assessed using Differential Interference Contrast (DIC) microscopy under 63× magnification as described in Lant and Derry (2013) (Lant and Derry, 2013). For each strain, at least 3 independent experimental replicates were performed with $n \geq 15$, where n = number of gonad arms, for each replicate.

RNA isolation and microarray preparation

Total RNA was purified from synchronized young adult worms grown at 20°C on OP50 bacteria. Total RNA was isolated using Tri-reagent (Molecular Research Center, Inc.) and purified with the RNeasy kit (Qiagen). cRNA synthesis/amplification, Cy3/Cy5 dye labeling, and

hybridization onto Agilent 4×44K *C. elegans* oligonucleotide microarrays were performed as previously described (Shaw et al., 2007a). One of three replicate arrays was dye-flipped.

Microarray analysis

The normalized expression data were uploaded onto the Princeton University MicroArray database (PUMA [http://puma.princeton.edu]). The raw data were retrieved by SUID (Sequence Unique IDentifier) then averaged by SEQ_NAME with any remaining SUIDs removed. Log2-transformed fold-change data were acquired after setting spot filter criteria, where genes with >80% good data were used. The data were analyzed and visualized using Cluster 3 and TreeView (de Hoon et al., 2004; Saldanha, 2004).

The log2 ratios of wt vs. *cep-1*(gk138) with or without UV treatment were obtained from Derry et al. (2007). The log2 ratios of wt vs. *cep-1* after gamma and X-ray irradiation data were obtained from Greiss et al. (2008). For the UV, gamma, and X-ray datasets, we averaged the intensity values of all three wt arrays for each treatment and used it as a reference. Then, we compared the results for each *cep-1* array for the same treatment to the reference to obtain the log2 ratio

SAM analysis

SAM analysis (Tusher et al., 2001) was used to identify gene sets that were similarly and differentially regulated in *isp-1* and *mev-1* mutants in a *cep-1*-dependent manner from our microarray data. Log2-transformed fold-change data with no cutoff were submitted to SAM. One class analysis was used to identify genes that similarly changed significantly and consistently in *isp-1* vs. *cep-1*;*isp-1* and *mev-1* vs. *cep-1*;*mev-1* datasets. To identify genes differently changed between *isp-1* vs. *cep-1*;*isp-1* and *mev-1* vs. *cep-1*;*mev-1* datasets, SAM two-class unpaired

analysis with a FDR = 1% was performed. The resulting gene list was compared with the gene list obtained from SAM one-class to exclude any duplicate genes. The unique 71 genes (Table 2.3) that were present only in SAM two-class analyses were considered differentially regulated between *isp-1* and *mev-1* mutants in a *cep-1*-dependent manner.

Gene Ontology classification

Worm Base IDs (WBID) of genes identified in SAM and K-mean clusters were input into the Functional annotation-clustering tool in DAVID (http://david.abcc.ncifcrf.gov/) (Huang et al., 2009) for gene annotation enrichment analysis. Functional annotation clustering was performed with the default criteria, and the enrichment score for each annotation cluster was determined.

Quantitative Reverse Transcription PCR (qRT-PCR):

Total RNA was isolated from synchronized young adult worms using Tri-reagent (Molecular Research Center, Inc.). cDNAs were synthesized with oligo-dT using the 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). act-1 was used as the internal control. The qRT-PCR experiments were performed at least in triplicate using independent RNA/cDNA preparations.

GFP microscopy

For GFP fluorescence images, worms at the L1-L2 stage were paralyzed with levamisole on an agar pad. The GFP signal was visualized at 60× magnification using a Leica DM 5000B microscope. All images were captured with the same intensity and exposure time using Open Lab software.

2.7 Acknowledgments

We thank David Gems for providing the P*ftn-1*::gfp line, the Caenorhabditis Genetics Center for providing several worm strains and the Cornell Functional Genomics Center for help with the microarray experiments. We thank members of the Lee lab for discussion and critical reading of the manuscript.

CHAPTER 3

C. elegans AAK-2/AMPK, CEP-1/p53, and CEH-23 Collaborate to Modulate Longevity in Response to Mitochondrial Electron Transport Chain Dysfunction[3]

3.1 Abstract

A decline in mitochondrial electron transport chain (ETC) function has long been implicated in aging and various diseases. Recently, moderate mitochondrial ETC dysfunction has been found to prolong lifespan in diverse organisms, suggesting a conserved and complex role of mitochondria in longevity determination. The molecular mechanisms by which reduced ETC function can extend lifespan are not well understood. Several nuclear transcription factors have been demonstrated to mediate the lifespan extension effect associated with partial impairment of the ETC, suggesting that compensatory transcriptional response to be crucial. In this study, we showed that the transcription factors CEP-1/p53 and CEH-23 act through a similar mechanism to affect longevity in response to defective ETC in *C. elegans*. Genome-wide gene expression profiling comparison revealed a new link between these two transcription factors and AAK-2/AMP kinase (AMPK) signaling. Further functional analyses indicated that CEP-1/p53 and CEH-23 act downstream of AAK-2/AMPK signaling to modulate organismal lifespan. Since AAK-2, CEP-1, and CEH-23 are all highly conserved, our findings provide important insights for understanding the organismal adaptive response

[3] Chapter 3 will be summarized as a manuscript. I conceived and performed all the experiments, except for Figure 3.6A, which is performed by Ara Hwang. The *isp-1* vs. *cep-1;isp-1* array was done by Ais Baruah. Sarah Gordon and Jennifer Chen assisted with developmental assay and paraquat resistance assay.

to mitochondrial dysfunction in diverse organisms and will likely be relevant to aging and pathologies with a mitochondrial etiology in human.

3.2 Introduction

The mitochondrial electron transport chain (ETC) produces the majority of ATP in cells and broadly influences diverse biological processes. Accordingly, perturbations in mitochondrial ETC function are usually detrimental. However, in some cases, moderate reduction of mitochondrial ETC function prolongs organismal longevity in organisms ranging from yeast to mice (Copeland et al., 2009; Dell'agnello et al., 2007; Dillin et al., 2002; Feng et al., 2001; Kirchman et al., 1999; Lee et al., 2003; Liu et al., 2005; Wong et al., 1994). In *C. elegans*, several mutants that harbor point mutations in distinct mitochondrial ETC subunits have been identified. These mutants show altered lifespan, with some living longer, while others living shorter, than wildtype worms (Feng et al., 2001; Hartman et al., 2001; Ishii et al., 1998; Kayser et al., 1999; Wong et al., 1994; Yang and Hekimi, 2010a). Moreover, RNAi knockdown of many different ETC subunits also impact lifespan (Dillin et al., 2002; Lee et al., 2003), and RNAi dilution experiments clearly demonstrating that the degree of knockdown of a single ETC subunit can determine whether the lifespan becomes extended or shortened (Rea et al., 2007). Together, these observations suggest that mitochondria play a crucial and complex role in determining the lifespan of an organism.

Proper communication between mitochondria and the nucleus is essential for organismal survival. Accumulating evidence indicates that the close communication between the two organelles is also crucial for aging and longevity. In yeast, a retrograde

signaling pathway relays signals from mitochondria to the nucleus and is key to the aging phenotypes associated with yeast cells with compromised mitochondrial ETC function (Jazwinski, 2013; Parikh et al., 1987). In *C. elegans*, mitochondrial ETC dysfunction is accompanied by specific changes in nuclear gene expression(Cristina et al., 2009; Falk et al., 2008; Yee et al., 2014), and particular transcription factors have been shown to mediate the altered longevity associate with the various ETC mutants (Khan et al., 2013; Lee et al., 2010; Ventura et al., 2010; Walter et al., 2011).

Our previous study demonstrated that the *C. elegans* homeodomain protein CEH-23 plays an important role in mediating lifespan extension associated with ETC dysfunction(Walter et al., 2011). Inactivation of *ceh-23* partially suppressed the prolonged lifespan of the *isp-1(qm150)* mutant without affecting its development and reproduction phenotypes, suggesting that CEH-23 specifically mediates the longevity of the *isp-1* mutant (Walter et al., 2011). *isp-1* encodes the Rieske iron sulphur protein, a key subunit of the mitochondrial ETC complex III, and the *isp-1* mutant harbors a point mutation that reduces the electron transport efficiency of complex III and extends lifespan by up to 50% (Feng et al., 2001). Interestingly, loss of *ceh-23* has no impact on the lifespan of wildtype animals or long-lived mutants in the insulin-like signaling and the *eat-2* caloric restriction pathways, suggesting that CEH-23 modulates longevity specifically in response to mitochondrial ETC dysfunction(Walter et al., 2011). How CEH-23 performs this function is unknown. Besides longevity modulation, earlier studies suggested a role for CEH-23 in neuronal differentiation (Altun-Gultekin et al., 2001),

although the *ceh-23(ms23)* null mutant has no obvious neuronal or behavioral defects (Walter et al., 2011).

In addition to CEH-23, CEP-1 is another transcription factor that is required for the longevity of the *isp-1* mutant (Baruah et al., 2014; Ventura et al., 2010). *cep-1* encodes the sole *C. elegans* ortholog of the mammalian p53 family, which acts as a transcription factor in response to various stresses (Baruah et al., 2014; Derry et al., 2001). The proteins of the mammalian p53 family are important tumor suppressors, as they hold key roles in maintaining genome integrity (Levine, 1997). Mammalian p53 is well-known to participate in DNA repair, cell cycle regulation, and apoptosis, processes that have also been implicated in aging (Levine, 1997). *C. elegans* CEP-1, similar to its mammalian ortholog, participates in apoptosis and cell cycle regulation (Derry et al., 2001; Greiss et al., 2008; Schumacher et al., 2005b). Recent studies in *C. elegans* revealed intriguing opposing roles of CEP-1 in the longevity of different ETC mutants; CEP-1 is required for promoting the extended lifespan of long-lived ETC mutants and also for shortening the lifespan of short-lived ETC mutants (Baruah et al., 2014; Ventura et al., 2010).

In this study, we investigated the possible collaboration of the transcription factors CEH-23 and CEP-1 in modulating longevity in response to ETC dysfunction. Our genetic analyses suggested that *ceh-23* and *cep-1* act in the same pathway to mediate the longevity of several ETC mutants. A comparison of the transcriptional outputs of CEH-23 and CEP-1 in response to ETC dysfunction revealed that these two transcription factors regulate a common set of target genes, which are overrepresented

by kinases and phosphatases of specific families, with likely roles in signal transduction. Intriguingly, the majority of the CEH-23 and CEP-1 co-regulated transcriptional targets are also transcriptional targets of activated AAK-2/AMPK signaling, suggesting a link between these two transcription factors and AAK-2/AMPK signaling. Indeed, our further data indicated that CEH-23 and CEP-1 play key roles downstream of AAK-2 to modulate stress response and lifespan. Since AMPK, CEP-1, and CEH-23 are all highly conserved, the findings reported here implicate the mammalian counterparts of these proteins in mediating the adaptive response to mitochondrial dysfunction.

3.3 Results

3.3.1 *ceh-23* and *cep-1* act in the same genetic pathway to modulate longevity of the ETC mutants

The homeodomain protein CEH-23 has been shown to be critical for the extended lifespan associated with the *isp-1* ETC mutant (Walter et al., 2011). Similarly, the p53 ortholog CEP-1 also plays a key role in modulating the lifespan of four different mitochondrial ETC mutants (Baruah et al., 2014; Ventura et al., 2010). We performed epistasis analysis to assess how these two transcription factors might interact to modulate the lifespan of mitochondrial ETC mutants. Consistent with previous results, we found that *ceh-23* and *cep-1* contribute to part of the extended lifespan of the *isp-1* mutant, as the absence of either *ceh-23* or *cep-1* only partially reduced *isp-1* mutant lifespan (Figure 3.1A, Table 3.1A). We found that the triple mutant *cep-1; ceh-23; isp-1* had a lifespan similar to the *cep-1; isp-1* and *ceh-23; isp-1* double mutants (Figure 3.1A). Because combining the *ceh-23* and *cep-1* mutations did not result in an additive

suppression of *isp-1* mutant lifespan, we conclude that *ceh-23* and *cep-1* act in the same genetic pathway to modulate *isp-1* mutant longevity.

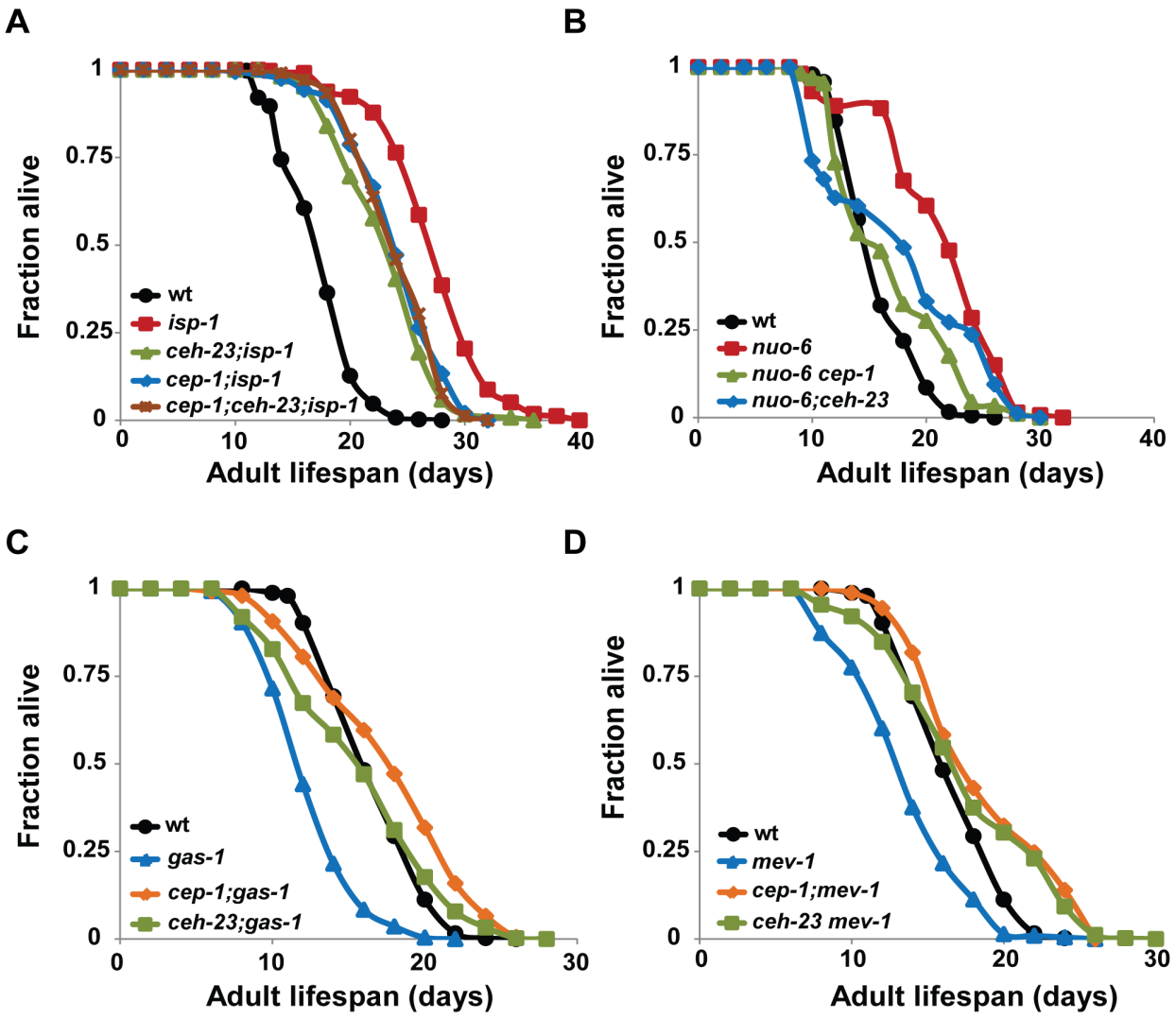


Figure 3.1 *ceh-23* and *cep-1* act in the same genetic pathway to modulate longevity when mitochondrial ETC function is impaired.
Both *ceh-23* and *cep-1* mutations partially suppressed the extended lifespan of the *isp-1*(qm150) mutant, and inactivation of *ceh-23* and *cep-1* did not additively suppress *isp-1* mutant lifespan, as the triple mutant lived as long as the double mutants (A). Moreover, *ceh-23* and *cep-1* are similarly required for mediating the longevity of several ETC mutants. Inactivation of *ceh-23* and *cep-1* partially suppressed the long lifespan of the nuo-6(qm200) mutant (B) and restored lifespan in the short-lived *gas-1*(fc21) (C) and *mev-1*(kn1) (D) mutants. Survival curves represent data pooled from multiple biological replicates. Quantitative data for individual experiments are shown in Table 3.1.

Table 3.1: Adult lifespan of all individual experiments.

Table 3.1. Epistasis between *ceh-23*(ms23) and *cep-1*(gk138) on *isp-1*(qm150) longevity

A

Strain	Total N	mean LS ± SEM (days)	% change to wt	*P-value vs ceh-23; isp-1*	*P-value vs cep-1; isp-1*
WT(N2)	97	20.21±0.21		.000	.000
isp-1(qm150)	91	27.72±0.31	37.19	.000	.000
ceh-23(ms23); isp-1(qm150)	96	25.44±0.40	25.89		.304
cep-1(gk138); isp-1(qm150)	96	25.00±0.33	23.66	.304	
cep-1(gk138); ceh-23(ms23); isp-1(qm150) iso.2	88	23.64±0.32	16.99	.000	.002
cep-1(gk138); ceh-23(ms23); isp-1(qm150) iso.22	75	24.00±0.41	18.78	.036	.091

B

Strain	Total N	mean LS ± SEM (days)	% change to wt	*P-value vs ceh-23; isp-1*	*P-value vs cep-1; isp-1*
WT (N2)	100	19.5±0.19		.000	.000
isp-1(qm150)	97	28.52±0.3	46.23	.000	.000
ceh-23(ms23); isp-1(qm150)	92	24.35±0.34	24.86		.329
cep-1(gk138); isp-1(qm150)	100	24.18±0.28	24.02	.329	
cep-1(gk138); ceh-23(ms23); isp-1(qm150) iso. 1	86	24.4±0.34	25.12	.931	.316
cep-1(gk138); ceh-23(ms23); isp-1(qm150) iso. 2	77	24.42±0.36	25.21	.736	.231
cep-1(gk138); ceh-23(ms23); isp-1(qm150) iso. 3	85	24.5±0.36	25.62	.494	.114

Table 3.2 Requirement of CEH-23 and CEP-1 in various ETC mutants

A

Strain	Total N	mean LS ± SEM (days)	% change to wt	*P*-value *vs wt*	*P*-value *vs nuo-6(qm200)*
WT(N2)	106	18.85±0.25			0.000
nuo-6(qm200)	80	30.88±0.67	63.84	0.000	
nuo-6(qm200); ceh-23(ms23) iso.1	98	27.99±0.74	48.52	0.000	0.005
nuo-6(qm200); ceh-23(ms23) iso.2	104	25.29±0.57	34.20	0.000	0.000
nuo-6(qm200); ceh-23(ms23) iso.3	107	27.5±0.59	45.92	0.000	0.000

B

Strain	Total N	mean LS ± SEM (days)	% change to wt	*P*-value *vs wt*	*P*-value *vs nuo-6(qm200)*
WT(N2)	98	14.96±0.29			0.093
nuo-6(qm200)	100	16.1±1.38	7.61	0.093	
nuo-6(qm200) cep-1(gk138) iso.1	93	15.3±1.12	2.24	0.387	0.450
nuo-6(qm200) cep-1(gk138) iso.2	90	13.78±0.6	-7.89	0.096	0.250
nuo-6(qm200); ceh-23(ms23)	94	16.46±1.22	10.02	0.033	0.998

C

Strain	Total N	mean LS ± SEM (days)	% change to wt	*P*-value *vs wt*	*P*-value *vs nuo-6*	*P*-value *vs mev-1*	*P*-value *vs gas-1*
WT(N2)	103	16.97±0.34			0.000	0.000	0.000
nuo-6(qm200)	153	22.85±0.33	34.65	0.000			
nuo-6(qm200) cep-1(gk138) iso.1	194	18.51±0.42	9.07	0.000	0.000		
nuo-6(qm200) cep-1(gk138) iso.2	123	15.88±0.46	-6.42	0.252	0.000		
nuo-6(qm200); ceh-23(ms23)	174	18.46±0.82	8.78	0.001	0.002		
mev-1(kn1)	108	14±0.36	-17.50	0.000			

ceh-23(ms23) mev-1(kn1)iso.1	107	15.86±0.37	-6.54	0.078		0.001	
ceh-23(ms23) mev-1(kn1)iso.2	96	15.21±0.41	-10.37	0.006		0.022	
cep-1(gk138); mev-1(kn1)	105	19.84±0.49	16.91	0.000		0.000	
gas-1(fc21)	106	13.07±0.28	-22.98	0.000			
ceh-23(ms23); gas-1(fc21) iso.1	41	13.46±0.58	-20.68	0.000			0.342
ceh-23(ms23); gas-1(fc21) iso.2	82	16.15±0.48	-4.83	0.378			0.000
cep-1(gk138); gas-1(fc21)	55	16.53±0.7	-2.59	0.751			0.000

D

Strain	Tot al N	mean LS ± SEM (days)	% change to wt	*P*-value *vs wt*	*P*-value *vs mev-1*	*P*-value *vs gas-1*
WT(N2)	108	16.18±0.27			0.000	0.000
cep-1(gk138)	89	17.63±0.32	8.96	0.000		
ceh-23(ms23)	104	16.04±0.28	-0.87	0.736		
mev-1(kn1)	107	13.92±0.38	-13.97	0.001		
cep-1(gk138); mev-1(kn1)	89	17.73±0.43	9.58	0.001	0.000	
ceh-23(ms23) mev-1(kn1)iso.1	106	17.85±0.53	10.32	0.000	0.000	
ceh-23(ms23) mev-1(kn1)iso.2	99	20.48±0.48	26.58	0.000	0.000	
ceh-23(ms23) mev-1(kn1)iso.3	88	21.92±0.56	35.48	0.000	0.000	
gas-1(fc21)	90	13.31±0.35	-17.74	0.000		
cep-1(gk138); gas-1(fc21)	101	18.82±0.55	16.32	0.000		0.000
ceh-23(ms23); gas-1(fc21) iso.1	93	17.46±0.45	7.91	0.000		0.000
ceh-23(ms23); gas-1(fc21) iso.2	72	15.26±0.63	-5.69	0.890		0.001
ceh-23(ms23); gas-1(fc21) iso.3	88	14.4±0.62	-11.00	0.609		0.012

E

Strain	Total N	mean LS ± SEM (days)	% change to wt	*P*-value *vs wt*	*P*-value *vs gas-1*
WT(N2)	98	17.44±0.37			0.000
ceh-23(ms23)	100	17.45±0.41	0.06	0.671	0.000
cep-1(gk138)	100	18.46±0.34	5.85	0.030	0.000

gas-1	84	11.81±0.32	-32.28	0.000	
gas-1; ceh-23	80	18.9±0.56	8.37	0.002	0.000
gas-1; cep-1	117	18.07±0.56	3.61	0.087	0.000

cep-1 has been shown to have opposing effects on longevity in different mitochondrial ETC mutants. It promotes longevity in the *nuo-6* (complex I) and *isp-1* (complex III) mutants, as *cep-1* inactivation suppresses the long life of these mutants. In contrast, *cep-1* limits the lifespan of the short-lived *gas-1* (complex I) and *mev-1* (complex II) mutants, as *cep-1* mutation restores a normal lifespan in these mutants (Baruah et al., 2014; Ventura et al., 2010). Because *ceh-23* and *cep-1* appear to act in the same genetic pathway to modulate longevity of the *isp-1* mutant, we expect that *ceh-23* would have similar opposing roles in the various ETC mutants. Consistent with our hypothesis, the *ceh-23* mutation suppressed the extended lifespan of the long-lived *nuo-6* mutant (Figure 3.1B, Table 3.1B) but restored lifespan in the short-lived *gas-1* and *mev-1* mutants (Figure 3.1C and D, Table 3.1B). Thus, *ceh-23* and *cep-1* are required for the extended or shortened lifespans of ETC mutants in genes in three different ETC complexes, suggesting that they perform a general role in modulating lifespan in responding to ETC dysfunctions.

3.3.2 CEH-23 and CEP-1 share a large group of transcriptional targets in response to mild ETC dysfunction

To elucidate the possible mechanism by which CEH-23 and CEP-1 modulate lifespan in the ETC mutants, we compared the transcriptional outputs of these transcription factors in the long-lived *isp-1* mutant. We previously identified the transcriptional responses regulated by CEP-1 in the *isp-1* mutant (Baruah et al., 2014). We found that CEP-1 regulates the expression of a broad array of genes in response to ETC stress, including those involved in phosphate metabolism, lipid modification, and neuropeptide signaling (Baruah et al., 2014). We also

demonstrated that the CEP-1-regulated gene *ftn-1* is required for the extended lifespan of the *isp-1* mutant (Baruah et al., 2014). To learn the transcriptional outputs of CEH-23 in response to ETC stress, we performed microarray analyses to profile the CEH-23 dependent transcriptomic response in the long-lived *isp-1* mutant by comparing the global gene expression profiles between *isp-1* and *ceh-23; isp-1* mutant worms synchronized at larval stage 4 (L4). We chose this developmental stage because it has been shown that mitochondrial ETC dysfunction during larval stage is critical for influencing adult lifespan (Dillin et al., 2002). Using the statistical tool SAM (Statistical Analysis of Microarray) (Tusher et al., 2001) with stringent parameters (False Discovery Rate (FDR) = 0.59% and a fold-change cut-off > 1.5 fold), we identified consistent changes in expression for 1,878 genes. Of these genes, 1,244 are up-regulated in the *isp-1* mutant with respect to *ceh-23; isp-1* double mutant, and 634 are down-regulated (Figure 3.2A). Gene Ontology (GO) analyses revealed that the 1,244 genes (upregulated in the *isp-1* mutant) were enriched for genes involved in many fundamental biological processes, in particular cell cycle, reproduction, lipid transport, and phosphate metabolism (Figure 3.2B). The 634 genes (downregulated in the *isp-1* mutant) were especially enriched for the biological function neuropeptide signaling (Figure 3.2B). These data suggest that CEH-23 regulates development, reproduction, metabolism and neuronal signaling. Our previous works showed that *ceh-23* deficiency in the *isp-1* mutant did not affect the slow development or reduce *isp-1* reproduction phenotypes (Walter et al., 2011); however, *cep-1* mutation partially restored normal development in the *isp-1* mutant (Baruah et al., 2014). Interestingly, we found that functional *ceh-23* is required for *cep-1* to modulate development in the *isp-1* mutant (Figure 3.2C). Exactly how these two factors interact in this context awaits further investigation.

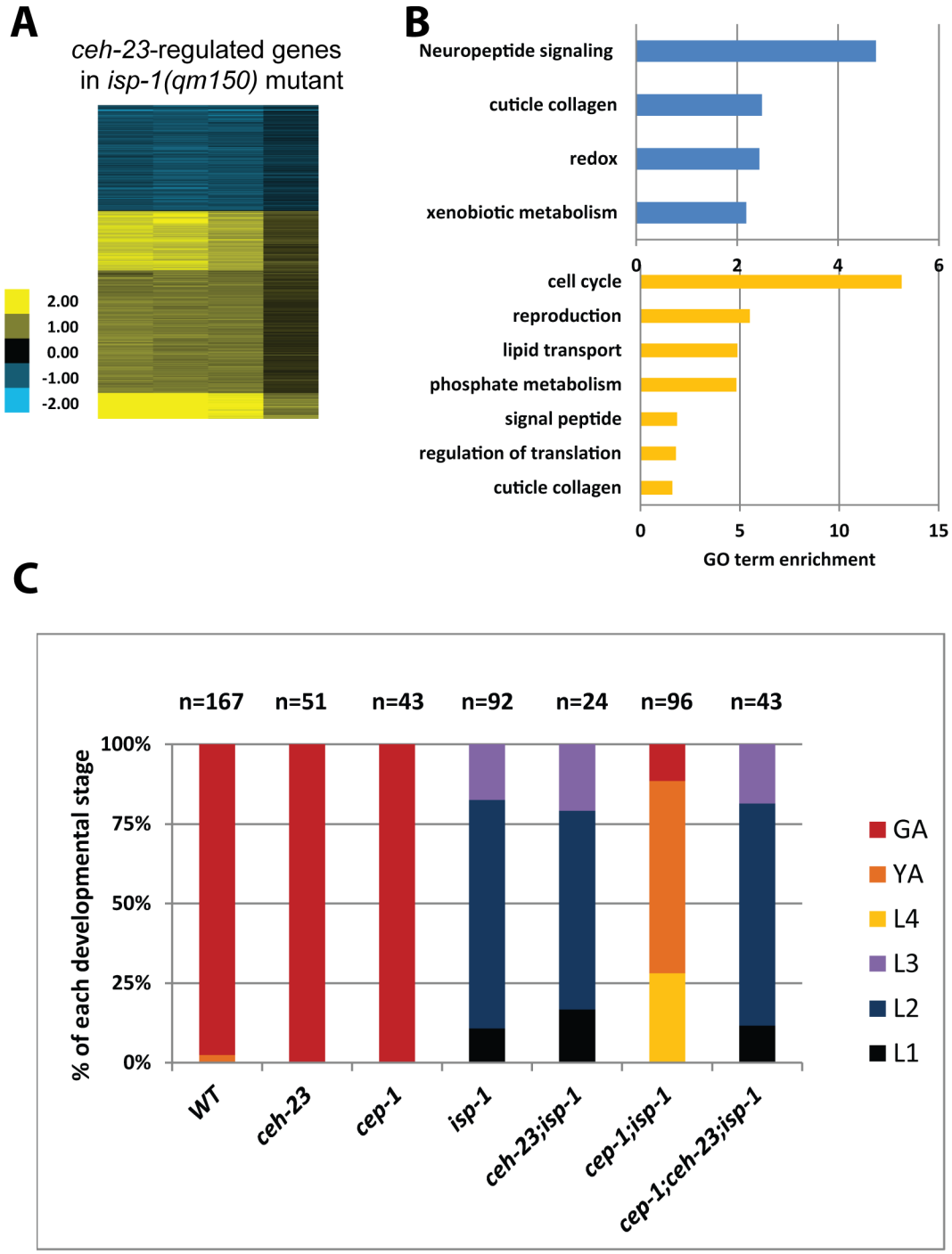


Figure 3.2 Transcriptomic targets of CEH-23.
(A) The heat map represents the expression levels of genes in the *isp-1*(qm150) mutant compared to the *ceh-23*(ms23); *isp-1*(qm150) double mutant. Gene changes were identified by SAM one-class analysis (FDR=0, fold-change>1.5). (B) The most enriched Gene Ontology (GO) terms among CEH-23-upregulated genes (yellow bars) and CEH-23-downregulated genes (blue bars). (C) Bar graph shows the fraction of each developmental stage (black: L1, blue: L2, purple: L3, yellow: L4, orange: YA and red: gravid adult) 60 hours after egg lay for each genotype. The numbers of worms scored for each genotype are listed on top of each bar.

Given that earlier epistasis analyses revealed that *ceh-23* and *cep-1* likely act in the same genetic pathway to modulate longevity in ETC mutants (Figure 3.1A), we hypothesized that these two factors would share a transcriptional outcome that is important for longevity determination during ETC dysfunction. We compared the CEH-23-dependent transcriptional response to *isp-1* mutation with the previously published CEP-1-dependent response (Baruah et al., 2014) to identify the genes that are similarly regulated by both transcription factors. Consistent with our hypothesis, statistical analyses with stringent criteria (FDR = 0%, fold-change > 1.5 fold) revealed a substantial number of genes that were upregulated in the *isp-1* mutant in a CEH-23 and CEP-1 dependent manner (897 genes). Interestingly, this analysis revealed very few genes that were downregulated in the *isp-1* mutant compared to the *ceh-23; isp-1* and *cep-1; isp-1* mutants (19 genes) (Figure3.3A Cluster A). We herein term the group of genes whose expression changed similarly when comparing *isp-1 vs. ceh-23; isp-1* or *isp-1 vs. cep-1; isp-1* the "CEH-23 and CEP-1 common target genes" (916 genes). GO term analyses revealed that the CEH-23 and CEP-1 common target genes are enriched with kinases and phosphatases (Figure 3.3B), suggesting that CEH-23 and CEP-1 regulate signaling networks to prolong *isp-1* mutant lifespan.

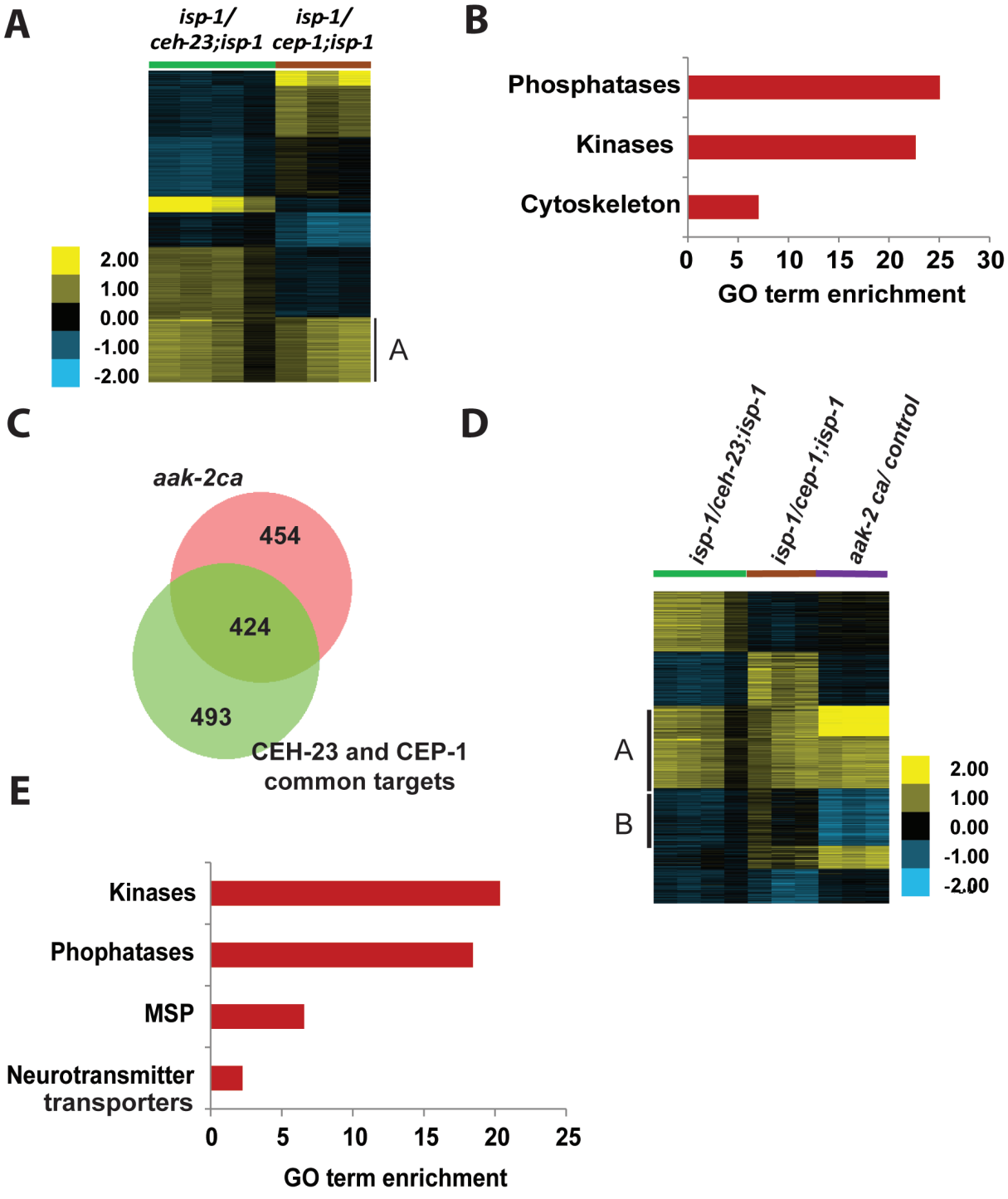

A
isp-1/ ceh-23;isp-1
isp-1/ cep-1;isp-1
2.00
1.00
0.00
-1.00
-2.00
A
B
Phosphatases
Kinases
Cytoskeleton
0 5 10 15 20 25 30
GO term enrichment
C
aak-2ca
454
424
493
CEH-23 and CEP-1 common targets
D
isp-1/ceh-23;isp-1
isp-1/cep-1;isp-1
aak-2 ca/ control
A
B
2.00
1.00
0.00
-1.00
-2.00
E
Kinases
Phophatases
MSP
Neurotransmitter transporters
0 5 10 15 20 25
GO term enrichment

Figure 3.3. Transcriptomic analyses revealed that CEH-23 and CEP-1 co-regulate a large set of genes in response to ETC dysfunction.
The heat map represents a genome-wide comparison of expression patterns between *isp-1*(qm150) and *ceh-23*(ms23); *isp-1*(qm150) or *cep-1*(gk138); *isp-1*(qm150). The 916 common targets of CEH-23 and CEP-1 in the *isp-1* mutant (identified by one class SAM analysis with FDR = 0%, fold-change > 1.5 fold) are indicated as cluster A. Gene clustering was done using K-mean clustering with K=7. For the heat maps, yellow depicts genes that are up-regulated, and blue depicts genes that are down-regulated, in the *isp-1*(qm150) mutant compared to the double mutants. The intensity of the heat map represents the log2 ratio of the expression comparison (A). The most enriched GO terms among the CEH-23 and CEP-1 common targets are kinases and phosphatases. X axis represents GO term enrichment scores (B). The Venn diagram shows a substantial (and highly significant) overlap between the genes that are commonly regulated by CEH-23 and CEP-1 in the *isp-1* mutant and those regulated by constitutively active AAK-2 (*aak-2* ca) (Representation factor: 7.0; p<0.000e+00) (C). The heat map represents a genome-wide comparison of expression patterns between *isp-1*(qm150) vs. *ceh-23*(ms23); *isp-1*(qm150) or *cep-1*(gk138); *isp-1*(qm150) and *aak-2*ca vs. wildtype. Genes without significant differences in any of the comparisons have been filtered out. Gene clustering was done using K-mean clustering with K=7. Cluster A highlights genes that are commonly regulated by CEH-23, CEP-1 and AAK-2, and cluster B highlights genes that are shared targets between CEH-23 and AAK-2. Yellow depicts genes that are upregulated, and blue depicts genes that are downregulated, in *isp-1*(qm150) or *aak-2*ca mutants compared to the double mutants or wildtype control as indicated (D). The most enriched GO terms among the CEH-23, CEP-1, and AAK-2 common targets are kinases, phosphatases, major sperm proteins (MSP) and neurotransmitter transporters. X axis represents GO term enrichment scores (E).

3.3.3 CEH-23 and CEP-1/p53 shared many transcriptional targets with active AAK-2/AMPK signaling

Although the CEH-23 and CEP-1 common transcriptional targets are enriched with kinases and phosphatases, these proteins have not been directly implicated in known longevity pathways. To further assess the roles of CEH-23 and CEP-1, we performed an unbiased search for factors that also regulate the expression of the CEH-23 and CEP-1 common target genes using the WormMine tool at www.wormbase.org. Interestingly, this analysis revealed a highly significant overlap between the genes that are upregulated by CEH-23 and CEP-1 in the *isp-1* mutant and the genes that are upregulated by constitutively active AMP kinase (AMPK) (424 Genes) (Figure 3.3C and Figure 3.3D, Cluster A) (Mair et al., 2011).

AMPK is a well-conserved kinase that serves as a cellular energy sensor. It regulates diverse biological processes in response to different environmental stresses and cellular energy levels, in particular a change in AMP:ATP ratio. AMPK has been implicated in several pathways that are key to organismal lifespan, including the mTOR pathway (Selman et al., 2009) and the insulin-like signaling pathway (Apfeld et al., 2004). Furthermore, overexpression of a constitutively active AAK-2, the *C. elegans* catalytic subunit of AMPK, is sufficient to extend *C. elegans* lifespan (Mair et al., 2011). Given that mitochondria are important for cellular fuel production, defects in the mitochondrial ETC are likely to trigger a cellular energy imbalance and thus activate AMPK. Indeed, AAK-2/AMPK activity appears upregulated in the *isp-1* mutant (Hwang et al., 2014). Consistently, *aak-2* is necessary for the extended lifespan of several ETC

mutants including *isp-1* (Curtis et al., 2006). Our finding that constitutively active AAK-2 signaling and transcription factors, CEH-23 and CEP-1, under mitochondrial stress regulate similar sets of target genes, and the fact that AAK-2 is known to be activated in mitochondrial ETC mutants (Curtis et al., 2006; Hwang et al., 2014), together suggest that AAK-2 signaling, CEH-23, and CEP-1 converge to modulate longevity in the *isp-1* mutant. GO term analyses of the genes that are commonly regulated by AAK-2, CEH-23, and CEP-1 revealed a highly significant enrichment for kinases, phosphatases, major sperm proteins (MSPs), and neurotransmitter transporters (Figure 3.3E). Therefore, AAK-2, CEP-1, CEH-23 may regulate signal transduction to promote longevity in mutant worms with mild ETC dysfunction. Although AAK-2/AMPK is known to modulate lifespan under a broad range of conditions (Burkewitz et al., 2014), the mechanism by which AAK-2 mediates the longevity of mitochondrial ETC mutants remains unclear. Our results revealed a new connection between AAK-2 and the transcription factors CEH-23 and CEP-1, and suggest a new regulatory module that likely mounts an adaptive response upon mitochondrial dysfunction.

3.3.4 CEH-23 and CEP-1 are potential downstream effectors of AAK-2 signaling

To further delineate the relationship of AAK-2, CEH-23, and CEP-1 in response to ETC stress, we assessed the effect of *aak-2* depletion in *isp-1* mutants lacking *ceh-23* or *cep-1*. First, we sought to confirm the role of *aak-2* in lifespan modulation. Consistent with previous findings (Curtis et al., 2006), we found that RNAi knockdown of *aak-2* slightly shortened the lifespan of wildtype animals and substantially suppressed the extended lifespan of *isp-1* mutant (Figure 3.4A, Figure 3.4B, Table 3.3), indicating

that AAK-2 signaling is essential for normal lifespan and required for the full lifespan extension of *isp-1* mutant. If AAK-2, CEH-23, and CEP-1 act together to modulate lifespan in the *isp-1* mutant, we would predict that loss of *aak-2* and *ceh-23* or *cep-1* would not additively suppress *isp-1* mutant lifespan. Indeed, we observed that *isp-1* mutants devoid of both *ceh-23* and *aak-2* (*ceh-23; isp-1; aak-2*(RNAi) lived as long as *isp-1* mutants with only *aak-2* depleted (Figure 3.4A). Interestingly, RNAi knockdown of *aak-2* in the *cep-1; isp-1* double mutants resulted in an intermediate lifespan, where *isp-1* mutants devoid of both *cep-1* and *aak-2* (*cep-1; isp-1; aak-2*(RNAi)) lived slightly longer than *isp-1* mutants with only *aak-2* depleted, but slightly shorter than the *ceh-23; isp-1* and *cep-1; isp-1* double mutants (Figure 3.4B). On more careful inspection, we found that *aak-2* RNAi was somewhat less efficient in strains without *cep-1* (~40% knockdown) compared to the other strains (~50% knockdown) (Figure 3.5B), which might explain the intermediate lifespan phenotype. Taken together, we interpreted these results to mean that inactivation of *ceh-23* or *cep-1* and depletion of *aak-2* do not additively suppress *isp-1* mutant lifespan, thus supporting the model that AAK-2/AMPK signaling acts with CEH-23 and CEP-1/p53 to prolong the lifespan of the *isp-1*mutant.

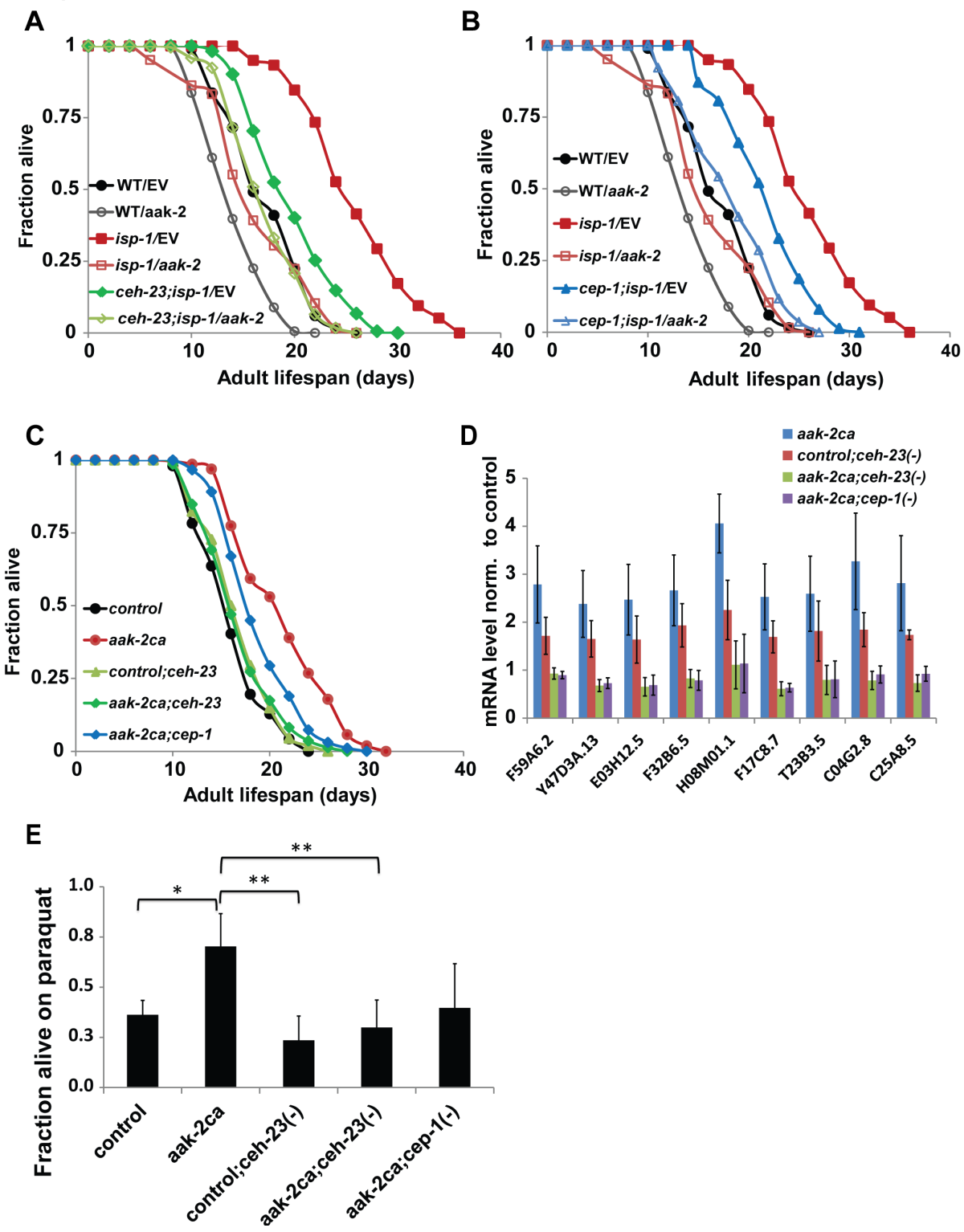

A
Fraction alive
Adult lifespan (days)
WT/EV
WT/aak-2
isp-1/EV
isp-1/aak-2
ceh-23;isp-1/EV
ceh-23;isp-1/aak-2
B
Fraction alive
Adult lifespan (days)
WT/EV
WT/aak-2
isp-1/EV
isp-1/aak-2
cep-1;isp-1/EV
cep-1;isp-1/aak-2
C
Fraction alive
Adult lifespan (days)
control
aak-2ca
control;ceh-23
aak-2ca;ceh-23
aak-2ca;cep-1
D
mRNA level norm. to control
aak-2ca
control;ceh-23(-)
aak-2ca;ceh-23(-)
aak-2ca;cep-1(-)
F59A6.2
Y47D3A.13
E03H12.5
F32B6.5
H08M01.1
F17C8.7
T23B3.5
C04G2.8
C25A8.5
E
Fraction alive on paraquat
control
aak-2ca
control;ceh-23(-)
aak-2ca;ceh-23(-)
aak-2ca;cep-1(-)

Figure 3.4 CEH-23, CEP-1, and AAK-2 act in the same pathway to mediate *isp-1* mutant lifespan.
aak-2 RNAi slightly shortens the lifespan of wildtype worms and substantially shortens the lifespan of *isp-1*(qm150) mutants. *ceh-23*; *isp-1* (A) and *cep-1*; *isp-1* (B) mutants treated with *aak-2* RNAi had similar lifespan as the *isp-1* single mutant treated with *aak-2* RNAi, suggesting that *ceh-23* and *cep-1* and *aak-2* act in the same genetic pathway to modulate *isp-1* mutant lifespan. Both *ceh-23* and *cep-1* mutations suppressed the extended lifespan (C), transcriptional response (D) and oxidative stress resistance (E) of *aak-2*ca worms. In (E), P value is represented in the following: *<0.05, **<0.01, ***<0.005. Although the difference in oxidative stress resistance between *aak-2*ca; *cep-1*(-) and *aak-2*ca is not significant (P value =0.13), we consistently observed this difference in all our trials. *aak-2*ca refers to WBM60 uthIs248[P*aak-2*::*aak-2* genomic (aa1-321)::GFP::unc54 3'UTR, Pmyo-2::tdTomato] and control refers to WBM59 uthIs272[Pmyo-2::tdTomato, unc-54 3'UTR].

Table 3.3 Longevity effects of knockdown *aak-2* on the *cep-1; isp-1* and *ceh-23; isp-1* mutants

A

Strain/RNAi	Total N	mean LS ± SEM (days)	% change to wt/control	P-value vs wt/control	% change to E.V. control	P-value vs E.V. control
N2/L4440	92	16.81±0.36				
N2/cep-1	73	16.58±0.39	-1.37	0.67	-1.382	.673
N2/aak-2	81	14.49±0.36	-13.80	0.00	-13.812	.000
isp-1 /L4440	25	23.84±1.12	41.82	0.00		
isp-1/cep-1	70	18.86±0.58	12.20	0.00	-20.901	.000
isp-1/aak-2	43	14.89±0.66	-11.42	0.08	-37.547	.000
cep-1; isp-1/L4440	73	19.47±0.46	15.82	0.00		
cep-1; isp-1/ cep-1	68	20.24±0.43	20.40	0.00	3.953	.391
cep-1;isp-1/ aak-2	47	17.22±0.54	2.44	0.49	-11.530	.001
ceh-23; isp-1/ L4440	61	19.12±0.63	13.74	0.00		
ceh-23; isp-1/ cep-1	79	19.01±0.47	13.09	0.00	-.565	.535
ceh-23; isp-1/ aak-2	69	17.33±0.41	3.09	0.26	-9.348	.006

B

Strain/treatment	Total N	mean LS ± SEM (days)	% change to wt/control	P-value vs wt/control	% change to E.V. control	P-value vs E.V. control
N2/L4440	91	18.17±0.43				
N2/cep-1	97	17.94±0.38	-1.29	0.40	-1.290	.405
N2/aak-2	88	14.18±0.31	-21.97	0.00	-21.974	.000
isp-1/L4440	91	26.61±0.49	46.43	0.00		
isp-1/cep-1	87	21.59±0.35	18.81	0.00	-18.861	.000
isp-1/aak-2	85	17.2±0.48	-5.37	0.25	-35.376	.000
cep-1; isp-1/L4440	78	24.16±0.36	32.93	0.00		
cep-1;isp-1/ cep-1	85	24.41±0.33	34.31	0.00	1.041	.761
cep-1; isp-1/ aak-2	90	19.17±0.5	5.51	0.01	-20.626	.000
ceh-23; isp-1/L4440	101	20.49±0.36	12.76	0.00		
ceh-23; isp-1/cep-1	87	21.04±0.36	15.77	0.00	2.669	.442
ceh-23; isp-1/aak-2	101	17.48±0.38	-3.80	0.11	-14.682	.000

Table 3.4 Requirement of CEH-23 and CEP-1 on longevity of the AAK-2 ca

A

Strains	Total N	mean LS ± SEM (days)	% change to WBM59	P-value vsWBM59	P-value vsWBM60
RFP control (WBM59)	109	16.08±0.39			0.000
aak-2ca (WBM60)	107	20.56±0.46	27.86	0.000	
WBM59; ceh-23(ms23)_1	105	17.11±0.35	6.41	0.210	0.000
WBM59; ceh-23(ms23)_2	105	17.2±0.4	6.97	0.057	0.000
WBM60; ceh-23(ms23)_1	108	16.24±0.4	1.00	0.841	0.000
WBM60; ceh-23(ms23)_2	110	16.94±0.43	5.35	0.252	0.000
WBM60; cep-1(gk138)_1	107	19.65±0.36	22.20	0.000	0.029
WBM60; cep-1(gk138)_2	104	19.34±0.35	20.27	0.000	0.006

B

Strains	Total N	mean LS ± SEM (days)	% change to WBM59	P-value vsWBM59	P-value vsWBM60
RFP control (WBM59)	106	16.63±0.35			0.000
aak-2ca (WBM60)	120	22.55±0.59	35.60	0.000	
WBM59; ceh-23(ms23)_1	107	16.54±0.38	-0.54	0.911	0.000
WBM59; ceh-23(ms23)_2	92	17.66±0.45	6.19	0.071	0.000
WBM60; ceh-23(ms23)_1	104	18.21±0.45	9.50	0.005	0.000
WBM60; ceh-23(ms23)_2	117	17.19±0.43	3.37	0.232	0.000
WBM60; cep-1(gk138)_1	98	17.64±0.41	6.07	0.044	0.000
WBM60; cep-1(gk138)_2	115	19.71±0.46	18.52	0.000	0.000

Since the microarray and epistasis data suggest that AAK-2, CEH-23, and CEP-1 act together to modulate lifespan of the ETC mutants, we tested whether CEH-23 and CEP-1 could influence AMPK activity in the mitochondrial ETC mutants. The activity of AMPK depends on the phosphorylation of the catalytic alpha subunit AAK-2. Consistent with previous reports (Hwang et al., 2014), we detected higher levels of phosphorylated AAK-2 in the *isp-1* mutant, which indicates increased AMPK activity (Figure 3.5A). This up-regulation of active AMPK in the *isp-1* mutant did not depend on functional *ceh-23* and *cep-1*, as neither *cep-1* nor *ceh-23* mutation reduced phospho-AAK-2 levels in *isp-1* mutants (Figure3.5A). Moreover, inactivation of CEH-23 or CEP-1 also had no detectable effect on the mRNA levels of *aak-2* in the *isp-1* mutant (Figure 3.5E). Taken together, our data suggest that CEP-1 and CEH-23 likely do not regulate AAK-2 activity in the *isp-1* mutant.

a

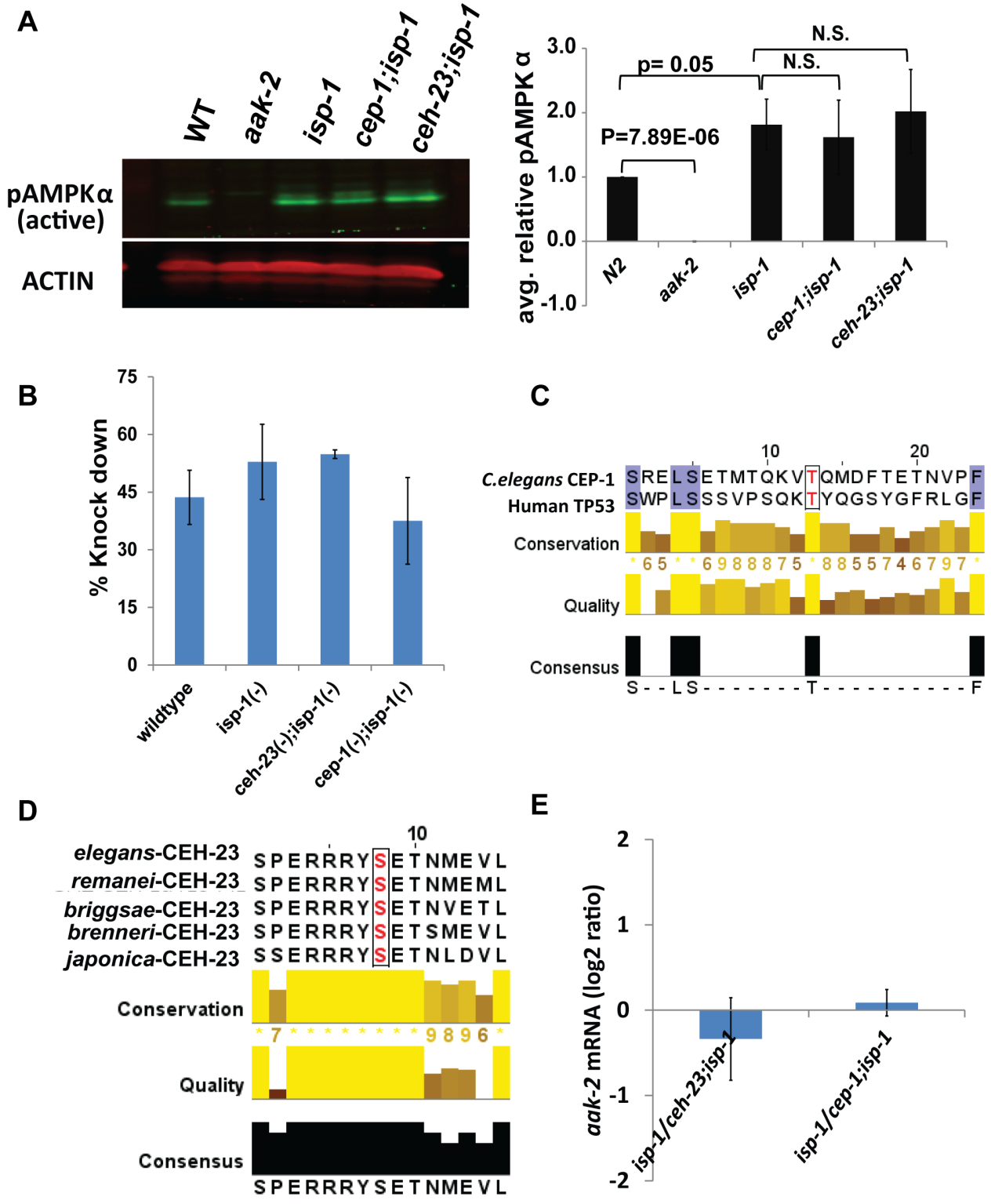

A
WT
aak-2
isp-1
cep-1;isp-1
ceh-23;isp-1
pAMPKα (active)
ACTIN
avg. relative pAMPK α
p= 0.05
N.S.
N.S.
P=7.89E-06
N2
aak-2
isp-1
cep-1;isp-1
ceh-23;isp-1
B
% Knock down
wildtype
isp-1(-)
ceh-23(-);isp-1(-)
cep-1(-);isp-1(-)
C
C.elegans CEP-1
Human TP53
Conservation
Quality
Consensus
D
elegans-CEH-23
remanei-CEH-23
briggsae-CEH-23
brenneri-CEH-23
japonica-CEH-23
Conservation
Quality
Consensus
E
aak-2 mRNA (log2 ratio)
isp-1/ceh-23;isp-1
isp-1/cep-1;isp-1

Figure 3.5 AAK-2 activity is not affected by CEH-23 or CEP-1.
(A) Western blot analysis shows that activated AAK-2 levels are similarly elevated in the *isp-1* single mutant and in the *ceh-23*; *isp-1* and *cep-1*; *isp-1* double mutants, suggesting that CEP-23 and CEP-1 are not required for AAK-2 activation. Quantified pAAK-2/AMPKα levels from three independent experiments are presented in the bar graph. (B) Knock down efficiency of *aak-2* in various mutants measured by RT-qPCR. (C) Multisequence alignment for *C. elegans* CEP-1 and human p53. Threonine marked in red represents a putative AMPK phosphorylation site. (D) Multisequence alignment for nematode CEH-23 homologs revealed a well-conserved putative AMPK site. The analyses were done using BLOSUM62. (E) *aak-2* mRNA level represented in averaged log2ratio of *isp-1* vs. *ceh-23*; *isp-1* or *cep-1*; *isp-1* derived from microarray experiments described in Figure 2.

We next investigated whether CEH-23 and CEP-1 could mediate the biological outputs of AAK-2. We tested whether functional CEH-23 and CEP-1 are required for the extended lifespan and the increased resistance to oxidative stress of worms with constitutively active AAK-2 (*aak-2ca*) (Hwang et al., 2014; Mair et al., 2011). The data showed that both *ceh-23* and *cep-1* are required for the extended lifespan of the *aak-2ca* animals (Figure 3.4C, Table 3.4), suggesting that CEH-23 and CEP-1 act downstream of AMPK signaling for longevity determination. Consistent with this finding, functional CEH-23 and CEP-1 are also required for the increased tolerance to oxidative stress of the *aak-2ca* animals (Figure 3.4E). Interestingly, *ceh-23* inactivation appeared to have a greater effect on suppressing the prolonged lifespan of the *aak-2ca* animals compared to *cep-1* inactivation. This observation suggests that CEH-23 mediates a greater portion of the longevity effect of AMPK than CEP-1, which might be reflected by the gene expression comparison where CEH-23, but not CEP-1, repressed the expression of a group of genes in the *isp-1* mutants that are also down-regulated in *aak-2ca* worms (Figure 3.3D, Cluster B). To further test whether CEH-23 and CEP-1 act downstream of AAK-2, we asked if CEH-23 and CEP-1 could mediate some of the transcriptional responses caused by constitutive active AAK-2. Microarray analyses revealed a group of genes that are regulated by CEH-23 and CEP-1 in the *isp-1* mutant and are up-regulated when AAK-2 is over active (Figure 3.3D, Cluster A). Quantitative PCR analyses demonstrated that the induction of these genes in the *aak-2ca* animals required *ceh-23* and *cep-1* (Figure 3.4D). Together, our data support the model that CEH-23 and CEP-1 likely act downstream of AAK-2 to modulate some of its biological

outputs, including longevity and oxidative stress response. Consistent with this idea, both CEH-23 and CEP-1 contain putative AMPK motifs that appear to be highly conserved (http://scansite3.mit.edu/#home). Mammalian p53 is a known substrate of AMPK (Adamovich et al., 2014), and multiple sequence alignment analysis revealed that *C. elegans* CEP-1 also contains a highly conserved AMPK motif (Figure 3.5C). Moreover, the predicted AMPK site on CEH-23 is conserved across various nematode species (Figure 3.5D), and EMX2, the putative mammlian homolog of CEH-23, also contains two predicted AMPK motifs. The conserved nature of the predicted AMPK motifs in both CEP-1 and CEH-23 suggests that these factors could be AMPK substrates in diverse organisms.

3.3.5 CEH-23 and CEP-1 are not required for ROS-mediated lifespan extension

The mechanism by which AAK-2 signaling affects lifespan when ETC function is compromised is not well understood. It was proposed that AAK-2 and HIF-1 play antagonistic roles to regulate ROS levels to promote lifespan in animals with impaired ETC function (Hwang et al., 2014). In this model, mitochondrial ETC dysfunction trigger mild elevation of mitochondrial ROS, and this ROS signal is further amplified by HIF-1 and repressed by AAK-2 to achieve an optimal pro-longevity effect (Hwang et al., 2014). Since our data link CEH-23 and CEP-1 to the function of AAK-2 in longevity, we wondered whether CEH-23 and CEP-1 have roles in regulating ROS levels similar to that of AAK-2. We therefore tested ROS levels in the *isp-1* mutants with or without *ceh-23* or *cep-1* using the same methodology. To our surprise, we found that *ceh-23* and *cep-1* mutations slightly reduced ROS levels in the *isp-1* mutant, but the effects were

marginal (Figure 3.6A). To further examine a possible role of CEH-23 and CEP-1 in ROS-mediated longevity, we turned to the model in which treating wildtype animals with a low-dose of the ROS-inducing chemical paraquat is sufficient to extend lifespan (Yang and Hekimi, 2010b). The loss-of-function *aak-2(ok524)* mutant responds only marginally to this pro-longevity paraquat treatment (Hwang et al., 2014), suggesting that AAK-2 is required for optimal ROS-mediated lifespan increase. We tested whether the *ceh-23* or *cep-1* loss-of-function mutants can respond to a similar low-dose paraquat treatment, and found that neither *ceh-23* nor *cep-1* inactivation had any impact on the extended lifespan of worms treated with low-dose paraquat (Figure 3.6B, Figure 3.6C, Table 3.5). Taken together, our data suggest that whereas CEH-23 and CEP-1 are likely to act downstream of AMPK to modulate longevity, they are not likely to do so by regulating ROS levels.

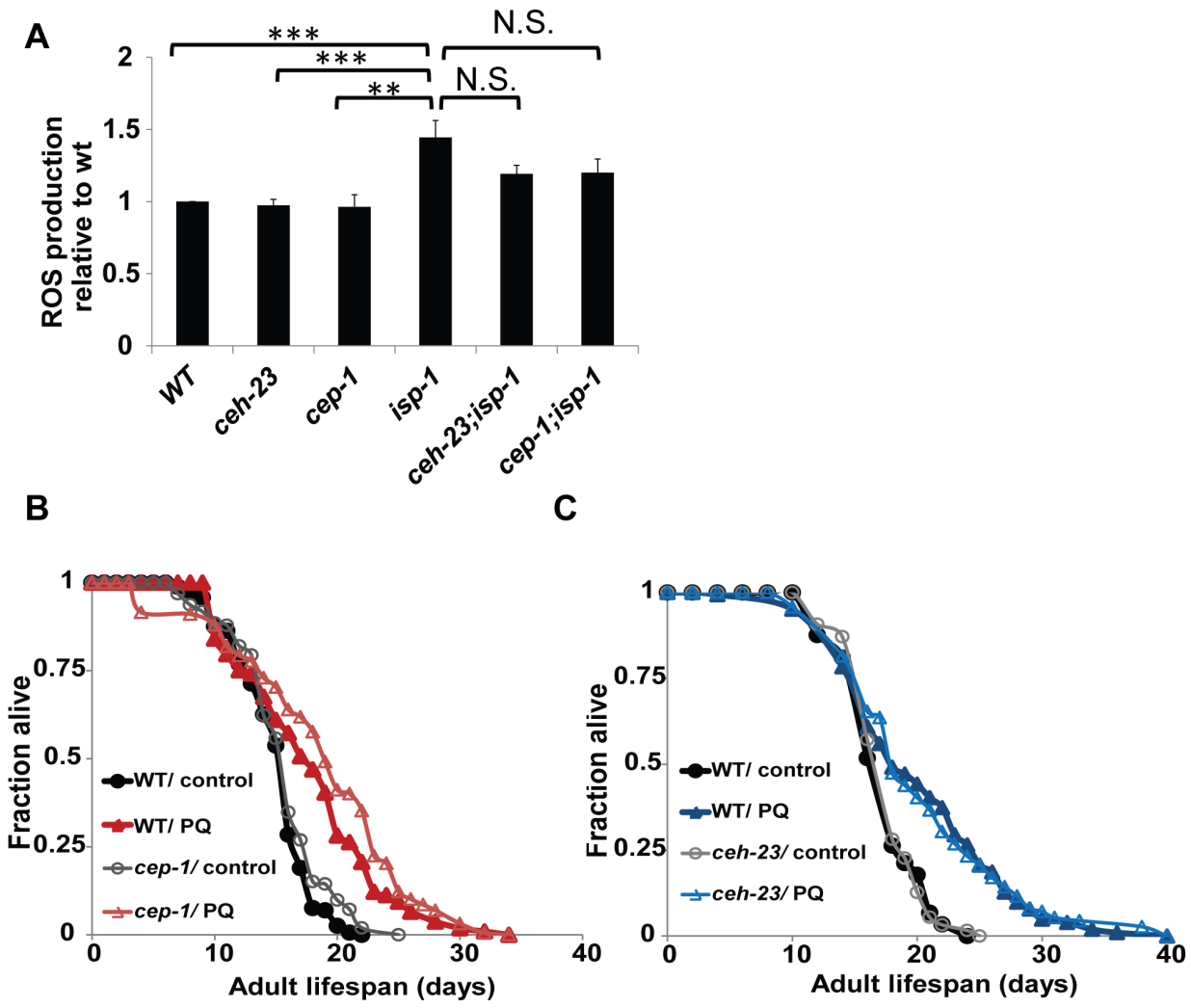


Figure 3.6. *ceh-23* and *cep-1* are not required for paraquat-induced lifespan extension. *isp-1* mutant exhibits mild elevation in ROS level measured by DCF-DA. *ceh-23* and *cep-1* mutations have marginal and statistical insignificant effects on ROS production of *isp-1* mutant (A) (P value: *<0.05, **<0.01, ***<0.005, and N.S= not significant). Treatment of 0.2 mM paraquat (PQ) extended the lifespan of wildtype worms. *ceh-23* and *cep-1* are dispensable for the PQ-mediated lifespan extension, as PQ treatments extended the lifespan of *cep-1* (B) and *ceh-23* (C) mutants to a similar extent as in wildtype worms. Survival curves represent data pooled from multiple biological replicates. Quantitative data for individual experiments are shown in Table 3.5.

Table 3.5 Effect of *ceh-23and cep-1* on PQ mediated lifespan extension

A

Strain/treatment	Total N	mean LS ± SEM (days)	% change to control treatment	*P*-value *vs control treatment*
ceh-23(ms23)/ control	86	19.09±0.44		
ceh-23(ms23)/0.05mM PQ	76	18.23±0.42	-4.510348705	.202
ceh-23(ms23)/ 0.1mM PQ	77	22.27±0.79	16.66772804	.000
ceh-23(ms23)/0.2 mM PQ	46	19.82±1.36	3.814539928	.128

B

Strain/treatment	Total N	mean LS ± SEM (days)	% change to control treatment	*P*-value *vs control treatment*
N2/ control	91	17.59±0.29		
ceh-23(ms23)/ control	88	17.75±0.36	0.91	0.819
N2/ 0.05mM PQ	102	19.41±0.46	10.36	0.002
ceh-23(ms23)/ 0.05mM PQ	90	18±0.57	2.34	0.368
N2/ 0.1mM PQ	91	20.71±0.64	17.75	0.000
ceh-23(ms23)/ 0.1mM PQ	103	19.1±0.62	8.60	0.006
N2/ 0.2mM PQ	71	21.45±0.7	21.92	0.000
ceh-23(ms23)/ 0.2mM PQ	69	20.58±1.04	16.99	0.000

C

Strain/treatment	Total N	mean LS ± SEM (days)	% change to control treatment	*P*-value *vs control treatment*
N2/ control	84	16.78±0.45		
ceh-23(ms23)/ control	82	17.18±0.38	2.41	.740
N2/ 0.1mM PQ	84	19.94±0.5	18.82	.000
ceh-23(ms23)/ 0.1mM PQ	87	19.42±0.45	15.73	.000
N2/ 0.2mM PQ	83	19.23±0.91	14.63	.027
ceh-23(ms23)/ 0.2mM PQ	87	20.33±0.77	21.18	.000
N2/ 0.3mM PQ	81	18.00±0.47	7.28	.074
ceh-23(ms23)/ 0.3mM PQ	77	17.31±0.45	3.18	.462

D

Strain/treatment	Total N	mean LS ± SEM (days)	% change to control treatment	*P*-value *vs control treatment*
N2/control	91	15.12±0.32		
cep-1(gk138)/control	83	15.55±0.45	2.84	
N2/0.1mM PQ	90	20.09±0.57	32.87	.000

cep-1(gk138)/0.1mM PQ	110	22.4±0.54	48.15	.000
N2_0.2mM PQ	28	19.68±1.27	30.16	.000
cep-1(gk138)/ 0.2mM PQ	83	21.82±0.69	44.31	.000

E

Strain/treatment	Total N	mean LS ± SEM (days)	% change to control treatment	*P*-value *vs control treatment*
N2/control	72	14.81±0.36		
cep-1(gk138)/control	70	15.86±0.35	7.09	
N2/0.1mM PQ	92	17.32±0.42	16.95	.000
cep-1(gk138)/0.1mM PQ	91	19.28±0.47	30.18	.000
N2/0.2mM PQ	70	18.65±0.55	25.93	.000
cep-1(gk138)/0.2mM PQ	95	18.99±0.47	28.22	.000

3.4 Discussion

Highly regulated communications between mitochondria and the nucleus are required for maintaining biological functions in response to compromised mitochondrial function. The transcription factors CEH-23 and CEP-1 have previously been identified as key mediators of the longevity extension phenotype of the mitochondrial ETC complex III mutant *isp-1(qm150)* (Baruah et al., 2014; Ventura et al., 2010; Walter et al., 2011). In this study, we investigated how these transcription factors mediate longevity specifically focusing on the possible collaboration between them. Epistasis analyses demonstrated that *ceh-23* and *cep-1* act through the same pathway to mediate the longevity of several different ETC mutants. This suggests that they are broadly required in adaptive responses to ETC dysfunctions, and are not limited to responding to defects in specific ETC complexes. Consistent with the genetic results, microarray analyses revealed that CEH-23 and CEP-1 share a large set of transcriptional targets that are enriched with kinases and phosphatases, in response to mitochondrial dysfunction. Intriguingly, the majority of the CEH-23 and CEP-1 co-regulated genes are also

transcriptional targets of constitutively active AAK-2 (Figure 3.3D and Figure 3.3E), thus pointing to a new link between CEH-23, CEP-1, and the AAK-2 signaling pathway. Further genetic analyses suggest that *ceh-23* and *cep-1* act downstream of AAK-2 to modulate lifespan. Interestingly, whereas the longevity effect of AAK-2 has been linked to ROS regulation, our data suggest that CEH-23 and CEP-1 likely act through other mechanisms, perhaps by regulating signal transduction, to modulate lifespan. Together, our data revealed a new regulatory relationship where CEH-23 and CEP-1 act downstream of AAK-2 to mediate the longevity phenotype of mutants with compromised mitochondrial ETC function. It is important to note that whereas we discovered a connection between AAK-2 and CEH-23 & CEP-1 by studying the mitochondrial mutant *isp-1*, our results with constitute active *aak-2* mutant suggest that CEH-23 and CEP-1 are likely downstream mediators of AAK-2 in additional contexts beyond mitochondrial dysfunction. Further elaboration of the molecular nature of the regulatory relationship among CEP-1, CEH-23, and AAK-2 will provide important mechanistic insights that will likely be broadly relevant.

In the context of mitochondrial dysfunction, inactivation of AAK-2 not only suppresses the extended lifespan of the *isp-1* mutant, but also results in worms that develop substantially slower than the *isp-1* mutant alone (Curtis et al., 2006). In contrast, while inactivation of CEH-23 and CEP-1 partially suppress the prolonged lifespan of the *isp-1* mutant, CEH-23 loss has no impact on the development of the *isp-1* mutant (Walter et al., 2011), and CEP-1 loss results in a slight but significant improvement on the development of the *isp-1* mutant (Ventura et al., 2010). These data suggest AAK-2,

CEH-23, CEP-1 have common as well as unique functions in mediating the physiological outputs of the *isp-1* mutant. Since all three factors appear important to mediate the extended lifespan of the *isp-1* mutant, we speculate that their shared transcriptional targets will be enriched for genes with roles in longevity determination. Among the 424 genes that showed similar expression changes in the constitutively active *aak-2* strain and in the *isp-1* mutant in a *ceh-23* and *cep-1* dependent manner, about half have no clear annotated function, whereas the other half are highly enriched for kinases (59), phosphatases (36), MSPs (37), and neurotransmitter transporters (6) (Figure 3.3E).

The *C. elegans* genome encodes 438 kinases, and our study revealed that CEH-23, CEP-1 and AAK-2 together regulate the expression of about 10% of the *C. elegans* kinases (59 out of 438). Among the 59 kinases, 40 of them are dual-specificity kinases, which can act as both tyrosine and serine/threonine kinases, and 18 of them are serine/threonine specific kinases. Strikingly, most of the CEH-23-, CEP-1-, and AAK-2-regulated kinases belong to two major kinase families: TTBKL (Tau-tubulin kinase like) serine/threonine kinases and FER (fps/fes related) tyrosine kinases. Interestingly, TTBK kinases have been associated with neurodegeneration in human, where TTBK1 is thought to phosphorylate Tau protein and promote the progression of Alzheimer's disease (Sato et al., 2006) and TTBK2 has been linked to another Tau related disease, spinocerebellar ataxia 11 (SCA11) (Ikezu and Ikezu, 2014). TTBK family is part of the CK1 superfamily and is one of the most expanded kinase families in *C. elegans*. Whereas *C. elegans* has a single TTBK ortholog, it has 31 kinases that display

sequence similarity to TTBK (G et al., 2002). Although the functions of most of these TTBK-like kinases are unknown, one has recently been implicated in the pathogenesis of a *C. elegans* model of the neurodegenerative disease amyotrophic lateral sclerosis (ALS) (Ikezu and Ikezu, 2014). Therefore, it is possible that other TTBK-like kinases may also have roles in neuronal functions. Indeed, many of the TTBK-like kinases identified in our study are expressed in neurons (Spencer et al., 2011). Further analyses of this subfamily of TTBK related kinases will likely provide insights pertinent to the adaptive response induced by mitochondrial dysfunction.

In addition to kinases, phosphatases are also overrepresented among the genes that are commonly regulated by CEH-23, CEP-1, and AAK-2. In particular, 11 of the 36 phosphatases are serine/ threonine specific phosphatases, and they share sequence similarity to the protein phosphatase I (PP1) catalytic subunit. PP1 has broad functions in diverse tissues, including neurons. Interestingly, PP1 has also been implicated in Alzheimer's disease (Braithwaite et al., 2012). Intriguingly, our analyses also revealed that several sodium:neurotransmitter symporters are overrepresented among the common transcriptional targets of CEH-23, CEP-1 and AAK-2. Taken together, it appears that neuronal signaling might represent a key downstream output of active AAK-2 and CEH-23 and CEP-1 in the *isp-1* mutant. Supporting this notion, more than 30% of the CEH-23, CEP-1 and AAK-2 commonly regulated genes are neuronally expressed (152 out of 424) (Spencer et al., 2011). In this regard, it is particularly interesting to note that ETC dysfunction in the neurons are thought to have a particularly key role in modulating longevity (Dillin et al., 2002). Furthermore, CEH-23, CEP-1 and

AAK-2 are all expressed in neurons (Mair et al., 2011; Spencer et al., 2011; Walter et al., 2011), and the action of AAK-2 in neurons has recently been demonstrated to play a particularly important role in modulating longevity (Burkewitz et al., 2015). An intriguing possibility is that the ETC dysfunction in the *isp-1* mutant induces the activation of AMPK and CEH-23 and CEP-1 in the neurons, which elicits a protective effect on lifespan.

Another interesting group of genes revealed as CEH-23, CEP-1 and AAK-2 common transcriptional targets encode MSPs. MSPs are the most abundant proteins found in *C. elegans* sperm, and have function in both signaling, as secreted hormones, and cell structure, as intracellular cytoskeletal proteins (Miller et al., 2001). During fertilization, secreted MSPs play key roles to promote oocyte maturation and MAPK signaling activation. Currently we do not understand why the MSPs are overrepresented in the common targets of CEH-23 and CEP-1, as we have not detected a substantial effect of CEH-23 and CEP-1 in the reproduction of the *isp-1* mutant (Baruah et al., 2014; Walter et al., 2011). However, it is worth noting that the MSP domain is highly conserved, and interestingly, a substitution mutation in the human MSP domain protein VAPB has been associated with ALS(Nishimura et al., 2004), suggesting a possible role of MSP-domain proteins in neuronal function.

We further compared the transcriptional profiles of the *isp-1* mutants with or without *ceh-23* or *cep-1* with that of wildtype worms (Figure 3.7). This comparison revealed a somewhat surprising pattern where the genes that are commonly upregulated in the *isp-1* mutant in a CEH-23- and CEP-1-dependent manner appear to

be downregulated in the *isp-1* mutant relative to wildtype worms (Figure 3.7, Cluster A), suggesting that functional CEH-23 and CEP-1 are required for maintaining the expression of these genes in the *isp-1* mutant, albeit at levels lower than wildtype, and inactivation of CEH-23 and CEP-1 result in further repression of these genes. Since the *isp-1* mutant is long-lived compared to the *ceh-23; isp-1* or *cep-1; isp-1* mutants, we proposed that activation of AAK-2, CEH-23, and CEP-1 in the *isp-1* mutant acts to buffer the downregulation of these genes, allowing the worms to live long, and the absence of AAK-2, CEH-23, CEP-1 results in further repression of these genes and a detrimental effect on lifespan.

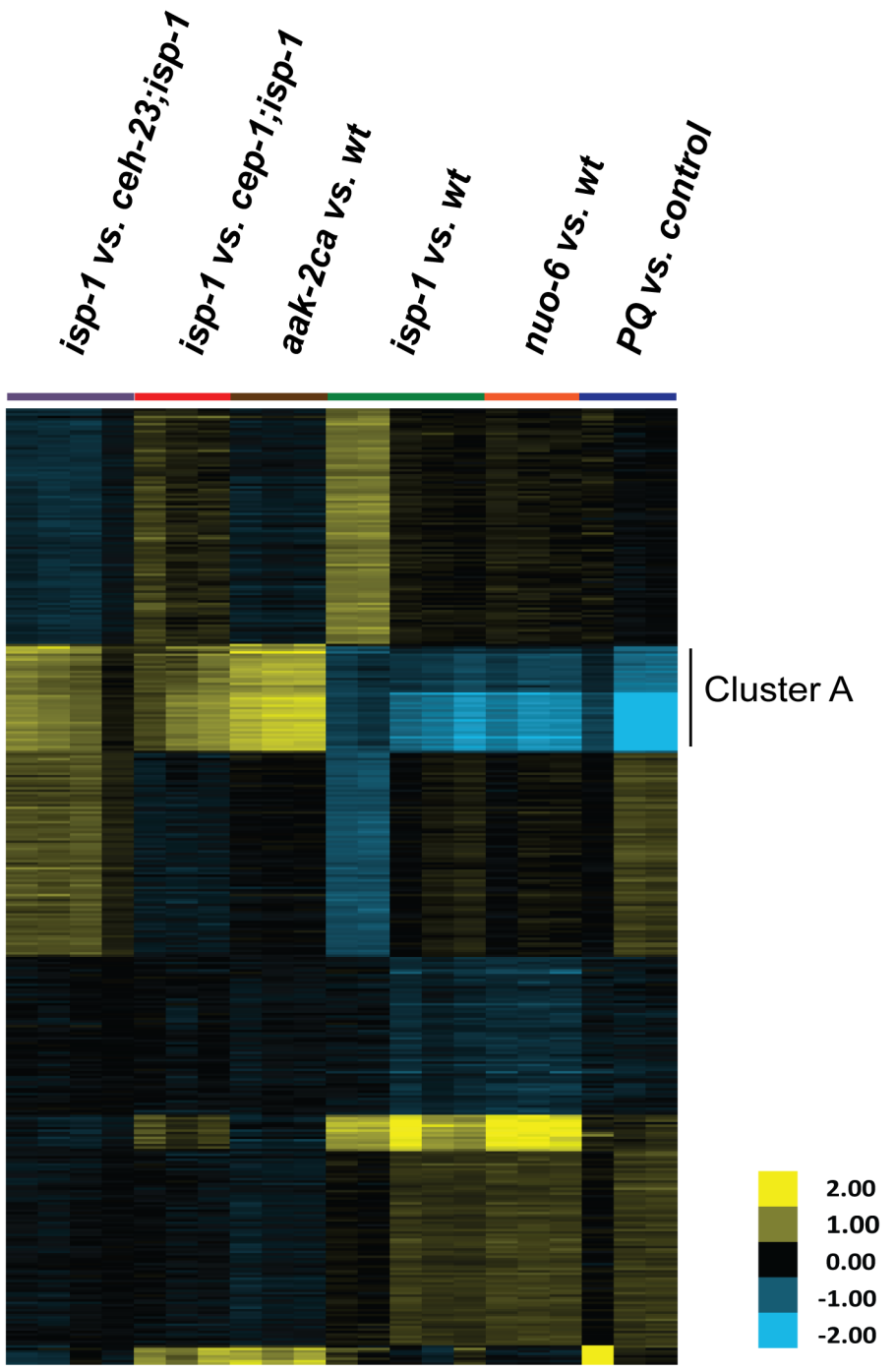


Figure 3.7. AAK-2, CEP-1, and CEH-23 might buffer the downregulation of a select set of genes in the mitochondrial ETC mutants.
The heat map shows a comparison of the indicated gene expression profiles. Yellow depicts genes that are upregulated, and blue depicts genes that are downregulated, in *isp-1*(qm150), *aak-2*ca, *nuo-6(qm200)*, or worms treated with pro-longevity dosage of paraquat as compared to the double mutants or wildtype or control, respectively. Cluster A represents the gene set commonly regulated by CEH-23, CEP-1, AAK-2 as discussed above.

It has been shown that the transcriptional response of the *isp-1* mutant overlaps with that of the long-lived ETC mutant *nuo-6(qm200)* and wildtype worms treated with pro-longevity dose of paraquat (Yee et al., 2014). Since slight elevation of ROS levels has been shown to contribute to the longevity of the *isp-1(qm150)* and *nuo-6(qm200)* mutants and worms treated with low dosage paraquat, their shared transcriptional response has been proposed to represent a molecular signature of pro-longevity ROS signaling (Yee et al., 2014). Similar to the comparison with *isp-1* vs. wildtype arrays, the genes that are upregulatead by in the *isp-1* mutant in a CEH-23- and CEP-1-dependent manner or in the constitutively active *aak-2* strain are also downregulated in the *nuo-6* mutant and in paraquat treated wildtype (Figure 3.7). This observation would be consistent with the model that active AAK-2 could dampen ROS levels (Hwang et al., 2014). However, our data indicate that CEH-23 and CEP-1 do not appear to modulate ROS levels in the *isp-1* mutant (Figure 3.6A). We do not yet understand what contributes to this striking opposite pattern in gene expression regulation. It is possible that in order to cope with ETC dysfunctions and oxidative stress, AAK-2, CEH-23 and CEP-1 are activated to buffer gene expression changes, and their loss led to further drastic change in gene expression that result in reduced lifespan. Further investigation into this interesting gene expression regulation will likely reveal important new insights about how cells and organisms adapt to mitochondrial dysfunction and ROS elevation. As a master regulator of energetic stress, AMPK is able to respond to other upstream signals, in particular a change in AMP/ADP/ATP ratio (Burkewitz et al., 2014), and

mediates its biological effects through different downstream substrates. Besides mild elevation of ROS, *isp-1* mutants also exhibit increased ADP:ATP ratio (Curtis et al., 2006). We hypothesize that CEH-23 and CEP-1 act downstream of AAK-2 in response to a stress signal that is independent of ROS and confer compensatory transcriptional changes that promote lifespan.

AMPK is a critical metabolic sensor and regulator and its deregulation has been linked to major diseases, such as cancer and neurodegenerative disease (Burkewitz et al., 2014; Cai et al., 2012). Likewise, mitochondrial ETC dysfunction is tightly linked to aging and many diseases (García-Escudero et al., 2013). AMPK has long been implicated as a key responder to mitochondrial dysfunction (Burkewitz et al., 2014; Curtis et al., 2006; Hwang et al., 2014), especially because of its ability to respond directly to altered AMP/ATP ratio and redox imbalance (Burkewitz et al., 2014). Our study reveals a new link between the transcription factors CEP-1/p53 and CEH-23 to AAK-2/AMPK and implicates additional downstream signaling, possibly involving the TTBK subfamily of kinases, as an adaptive response that leads to prolonged lifespan in mutants with reduced ETC function. Importantly, our finding suggests CEH-23 and CEP-1/p53 are key effectors of AAK-2/AMPK in contexts beyond mitochondrial ETC dysfunction. Further investigation of the molecular relationship of AAK-2/AMPK, CEH-23, and CEP-1/p53 will likely provide important insights that are broadly relevant to aging and age-related diseases in diverse organisms.

3.5 Materials and Methods

C. elegans strains

All strain stocks were maintained on OP50 Escherichia coli at 20^{o}C and were handled under standard growth conditions. We used the following strains: Wildtype N2 Bristol, *ceh-23(ms23), cep-1(gk138), isp-1(qm150)*, *ceh-23(ms23)*; *isp-1(qm150)*, *cep-1(gk138); isp-1(qm150)*, *cep-1(gk138); ceh-23(ms23)*; *isp-1(qm150)*, *nuo-6(qm200)*, *mev-1(kn1)*, *mev-1(kn1); cep-1(gk138*), *gas-1(fc21)*, *cep-1(gk138); gas-1(fc21)*. Standard genetic methods were used to construct the following strains: *nuo-6(qm200); ceh-23(ms23)*, *ceh-23(ms23) mev-1(kn1)*, *ceh-23(ms23); gas-1(fc21)*, *uthIs248; ceh-23(ms23), uthIs248; cep-1(gk138),* and *uthIs272; ceh-23(ms23)*. We failed to generate *uthIs272; cep-1(gk138)* using the same cross strategy possibly because the transgene and *cep-1(gk138)* is linked. *nuo-6(qm200) cep-1(gk138)* was generously provided by Dr. Siegfried Hekimi at McGill University. WBM60 uthIs248[P*aak-2*::*aak-2* genomic (aa1-321)::GFP::unc54 3'UTR, Pmyo-2::tdTomato] is the transgenic strain with overexpressed *aak-2ca* and WBM59 uthIs272[Pmyo-2::tdTomato, unc-54 3'UTR] in the transgenic control. Both WBM59 and WBM60 were generous gifts from Dr. Willian B. Mair at Harvard University.

Lifespan analysis

All lifespan experiments were performed at 20°C on Nematode Growth Media (NGM) plates seeded with E. coli OP50 or HT115 for RNAi experiments. For experiments with OP50, bacteria was cultured overnight at 37°C, the OD600 was measured, and the overnight culture was concentrated to OD600=4.0. One hundred and fifty microliters of concentrated culture was seeded onto 35 mm NGM plates. Most lifespan experiments were performed without FUDR, unless otherwise noted. All lifespan plates were seeded

and dried at room temperature and stored at 4°C until use. For NAC treatment, NAC was added to the bacterial lawn to a final NAC concentration of 1 mM at least 8 hours before use. For RNAi experiments, HT115 bacteria containing vectors expressing dsRNA were grown at 37°C in LB with 100 ug/ml carbenicillin and 15 ug/ml tetracycline to OD600=0.8 and concentrated to OD600=4. IPTG (4 mM) was added to RNAi bacteria-seeded plates at room temperature overnight prior to use to induce dsRNA expression. Well-fed gravid adult worms were allowed to lay eggs at 20°C, and the progeny were grown at 20°C. The synchronized adult worms were transferred to new lifespan plates every other day until day 12. The adult worms were scored for survival every other day. The survival curves of each population were estimated using the Kaplan-Meier method, and a log-rank test was performed for statistical analysis. A $P \leq 0.01$ was considered significantly different from the control population. The independent trials were analyzed both separately and pooled between independent trials, and the pooled experiments are presented in the figures.

Oxidative stress assay

L4 worms were transferred to NGM plates with 10mM paraquat or vehicle control (M9). Survival of worms was scored 96 hours after exposure to paraquat. The experiment was done at 20°C.

Measurement of total ROS levels by using 2',7'-dichlorofluorescein diacetate (DCF-DA).

To measure the total ROS levels, a DCF-DA (Molecular Probes, Carlsbad, CA, USA) assay was used as described previously(Lee et al., 2010).

Microarray

Microarray sample preparation. *isp-1(qm150) and ceh-23(ms23); isp-1(qm150)* mutant worms were grow on NGM plates with live OP50 bacteria. Worms were harvested when the majority of the population reached mid-L4 stage. Total RNA was isolated using Tri-reagent (Molecular Research Center, Inc.) and purified with the RNeasy kit (Qiagen). cRNA synthesis/amplification, Cy3/Cy5 dye labeling, and hybridization onto Agilent 4X44K *C. elegans* oligonucleotide microarrays (v2) were performed as previously described (Shaw et al., 2007b). Two out of the four arrays were dye-flip replicates, and three independent biological experiments were performed.

Microarray analysis. Raw microarray data were normalized using Agilent feature extraction software. The normalized data were uploaded onto to the Princeton University MicroArray database (PUMA [http://puma.princeton.edu]). The raw data were retrieved by SUID (Sequence Unique IDentifier) then averaged by wormbase ID. Log2-transformed fold-changes were acquired after filtering out genes with <80% good data. The data were analyzed and visualized using Cluster 3 and Tree View (Saldanha, 2004; Tusher et al., 2001). Gene sets that were regulated by CEH-23 in the *isp-1(qm150)* mutant were determined by SAM 1 class analysis (Tusher et al., 2001)with a FDR=0.59% and 1.5 fold gene cutoff. Ten genes were randomly selected to validate the quality of this gene list using RT-qPCR. Similar 1 class analysis was performed with

stringent criteria (FDR=0% with 1.5 fold gene cutoff) using the *isp-1 vs. cep-1; isp-1* and *isp-1 vs. ceh-23; isp-1* datasets to identify genes that are commonly regulated by CEH-23 and CEP-1 under ETC stress. We identified 916 common targets of CEH-23 and CEP-1 from this analysis. genome-wide CA-AAK-2 vs. wildtype microarray data were obtained from GEO (GSE25513) and 1 class SAM analysis was used to identify the genes that show significant changes in gene expression in CA-AAK-2 animals compare to wildtype. Genes that are common regulated by CEH-23, CEP-1 and AMPK represent the overlap between the CEH-23 and CEP-1 commonly regulated gene list and the CA-AAK-2 regulated gene list described above.

Gene Ontology classification. Gene sets identified by SAM analysis were input into the Functional annotation clustering tool in DAVID (http://david.abcc.ncifcrf.gov/) (Huang et al., 2009) for gene annotation enrichment analysis. Functional annotation clustering was performed with the default setting. Annotated clusters with enrichment scores were reported from this analysis. Kinases and phosphatases classification were based on GO term molecular function.

Statistical significance of overlap between gene lists. The statistical significance between CEH-23 and CEP-1 common target genes and AAK-2 CA targets or ROS-responsive targets were determined using the web-based tool (http://nemates.org/MA/progs/overlap_stats.html). A representation factor and p-value (determined by hypergeometric probability test) were reported.

Western Blot

Worms were grown on 10 cm NGM plates seeded with OP50 E. coli, synchronized at the mid-L4 stage, and harvested in M9 buffer. Worms were lysed by boiling and vortexing for 10 minutes each and were centrifuged at 13,000 rpm for 15 minutes to remove insoluble debris. The protein concentrations were quantified using Quick Start Bradford Protein Assay (Biorad). One hundred nanograms of total protein was loaded on 8% SDS-PAGE, electrophoresed, and transferred to a nitrocellulose membrane. The nitrocellulose membrane was blocked in 5% BSA in TBST and subsequently incubated with primary antibody, anti-actin (mouse, Chemicon) and anti- phospho-AMPKα (rabbit, #4188 Cell Signaling Beverly, MA). Anti-phopho- AMPKα incubation was done at 4^{o}C for 6-9 hours, and Anti-actin incubation was done at room temperature for 1 hour. Anti-mouse or anti-rabbit secondary antibodies conjugated to fluorescent dyes were used for anti-ACTIN and anti-phopho-AMPKα, respectively. The western blot images were obtained with the Odyssey infrared imaging system and quantified using Image Studio ver. 2.0. Phospho-AMPKα levels were normalized to actin as an internal control and normalized to WT.

Development assay

Gravid adult worms were placed onto 60 mm NGM plates seeded with OP50 E. coli and allowed to lay eggs for 3-5 hours. Subsequently, the adults were removed, and the embryos were allowed to develop at 20^{o}C. The developmental stages were scored 60 hours after egg lay. The L1-L3 larval stages were scored based on gonad structure using a DIC microscope (Leica DM 5000B microscope). The L4 larval stage and adult worms were identified based on vulval morphology.

Accession Number

The GEO accession number for the *isp-1(qm150) vs. ceh-23(ms23); isp-1(qm150)* microarray dataset in this paper is GSE67754

3.6 Acknowledgments

We thank the *Caenorhabditis* Genetics Center for providing some of the strains used in this study, Dr. Willian B. Mair at Harvard University for the *aak-2ca* strain, and Dr. Siegfried Hekimi at McGill University for the *nuo-6 cep-1* strain. We thank Dr. Erich Schwarz and Lee lab members at Cornell University for helpful discussion and suggestions. We also thank Dr. Kenneth Kemphues and Dr. Thomas D. Fox at Cornell University for helpful discussion and critical reading of the manuscript.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Mitochondrial electron transport chain (ETC) function has been tightly link to aging and longevity. Interestingly, mild defects in the mitochondrial ETC extend lifespan in various organisms, suggesting that the ETC modulates longevity through a conserved mechanism. The detailed mechanism of how ETC function affects organismal lifespan is not fully understood. Mitochondria and the nucleus have coevolved throughout millions of years, and the functions of these two organelles are highly interdependent. Precisely coordinated communication between mitochondria and the nucleus ensure proper cellular function and organismal survival. Retrograde signaling was first described in yeast, where impaired mitochondrial function triggered a transcription response in the nucleus to cope with the mitochondria defects (Kirchman et al., 1999). A similar communication platform relays signals from mitochondria to the nucleus in other organisms. In *C. elegans*, several microarray analyses revealed transcriptional changes in response to ETC dysfunctions and identified several transcription factors (TFs) required for the longevity of these mitochondrial ETC-impaired animals, suggesting retrograde signaling is essential for coping with ETC dysfunction (Cristina et al., 2009; Falk et al., 2008). The longevity effects of some TFs have been characterized (Baruah et al., 2014; Lee et al., 2010; Walter et al., 2011), but whether these TFs function collaboratively has been understudied. My work has focused on determining whether the transcription factors CEH-23 and CEP-1 modulate the longevity of the ETC-impaired

worms in a collaborative manner and the possible mechanisms that these transcription factors may engage to contribute to aging.

Using genetic and functional genomic approaches, I demonstrated that CEH-23 and CEP-1 likely collaborate to modulate the lifespan of ETC mutants. Consistent with this finding, CEH-23 and CEP-1 trigger similar transcriptional responses under ETC stress. Surprisingly, CEH-23 and CEP-1 commonly regulated genes are not enriched with many known aging genes but rather with kinases and phosphatases, suggesting possible novel aging genes involved in cell signaling might be uncovered by my analyses. I further demonstrated that CEH-23 and p53/CEP-1 mediated transcriptional reprogramming has a tremendous overlap with transcriptional responses induced by constitutively active AMPK without any ETC dysfunctions suggesting a possible collaboration among CEH-23, p53/CEP-1 and AMPK. Supported by several functional and bioinformatics data, we proposed a model in which CEH-23 and p53/CEP-1 act downstream to the AMPK when AMPK activity is further induced by ETC dysfunctions. In mammal, p53 has been shown to be one of the AMPK substrates (Adamovich et al., 2014) suggest our observation between AMPK with CEH-23 and p53/CEP-1 is likely to be conserved.

4.1 Modes of interaction between CEH-23 and CEP-1 and their tissue specificity requirements

My data showed that CEH-23 and CEP-1 are epistatic to each other in modulating the longevity of the *isp-1(qm150)* mutant. Precisely how these TFs collaborate remains to be explored. As CEH-23 and CEP-1 share a significant amount

of common transcriptional targets, they likely interact at a molecular level. However, based on data from transgene expression, CEH-23 and CEP-1 do not appear to be coexpressed in any cell types; CEH-23 is expressed in neurons and intestine, whereas CEP-1 is expressed in the pharynx and germline. However, because transgenes are frequently silenced in the germline, it is possible that the two proteins are co-expressed in the germline. Supporting this notion, germline-expressed genes are overrepresented in *ceh-23* and *cep-1* shared gene targets in my microarray studies. Nonetheless, these two TFs are not likely to act together to activate their targets in pharynx, neurons and intestine since CEH-23 and CEP-1 are not co-expressed in these tissues. Lack of the co-expressed tissue lead to the speculation that *ceh-23* and *cep-1* is likely to regulate gene expressions of their target genes cell non-autonomously. Future work on dissecting the real expression pattern using the single copy tagged gene will allow us to determine whether CEH-23 is expressed in the germline, where CEH-23 and CEP-1 can potentially physically interact to commonly regulate their downstream transcriptional targets. It is also important to investigate the possibility that CEH-23 and CEP-1 can act cell non-autonomously to regulate gene expression at the distal tissue. Understanding tissue specificity requirement or sites of action of CEH-23 and CEP-1 will further our understanding on how these two TFs interact and how they response to different tissue.

4.2 CEH-23 and CEP-1 specific transcriptional targets revealed the distinct biological processes that are regulated by these two TFs.

My work suggests that CEH-23 and CEP-1 modulate the lifespan of ETC mutants through a similar mechanism possibly through their common targets. Although CEH-23

and CEP-1 shared a large group of common targets, they also have their own distinct targets. Very little is known about the biological function of CEH-23 except for its role in regulating neuronal differentiation markers and in regulating longevity in ETC-defective worms. My microarray results uncovered novel biological processes that may involve CEH-23. For example, CEH-23 specifically regulates many cell cycle genes involved in both mitosis and meiosis, suggesting that CEH-23 might participate in reproduction or development. Although *ceh-23* mutation does not appear to affect the development of *isp-1(qm150)* mutants, as discussed in Chapter 3, CEH-23 does impact the development of *cep-1(gk138);isp-1(qm150)* double mutants. Interestingly, CEH-23 and CEP-1 exhibit different relationships in regulating development and lifespan. It is possible that CEH-23 and CEP-1 collaborate via complementary temporal stages to regulate development and longevity, respectively. Besides cell cycle regulation, my work also revealed a potential role of CEH-23 in regulating neuronal function, as it regulates expression of several neuropeptides. Although the *ceh-23* mutant does not exhibit major defects in neuronal morphology, its behavioral phenotypes associated with neuronal function have not been fully explored.

Like CEH-23, CEP-1 also responds to ETC stress, as discussed in Chapter 2. Besides regulating longevity, CEP-1 regulates apoptosis and is important for coping with irradiation stress(Derry et al., 2001). The differential functions of CEH-23 and CEP-1 can be further demonstrated by comparing the CEH-23 and CEP-1 transcription targets with the transcriptional output of other known signaling pathways. Interestingly, my analyses revealed that CEH-23 and CEP-1 may functionally engage mitogen-activated

protein kinases (MAPKs), MPK-1/ERK, and PMK-1/p38, as they shared significant overlap in their transcriptional targets (Described in Appendix I). Both MPK-1/ERK and PMK-1/p38 have been implicated in longevity modulation (Okuyama et al., 2010; Troemel et al., 2006), and both MAPKs respond to ROS (Schmeisser et al., 2013b; Yang et al., 2010b), highlighting the relevance of MAPK signaling to mitochondrial ETC longevity. Although both ERK and p38 MAPKs are attractive candidates for CEH-23 and CEP-1 partners for modulating organismal lifespan in ETC-impaired animals, these two MAPKs likely engage independent regulatory circuits. However, this hypothesis is inconsistent with the observation that CEH-23 and CEP-1 act through a similar mechanism. However, phosphatases in mammals have been shown to mediate crosstalk between ERK and p38 (Wang et al., 2006). Further investigation into whether a similar crosstalk exists in *C. elegans* and possible roles that CEH-23 and CEP-1 might have in this pathway is merited.

4.3 Implications in interactions among CEH-23, CEP-1 and AMPK

The link between CEH-23 and p53/CEP-1 to AMPK is very is very exciting. Other than ETC dysfunctions, AMPK is also activated by other stresses with imbalanced energy states and plays crucial roles for organismal survival under these stresses. As an important regulator for cellular energy and metabolism, AMPK has been implicated in several human diseases. It is interesting that *ceh-23* and *cep-1* are required for several phenotypes that are associated with constitutive active AMPK animals (AAK-2ca), which have relatively normal mitochondrial ETC function compared to the ETC mutants. The requirement of CEH-23 and CEP-1 for the AAK-2ca associated phenotypes suggest the

relationship between CEH-23, CEP-1 and AMPK is not limited to the ETC stress. Consistent with this hypothesis, it has been shown that AMPK regulates the activity of p53 in liver cancer cell (Lee et al., 2012) suggest that the relationship among CEH-23, p53/CEP-1, and AMPK we observed in the ETC mutant worms can be extended to the mammalian cancer cells. Human EMX2 is a putative human CEH-23 homolog, and its down-regulation in expression has been correlation with tumor growth of lung cancer and edometrical cancer (Okamoto et al., 2010; Qiu et al., 2013). Similar to p53 and AMPK, EMX2 also possesses tumor suppressor function as overexpress EMX2 can suppresses tumor growth in human (Li et al., 2012). Very little is known about the relationship between AMPK and EMX2 in mammal, but it has been shown that the decreased EMX2 transcription can be restored by AMPK activator, resveratrol (Cao et al., 2014). Given the relationship of AMPK, EMX2/CEH-23, and p53/CEP-1 and their roles in cancer, further investigation of EMX2/CEH-23 and p53/CEP-1 on AMPK mediated cancer pathway will be an interesting topic for future research.

Interestingly, my microarray analyses revealed that many of the kinases that are commonly regulated by CEH-23, CEP-1 and constitutively active AAK-2 (Figure 3.3D, ClusterA) are homologous to the tau tubulin kinases (TTBK) in human. Human TTBK has two isoform TTBK1 and TTBK2 both of them has been implicated in the neurodegenerative diseases (Liachko et al., 2014; Sato et al., 2006). In both mice and fly models, several lines of evidence implied that AMPK activity has protective roles against neurotoxicity diseases such as Alzheimer's disease and Parkinson's disease (Ng et al., 2012; Vingtdeux et al., 2010) while *C.elegans* TTBK protein has been

implicated in amyotrophic lateral sclerosis (ALS). Together these observations hint at an intriguing model in which CEH-23 and CEP-1 work downstream to AMPK and transcriptionally regulated tau tubulin kinases like proteins (TTBKLs) to cope for neurotoxicity. Further analyses to study the roles of the CEH-23, CEP-1 and AMPK commonly regulated TTBKLs in neurodegenerative disease progression will be crucial to test this model.

In conclusion, I suggest the following model based on my work: When the mitochondrial ETC is impaired, one or more stress signals is released from defective mitochondria to the nucleus and triggers the activation of TFs, such as CEH-23 and CEP-1. In this context, AMPK signaling pathway can be activated in response to the ETC stress signals and led to activation of the several transcription factors including CEH-23 and CEP-1 in the nucleus. Together, the concerted action of signaling pathway and transcription network modulate the lifespan of organisms with impaired mitochondrial ETC function. Mitochondria have been associated with aging and longevity for decades, but our knowledge about how these organelles function influence on lifespan in the whole organism remains incomplete. This work attempts to integrate multiple entities that are known to affect lifespan when mitochondrial ETC function is aberrant. Additionally, my work also revealed an exciting relationship between the transcription factors CEH-23 and CEP-1 to AMPK signaling and suggested that this observation can be extended beyond the ETC dysfunctions. The findings from my work demonstrated a sophisticated collaboration between cell signaling pathway and transcriptional network to cope for cellular stress. My study will pave the way for future investigation into how

does transcriptional reprogramming influence on lifespan under ETC stress and the possibility of manipulating their transcriptional targets to promote healthy aging in the absence of ETC dysfunction.

APPENDIX I

CEH-23 AND CEP-1 MAY COLLABORATE WITH MAPKS MPK-1/ERK AND PMK-1/P38, RESPECTIVELY.

As discussed in Chapter 3, the microarray analyses revealed that CEH-23 and CEP-1 regulate expression of many kinases and phosphatases when the mitochondrial ETC is impaired. During my investigation for possible lifespan effects of these phosphate metabolic genes, I observed that four of the CEH-23 and CEP-1 coregulated phosphatases were linked to *C. elegans* MAPKs through interactome analyses (Figure AI.1).

The mitogen-activated protein kinases (MAPKs) are highly conserved, and their function is important for many cellular functions such as cell proliferation, cell differentiation, apoptosis, and cell survival. The MAPK family is comprised of ERK, p38, and JNK1. The interactome analyses linked the CEH-23 and CEP-1 coregulated tyrosine phosphatases to MPK-1, which is the homolog of *C. elegans* ERK, and PMK-1 and PMK-2, which are the *C. elegans* orthologs of p38. We further investigated the possible relationship between CEH-23, CEP-1, and these MAPKs by comparing the transcriptional targets of CEH-23, CEP-1, MPK-1, and PMK-1. MPK-1 targets were identified using microarray analysis of the temperature-sensitive mpk-1(ad111) mutant, which revealed that ERK signaling was active in the germline (Leacock and Reinke, 2006). The PMK-1 targets were also identified by microarray aimed at identifying PMK-1-responsive genes in the long-lived daf-2 mutant (Troemel et al., 2006). To our surprise, when we compared the transcription outputs of CEH-23, CEP-1, and MAPK, we

observed that CEH-23 and CEP-1 did not coregulate the expression of either MPK-1/ERK or PMK-1/p38 transcription targets. Instead, CEH-23 transcriptional targets significantly overlapped with MPK-1/ERK-responsive genes (Figure AI.2A), whereas CEP-1 and PMK-1/p38 shared more transcriptional targets (Figure AI.2B).

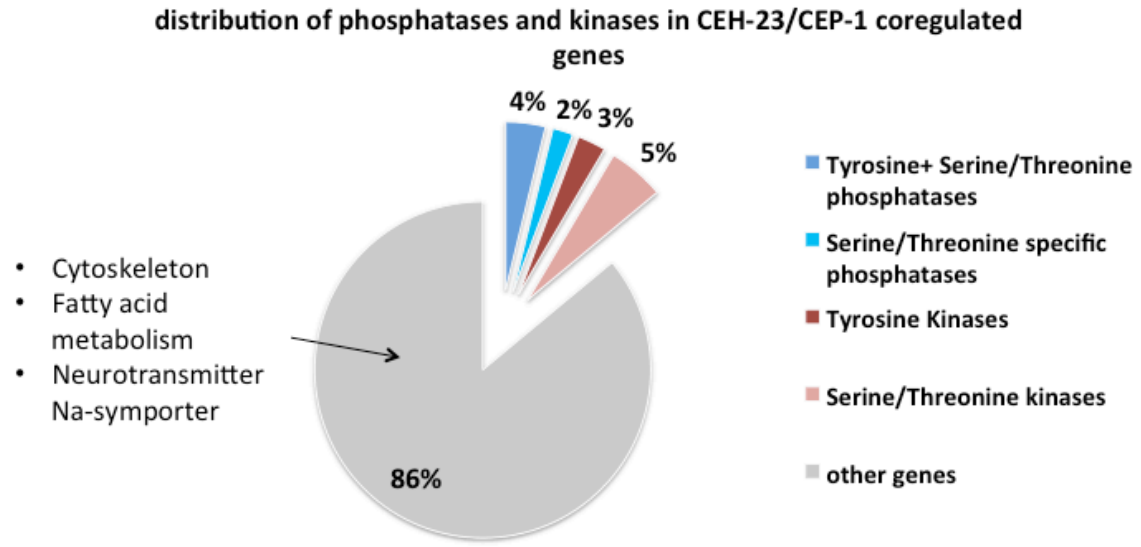
Furthermore, the expression pattern of CEH-23, CEP-1, and MAPK revealed another interesting insight into their regulatory functions. CEH-23 is coexpressed with PMK-1 and MPK-1 in neurons and the intestine, which are organs important for regulating longevity in various organisms. CEP-1 is coexpressed in the germline with MPK-1. It is possible that CEH-23 interacts with MPK-1 in neurons and the intestine to activate the expression of their common targets, and the activation of these genes might contribute to lifespan determination. The CEP-1 expression highlights a predicament about how CEP-1 and PMK-1 may interact, as they are not expressed in the same cell type. One possibility is that CEP-1 or PMK-1 act through a cell non-autonomous mechanism to activate PMK-1 activity in neurons or the intestine.

MAPK regulates development, so I further tested whether there was a temporal requirement for CEH-23 and CEP-1 to regulate the expression of MAPK target genes. My results revealed that CEP-1 specifically regulates the expression of PMK-1 targets at both the fourth larval (L4) and young adult (YA) stages, as the expression of selected PMK-1 target genes were reduced in the *cep-1*(gk138);*isp-1*(qmq150) double mutant but unaffected in the *ceh-23*(ms23);*isp-1*(qm150) double mutant (Figure AI.4A, B). Furthermore, my qPCR results demonstrated that both CEH-23 and CEP-1 regulate the lifespan of mpk-1 at the L4 stage (Figure AI.4C). This regulation is temporally controlled,

as the difference in mpk-1 targets gene expression between the *isp-1*(qm150) single mutant and the double mutants diminished in the young adult stage (Figure AI.4D). With available reagents to probe for active forms of PMK-1/p38, my data showed that although CEP-1 function is required for proper expression of PMK-1 targets at both L4 and YA stages, CEP-1 is not required for activation of PMK-1, as the active form of PMK-1 is not reduced in the *cep-1*(gk138);*isp-1*(qm150) double mutant. Similarly, CEH-23 is not required to activate PMK-1 (Figure AI.5)

In conclusion, the transcriptional reprogramming regulated by CEH-23 and CEP-1 and MAPKs signaling appear curiously connected. MPK-1/ERK signaling is important for germline development (Church et al., 1995; Hsu et al., 2002; Lackner and Kim, 1998). Both CEH-23 and CEP-1 share many MPK-1 targets during the larval stage, which coincides with germline proliferation. Therefore, the regulation of CEH-23 and CEP-1 may be specific to their functions in the germline. The p38 MAPK plays an important role in stress response and regulates innate immunity, which is important for organismal survival under stressful conditions. Similar to CEP-1, my survival analyses indicated that PMK-1 was also required for the extended lifespan of the *isp-1*(qm150) mutant, as knocking down pmk-1 greatly reduced the lifespan of the long-lived *isp-1*(qm150) mutant (Figure AI.6). Further investigation is need to assess whether CEP-1 and PMK-1 modulate *isp-1*(qm150) lifespan through a similar mechanism. However, the fact that CEH-23 is not involved in PMK-1 signaling suggests that a shared mechanism that engages both CEH-23 and CEP-1 to promote lifespan extension is unlikely.

A



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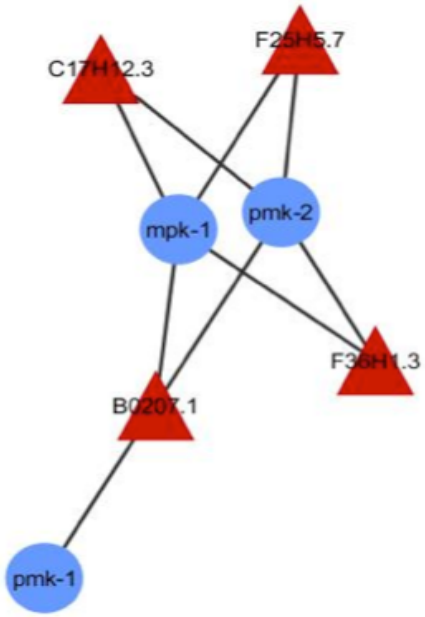


Figure AI.1 CEH-23 and CEP-1 regulate many common phosphatase metabolic genes and are connected to MAPK signaling pathways.
(A) The pie chart illustrates that 14% of the genes regulated by both CEH-23 and CEP-1 are phosphate metabolic genes, wherein kinases and phosphatases are equally distributed. (B) Interactome analysis links four CEH-23 and CEP-1 coregulated phosphatases to MAPKs.

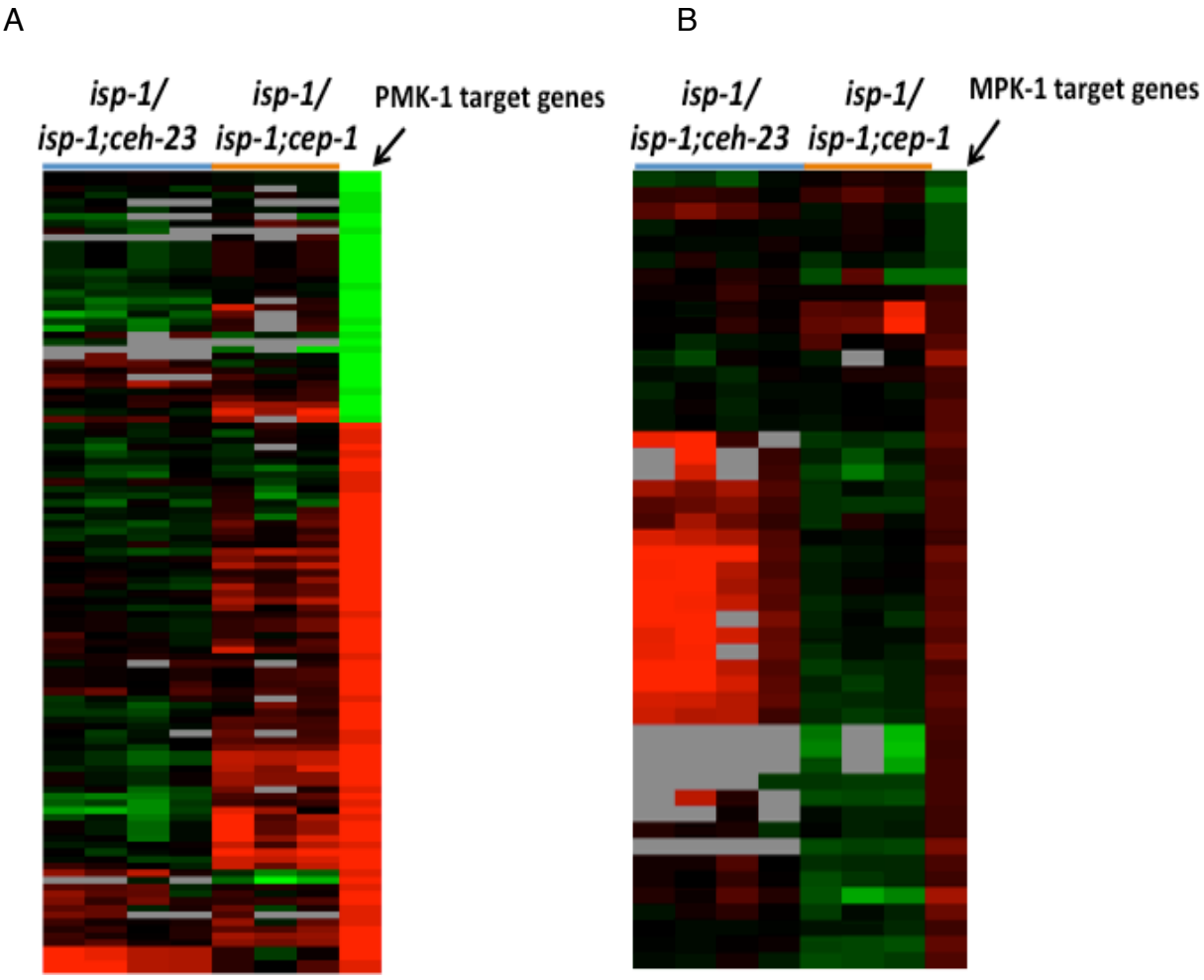


Figure AI.2 CEP-1 and CEH-23 share a common transcriptional output with MAPKs PMK-1 and MPK-1, respectively.

(A) The heat map illustrates the PMK-1 transcriptional targets identified in the daf-2(e1368) mutant. Forty out of 116 PMK-1 targets are also regulated by CEP-1 (hypergeometric probability test: representation factor 2.6; p <6.690e-05). (B) The heat map illustrates MPK-1 transcription targets in the germline. Twenty out of 50 known MPK-1 targets are also regulated by CEH-23 (hypergeometric probability test: representation factor 2.4; p <0.002).

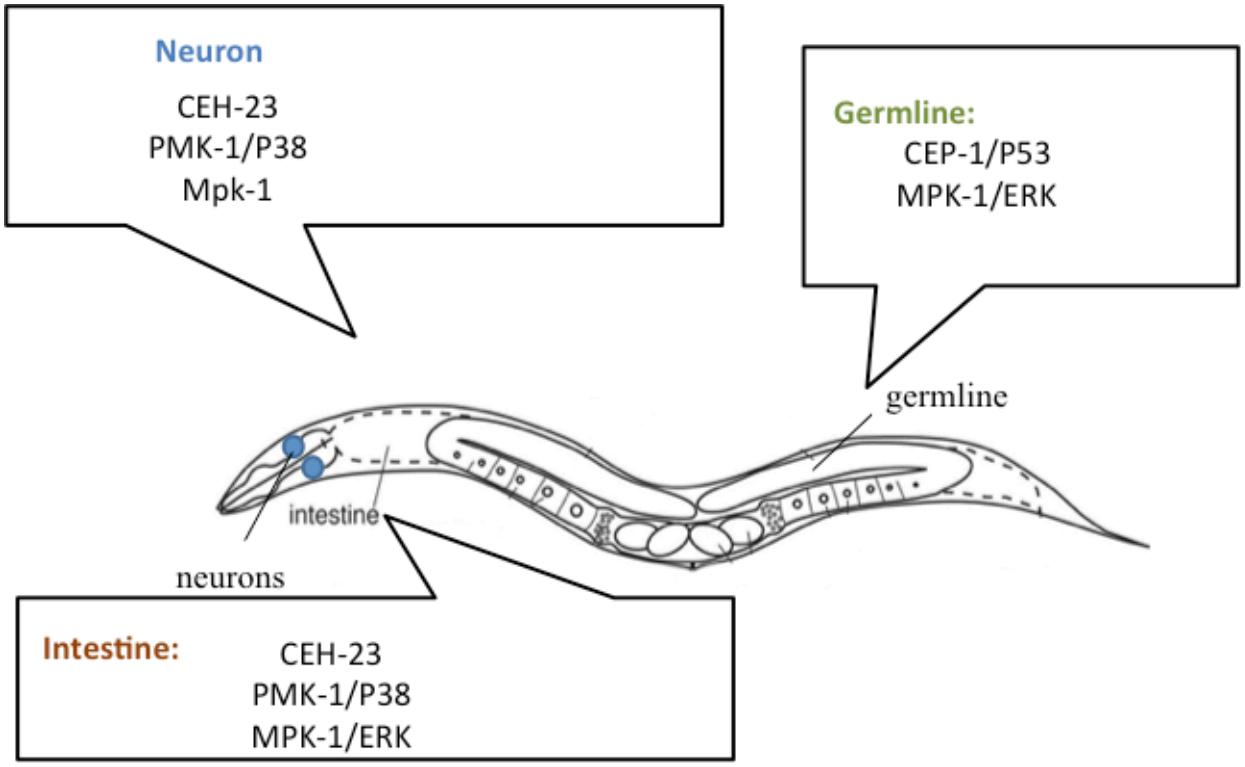
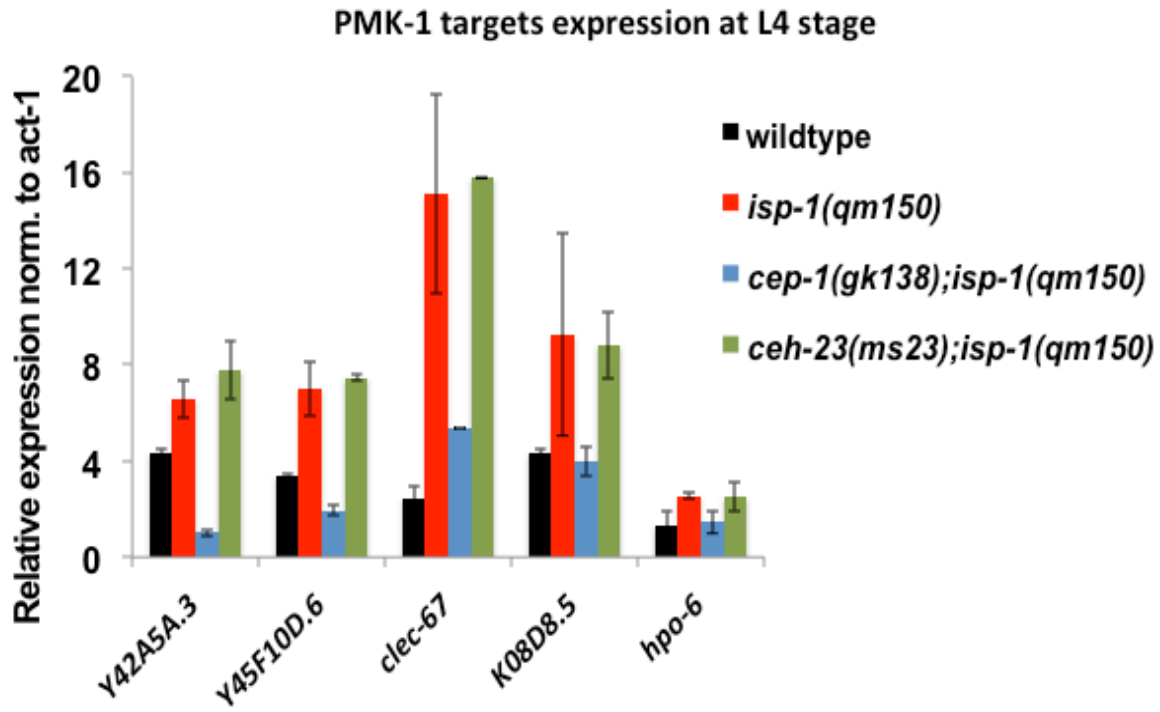
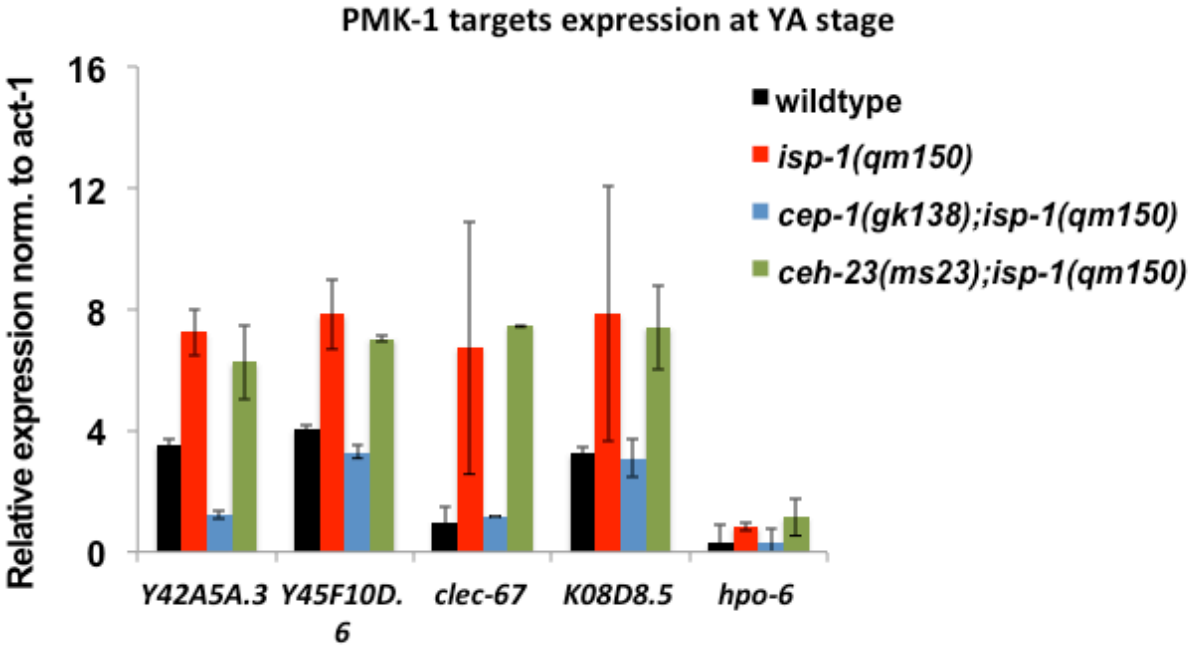


Figure AI.3 Expression patterns of CEH-23, CEP-1, PMK-1, and MPK-1.
CEH-23 is coexpressed with PMK-1 and MPK-1 in neurons and the intestine. CEP-1 is coexpressed with MPK-1 in the germline.

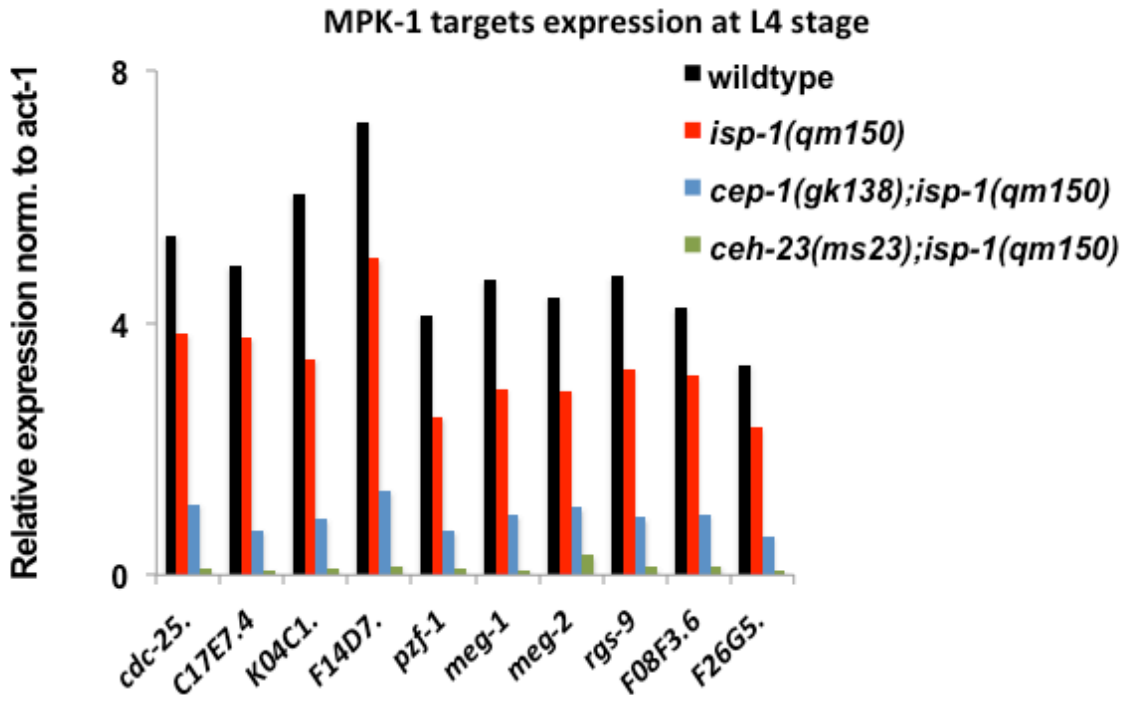
A


PMK-1 targets expression at L4 stage
Relative expression norm. to act-1
20
16
12
8
4
0
wildtype
isp-1(qm150)
cep-1(gk138);isp-1(qm150)
ceh-23(ms23);isp-1(qm150)
Y42A5A.3
Y45F10D.6
clec-67
K08D8.5
hpo-6

B


PMK-1 targets expression at YA stage
Relative expression norm. to act-1
16
12
8
4
0
wildtype
isp-1(qm150)
cep-1(gk138);isp-1(qm150)
ceh-23(ms23);isp-1(qm150)
Y42A5A.3
Y45F10D.6
clec-67
K08D8.5
hpo-6

C


MPK-1 targets expression at L4 stage
Relative expression norm. to act-1
8
4
0
wildtype
isp-1(qm150)
cep-1(gk138);isp-1(qm150)
ceh-23(ms23);isp-1(qm150)
cdc-25.
C17E7.4
K04C1.
F14D7.
pzf-1
meg-1
meg-2
rgs-9
F08F3.6
F26G5.

D

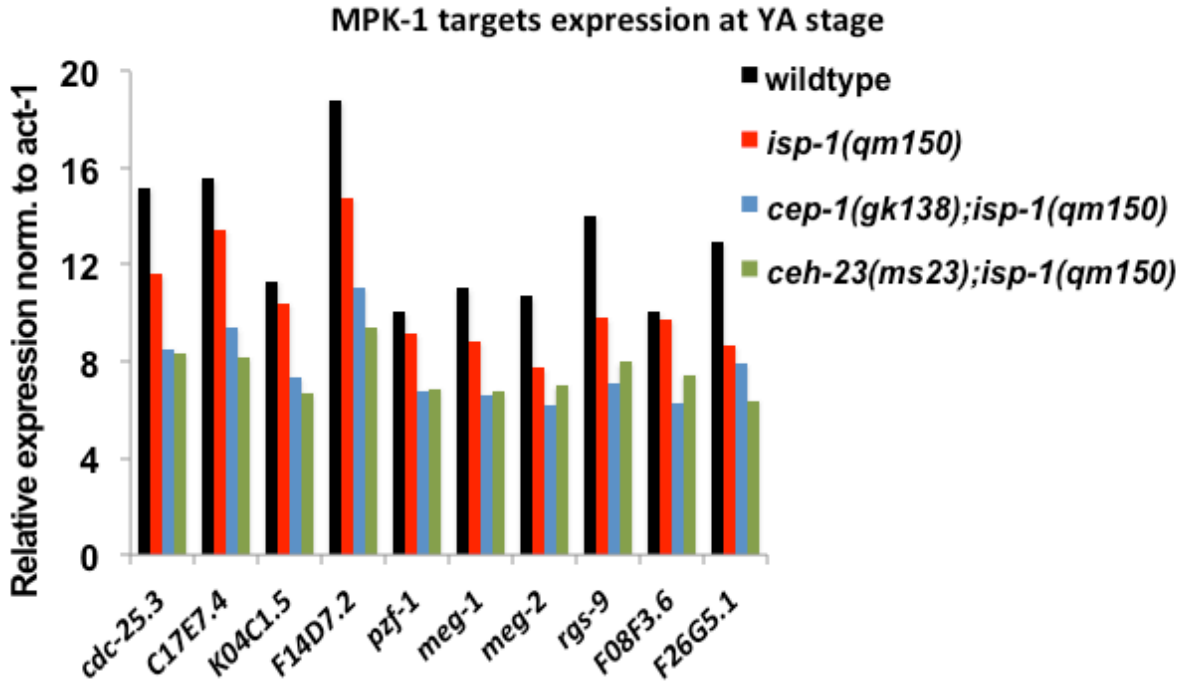
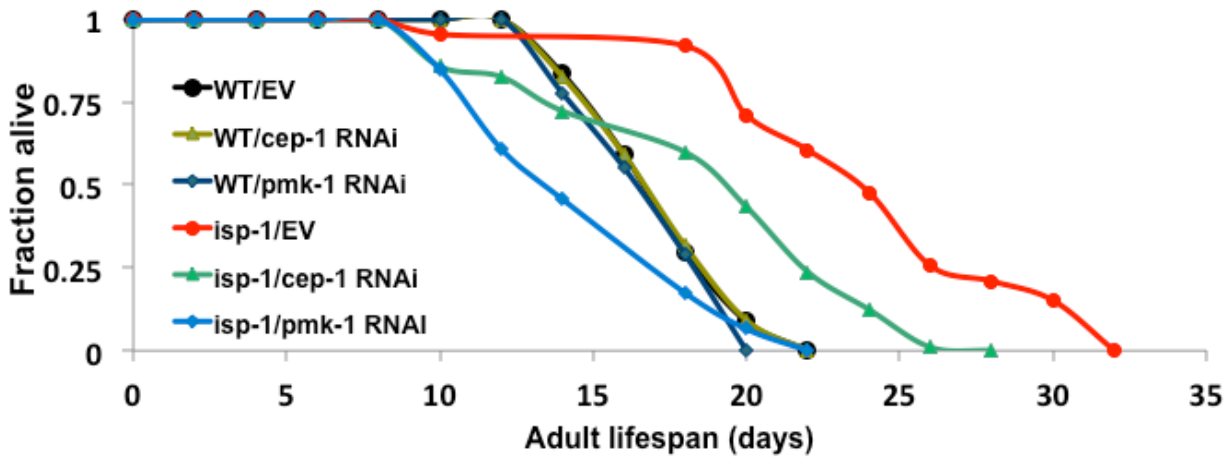

MPK-1 targets expression at YA stage
Relative expression norm. to act-1
20
16
12
8
4
0
wildtype
isp-1(qm150)
cep-1(gk138);isp-1(qm150)
ceh-23(ms23);isp-1(qm150)
cdc-25.3
C17E7.4
K04C1.5
F14D7.2
pzf-1
meg-1
meg-2
rgs-9
F08F3.6
F26G5.1

Figure AI.4 CEH-23 and CEP-1 regulate the transcriptional targets of MAPKs at the L4 and young adult stages.
The gene expression of PMK-1 transcriptional targets is regulated by CEP-1, but not CEH-23, at both L4 (A) and young adult (YA) (B) stages. MPK-1 transcriptional targets are affected by both CEP-1 and CEH-23 only during L4 (C), and the change is attenuated in YAs (D). The data for the PMK-1 target experiment is an average of two independent experiments. The MPK-1 target experiment was performed twice. Both experiments showed a similar trend, but the absolute value of the two experiments differed vastly, so one representative experiment is presented.

A



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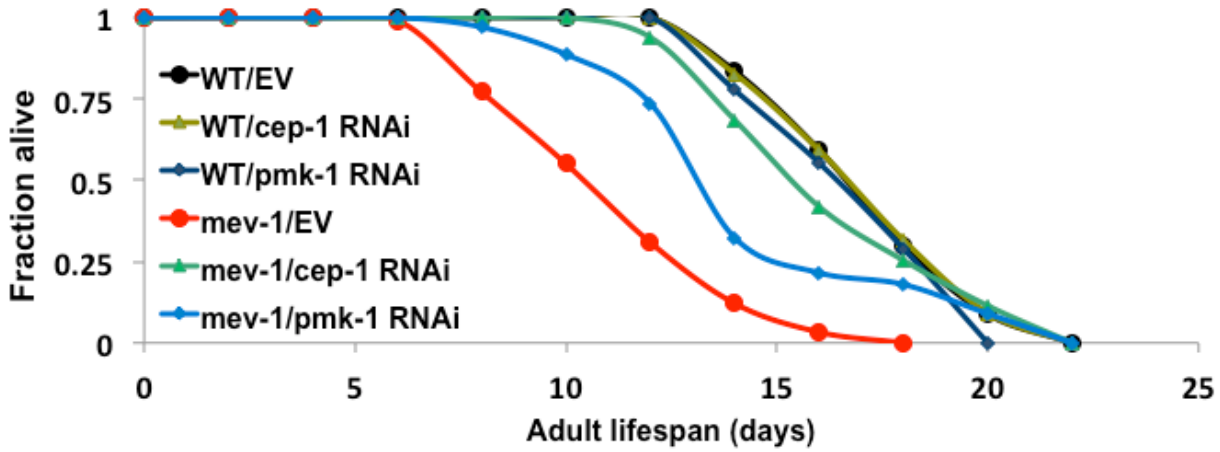


Figure AI.5 pmk-1 knock down phenocopies the *cep-1* mutation.
Similar to the *cep-1* mutation, RNAi-mediated knock down of pmk-1 also shortens the lifespan of the *isp-1*(qm150) mutant (A) and restore the lifespan of the *mev-1*(kn1) mutant (B).

APPENDIX II

OVEREXPRESSION OF CEH-23 PROMOTES LIFESPAN THROUGH A PATHWAY THAT IS LIKELY DISTINCT FROM THAT TRIGGERED IN RESPONSE TO MITOCHONDRIAL ETC DYSFUNCTION.

The role of CEH-23 in lifespan determination was discovered in an ETC mutant background, as functional CEH-23 was required for the full extended lifespan of the *isp-1*(qm150) mutant. Moreover, overexpression of *ceh-23* is sufficient to extend the lifespan of wildtype worms (Walter et al., 2011) (Figure AII.1). These findings suggest a model, wherein upon mitochondrial ETC dysfunction, CEH-23 levels are increased, which, in turn, activates the expression of downstream genes that promote long lifespan. Since the presence of CEH-23 is required for long life in ETC mutants, and CEH-23 overexpression leads to a longer lifespan, we hypothesized that a CEH-23 regulated mechanism that modulates lifespan is shared by both ETC mutants and *ceh-23*oe animals.

This hypothesis motivated us to perform microarray analyses to identify CEH-23 transcription targets that are important for longevity in both long-lived *isp-1*(qm150) mutants and *ceh-23* overexpressed transgenic worms (*ceh-23*oe). We performed three microarray experiments to compare global gene expression profiles between the following strains: (1) wildtype vs. *ceh-23(ms23)* revealed CEH-23-responsive transcriptional targets under basal conditions, (2) *isp-1(qm150)* vs. *ceh-23(ms23);isp-1(qm150)* identified CEH-23 targets in response to ETC dysfunction, and (3) *ceh-23*oe vs. an RFP transgenic control identified transcriptional changes that might contribute to the long lifespan of the *ceh-23*oe animals. Comparing the arrays described above, we

found that under conditions without stress, few genes were robustly altered across different biological replicates. In contrast, the changes in gene expression regulated by CEH-23 were more robust *isp-1*(qm150) mutants. To our surprise, *ceh-23*oe did not exhibit a similar transcriptional profile to *isp-1*(qm150) mutants (Figure AII.2A), except for a small subset of genes that appeared repressed by CEH-23 in both *isp-1*(qm150) and *ceh-23*oe animals. This group of genes harbors molting cycle genes, collagen genes, and cuticle genes but no genes with known functions in aging or longevity regulation. Using SAM (Significant Analysis of Microarray), I identified 105 genes with significantly altered expression in the *ceh-23*oe animals, most of which were repressed by CEH-23 (Figure AII.2B). GO term analyses reveal that some of the genes that are regulated by overexpressed *ceh-23* are genes that are important for molting process and collagen. Because GO analyses is not sufficient to understand the biological mechanisms that lead to extended lifespan upon *ceh-23* overexpression, further analysis of one of the CEH-23-repressed genes, set-15, may contribute to such an answer. set-15 encodes a SET domain-containing protein that is likely to be a methyltransferase. Interestingly, knocking down set-15 has been shown to promote lifespan (Hamilton et al., 2005). Therefore, I speculate that in the *ceh-23*oe animal, the expression of set-15 is suppressed by overexpressed *ceh-23*, which mimics the effect of set-15 RNAi treatment and extends lifespan. Although the expression of set-15 is altered when *ceh-23* is overexpressed, it is expressed at a level between *isp-1*(qm150) and *ceh-23*(ms23);*isp-1*(qm150), suggesting that set-15 may not be a common lifespan modulator under ETC stress or when *ceh-23* is overexpressed. However, this model is

pure speculation, as further characterization of set-15 and *ceh-23* is required to understand why overexpressing *ceh-23* extends lifespan. Understanding this concept will elucidate the role CEH-23 plays in regulating organismal longevity.

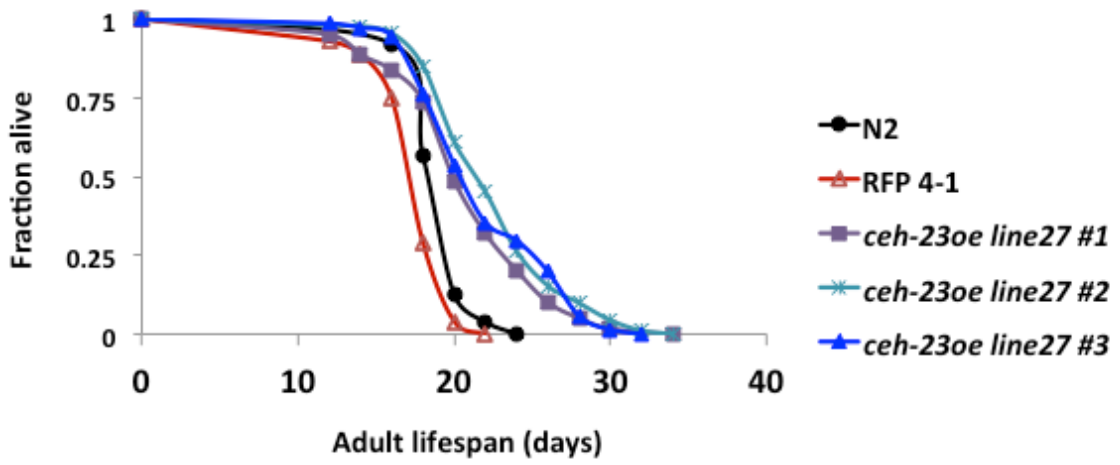


Figure AII.1 Overexpression of *ceh-23* is sufficient to promote lifespan.
The survival curves represent the lifespan of wildtype (N2), transgenic control (RFP 4-1), and three isolates of the integrated *ceh-23* overexpression transgenic lines. All three *ceh-23*oe lines live longer than transgenic control and wildtype animals.

A

B

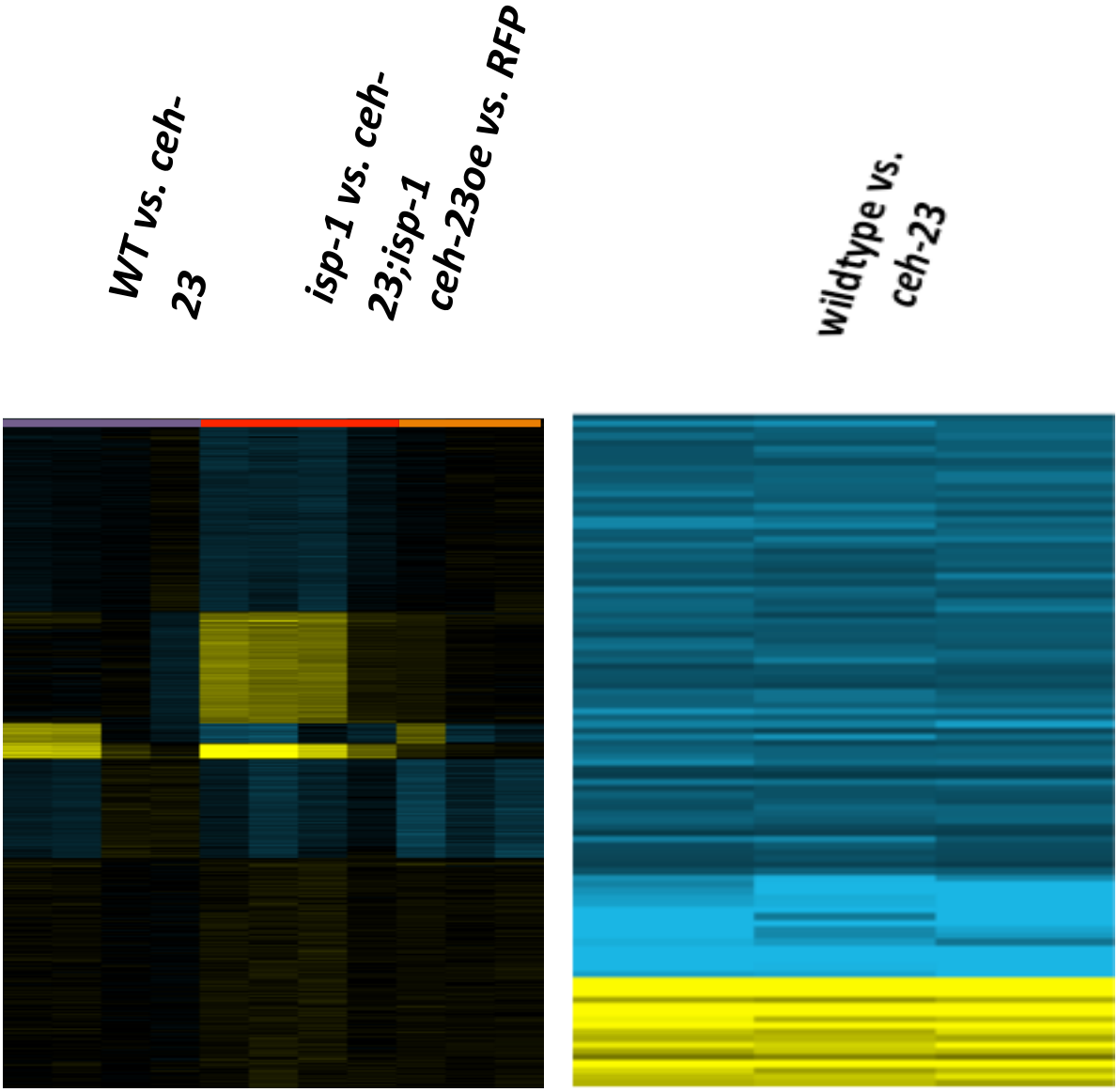


Figure AII.2 Overexpression of *ceh-23* stimulates a transcription network that regulates lifespan that is distinct from that of the ETC mutant.
(A) The heat map represents a genome-wide comparison of the transcriptional outputs of CEH-23 under basal conditions, ETC dysfunction and overexpressed CEH-23. (B) The heat map represents 105 genes with altered gene expression in *ceh-23*oe animals compared to the transgenic control. Yellow depicts genes that are up-regulated, and blue depicts genes that are down-regulated, in the *isp-1*(qm150) mutant compared to the double mutants. The intensity of the heat map represents a log2 ratio of the expression comparison.

APPENDIX III

A COMPLEX RELATIONSHIP BETWEEN CEH-23, CEP-1, HIF-1 AND ROS IN MEDIATING LIFESPAN EXTENSION AND TRANSCRIPTIONAL REPROGRAMMING IN RESPONSE TO ETC DYSFUNCTIONS

As discussed in Chapter 1, CEH-23 and CEP-1 are not the only transcription factors that affect lifespan of the ETC mutants. HIF-1 also has a role in mediating the extended lifespan of the ETC mutants. Hypoxia-inducible factors (HIFs) comprise a highly conserved transcription activator complex that allows cell to adapt to a low-oxygen environment (Jiang et al., 2001). *C. elegans hif*-1 encodes the ortholog of the mammalian HIF-1α, the oxygen-regulated subunit of the HIF-1 complex. The stability of HIF-1α is one of the major determinants of HIF-1 activity. *C. elegans* HIF-1 is a crucial modulator of organismal lifespan in response to different environmental cues, including low oxygen and temperature (Jiang et al., 2001; Leiser et al., 2011). HIF-1 was recently found to be required for the extended lifespan of several ETC mutants and is proposed to act by regulating ROS signaling (Lee et al., 2010).

To assess if HIF-1 collaborates with CEH-23 and CEP-1 to modulate the lifespan of mitochondrial ETC mutants, I performed epistasis studies for their effects on *isp-1* mutant lifespan. While lacking functional *hif-1, ceh-23* and *cep-1* only partially suppress the extended lifespan of *isp-1* mutant, it is important to note that the *hif-1(ia4)* mutation suppressed *isp-1* longevity to a greater extent than the *ceh-23(ms23)* and *cep-1(gk138)* mutations (Figure AIII.1A, B). Evaluation on pair-wise epistatic relationships between *hif-1* with *ceh-23* and *cep-1* for their effects on *isp-1* mutant longevity revealed *cep-1; isp-1;hif-1* and *ceh-23;isp-1;hif-1* triple mutants lived as long as the *isp-1;hif-1* double

mutant, which had a slightly shorter lifespan than the *cep-1;isp-1* and *ceh-23;isp-1* double mutants (Figure AIII.1 A, B). Together, these data suggests that *cep-1*, *ceh-23*, and *hif-1* act in a similar genetic pathway to modulate *isp-1* mutant longevity. In addition, *hif-1* appeared to have the most robust suppression of *isp-1* mutant longevity, and that loss of either *cep-1* or *ceh-23* was epistatic to *hif-1* inactivation in the *isp-1* mutant. Further supporting this idea, I constructed the *cep-1;ceh-23;isp-1;hif-1* quadruple mutant and found that it had a lifespan similar to the *isp-1;hif-1* double mutant (Figure AIII.1C).

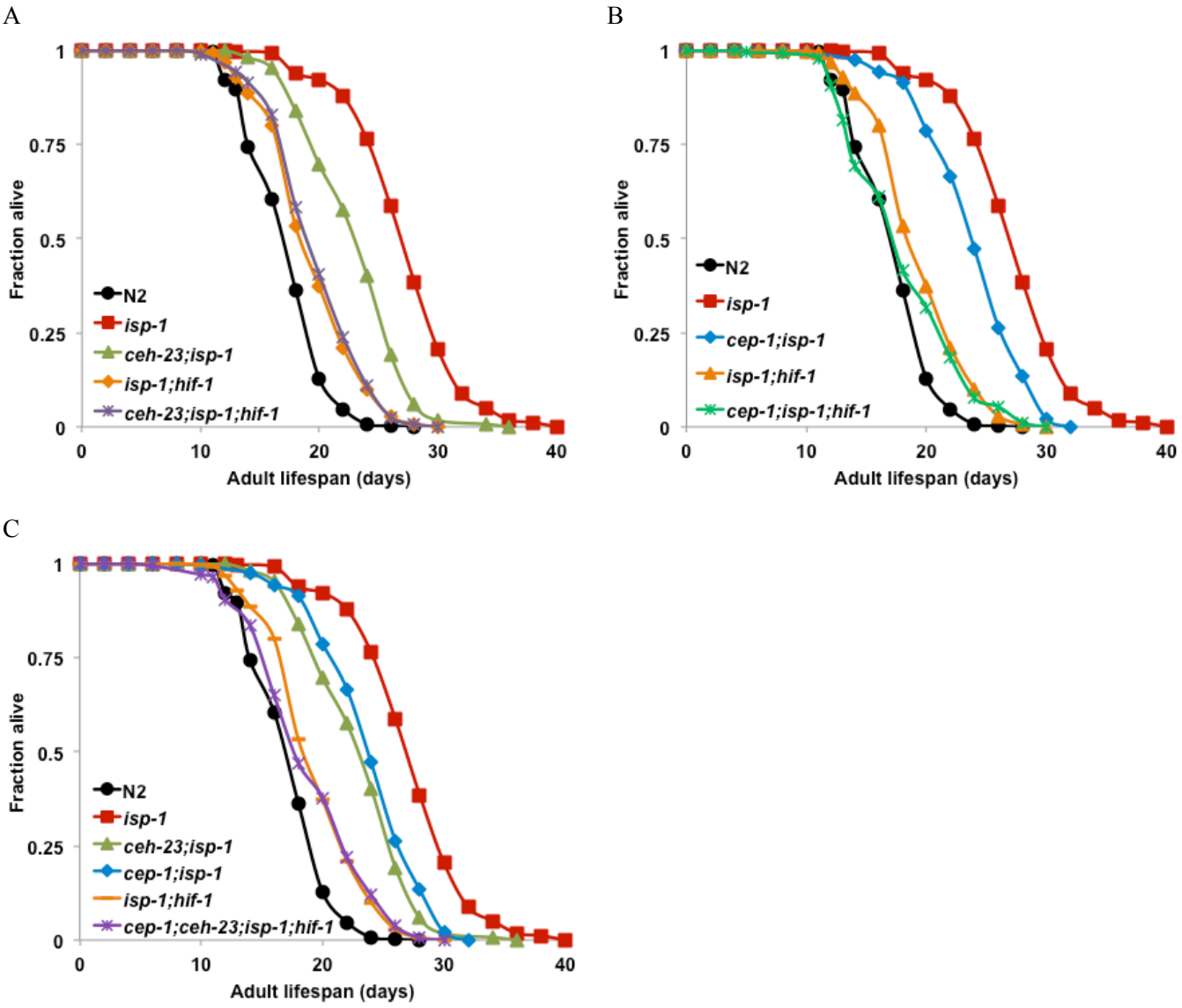


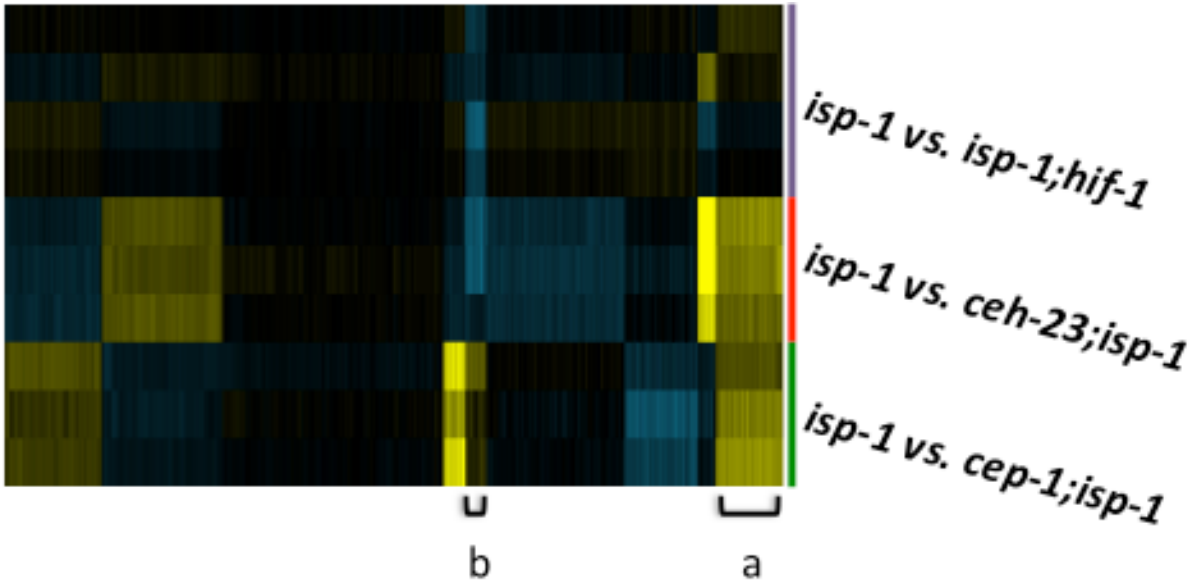
Figure AIII.1 *ceh-23*, *cep-1*, and *hif-1* act in the same genetic pathway to promote longevity in the *isp-1(qm150)* mutant.

The lifespans of several ETC mutants were assessed to examine the epistasis relationship between *ceh-23, cep-1,* and *hif-1* in the *isp-1(qm150)* mutant. *ceh-23, cep-1, or hif-1* mutation each partially suppressed the extended lifespan of the *isp-1(qm150)* mutant, and *hif-1* mutation displayed the strongest suppression effect. Inactivation of *ceh-23* and *hif-1* (A), or *cep-1* and *hif-1* (B) do not additively suppress *isp-1* mutant lifespan, as the triple mutant lives as long as the respective double mutants. Similarly, the *cep-1;ceh-23;isp-1;hif-1* quadruple mutant lives as long as the *isp-1;hif-1* double mutant (C). Survival curves represent data pooled from two biological replicates.

Given their roles as transcription factors, we hypothesized that they would exhibit common transcriptional outputs that may be important for lifespan determination. To identify the common transcriptional targets, I compared the global gene expression profile changes after removal of either *ceh-23*, *cep-1*, or *hif-1* in the *isp-1(qm150)* mutant. Surprisingly, I did not identify any genes commonly regulated by CEH-23, CEP-1, and HIF-1 (Figure AIII.2A). The lack of common targets further complicates the possibility that these TFs may collaborate to promote the long lifespan of *isp-1(qm150)*. While not able to identify genes commonly regulated by the three TFs, I did identify genes that were regulated by two of these TFs. As described in Chapter 3, CEH-23 and CEP-1 commonly regulate many kinases and phosphatases. CEH-23 and HIF-1 both repress signaling peptides and neuropeptides, thus these transcription factors might function in neurons (Figure AIII.2B). Interestingly, the transcriptional outputs of HIF-1 and CEP-1 do not display much overlap, which evokes a very crucial question: how do HIF-1 and CEP-1 collaborate to modulate the lifespan of *isp-1(qm150)* if not through their transcription outputs? One possibility is that CEP-1 and HIF-1 both act through CEH-23 to regulate lifespan. I speculate that aberrant mitochondrial ETC function triggers a transcriptional reprograming, in which CEH-23 and CEP-1 work in concert to activate the expression of phosphate metabolism genes while CEH-23 collaborates with HIF-1 to repress a small subset of neuropeptide genes. Proper regulation of these groups of genes is important for optimizing longevity of the *isp-1* mutant. However, one caveat remains with this model. CEH-23 and CEP-1 do not have overlapping expression patterns; therefore, it is less likely that CEH-23 and CEP-1 physically interact to regulate

their downstream targets. Alternatively, given that CEH-23 might be expressed in the germline, it may physically interact with CEP-1 in that tissue to regulate common targets. The expression pattern of CEH-23 was determined by a *ceh-23::gfp* reporter that is introduced as a multi-copy transgene that forms extrachromosomal arrays, which are often silenced in the germline (Kelly et al., 1997; Reese et al., 2000). As a result, the silenced transgenic reporter may artificially mask the germline expression of CEH-23. Many novel tools have been recently developed to allow single transgene expression in the germline (Dickinson et al., 2013; Frøkjaer-Jensen et al., 2008), which will allow us to more accurately assess the expression patterns of the three TFs and uncover whether these TFs interact with each other. Moreover, if the cell type-specific expression of these TFs is more definitively established, identifying whether these TFs interact with each other will provide crucial hints about their relationships in modulating longevity.

A


isp-1 vs. isp-1;hif-1
isp-1 vs. ceh-23;isp-1
isp-1 vs. cep-1;isp-1
b
a

B

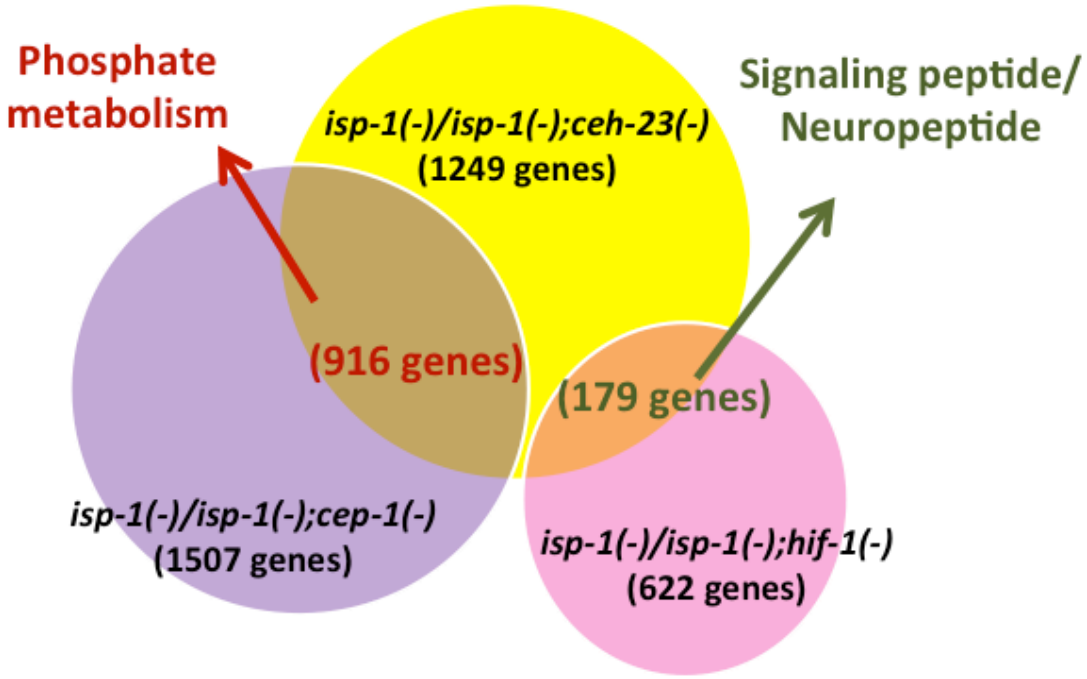

Phosphate metabolism
isp-1(-)/isp-1(-);ceh-23(-)
(1249 genes)
Signaling peptide/ Neuropeptide
(916 genes)
(179 genes)
isp-1(-)/isp-1(-);cep-1(-)
(1507 genes)
isp-1(-)/isp-1(-);hif-1(-)
(622 genes)

Figure AIII.2 CEH-23, CEP-1, and HIF-1 do not share transcriptional targets.
(A) The heat map represents a genome-wide comparison of the transcription outputs of CEH-2, CEP-1, and HIF-1. Yellow depicts genes that are up-regulated, and blue depicts genes that are down-regulated, in the *isp-1*(qm150) mutant compared to the double mutants. The intensity of the heat map represents a log2 ratio of the expression comparison. Brackets specify genes that are commonly regulated by CEH-23 and CEP-1(a) and CEH-23 and HIF-1 (b). (B) The Venn diagram illustrates an overlap between CEH-23-regulated, CEP-1-regulated, and HIF-1-regulated genes. SAM analyses identified 916 genes that are commonly regulated by CEH-23 and CEP-1 (FDR=0%, 1.5 fold gene cutoff), which are highly enriched for phosphate metabolism. SAM analyses identified 179 genes (FDR=0.21%, 1.5 fold gene cutoff) commonly regulated by CEH-23 and HIF-1, which are enriched for neuropeptides.

APPENDIX IV

ACTIVATED MITOCHONDRIAL UPR AND DO NOT REQUIRE CEH-23.

Mitochondrial function is essential for survival; therefore, it is important to maintain mitochondrial quality. The mitochondrial unfolded protein response (mitoUPR) is a mechanism that protects mitochondria from aberrant unfolded protein stress. When the mitochondrial ETC is impaired, the mitoUPR is activated and it has been shown knocking down *ubl-5* and *atfs-1*, which are two of the key component for mitoUPR activation (Durieux et al., 2011; Haynes et al., 2010). *ubl-5* encodes a transcription factor that regulates the expression of downstream mitoUPR effectors, including many mitochondrial protein chaperones (Benedetti et al., 2006). ATFS-1 serves as a sensor to monitor mitochondrial quality. Upon mitochondrial dysfunction, ATFS-1 translocates from the mitochondria to the nucleus and activates the expression of several mitochondrial protein chaperones (Nargund et al., 2012b).

Interestingly, both mitoUPR activation and the prolongevity effect of ETC dysfunction have been shown to have a cell non-autonomous nature (Durieux et al., 2011). *ceh-23* is expressed in neurons and the intestine, which are both key cell types for mitoUPR activation and longevity regulation in response to ETC dysfunction (Walter et al., 2011). Since the mitoUPR is activated in the same tissues as CEH-23 expression, we hypothesized that CEH-23 may participate in regulating mitoUPR activation in the presence of mitochondrial stress. My results demonstrated that functional CEH-23 is dispensable for the activation of the mitoUPR (Figure AIV.1). Knocking down the mitoUPR components ubl-5 and atfs-1 greatly reduced *isp-1(qm150)* mutant lifespan,

suggesting that a proper mitoUPR response is important for survival. Although, in both cases, *ceh-23* mutation did not further suppresses the lifespan of *isp-1(qm150)* mutant with attenutated mitoUPR components. With these results, it is difficult to differentiate between a scenario where CEH-23 is epistatic to the mitoUPR or whether *isp-1(qm150)* mutant animals become extremely sick without a proper mitoUPR, so the absence of *ceh-23* is insignificant (Figure AIV.2). It is important to note that knock down of atfs-1 in the *isp-1(qm150)* mutant yields more censored animals, even more than *ceh-23(ms23);isp-1(qm150)* mutants. The combination of *ceh-23* mutation and *atfs-1* RNAi reduces animal healthspan, suggesting that *ceh-23* and atfs-1 might engage in regulating similar biological process but not necessary aging or the mitoUPR. Together, my data demonstrated that CEH-23 is unlikely to modulate lifespan through mitoUPR.

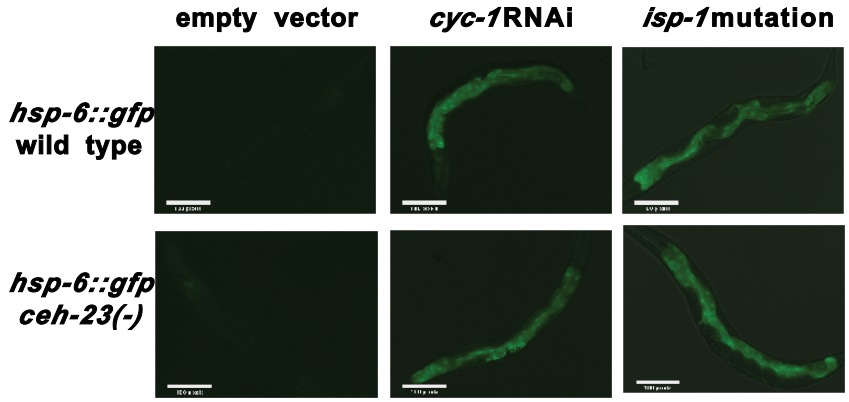
As described in Chapter 1, accumulating evidence supports a model in which mitochondria regulates longevity through ROS signaling. We have investigated the roles of CEH-23 and CEP-1 in ROS signaling in Chapter 3. We found that both CEH-23 and CEP-1 are likely to act upstream of ROS production, whereas hif-1 acts downstream of ROS. *sod-2* encodes one of the two mitochondrial superoxide dismutases (SODs) in *C. elegans*, wherein it acts to break down superoxide in the mitochondria. The *C. elegans* genome contains five SODs, and only one has a longer lifespan when inactivated (*sod-2*) (Van Raamsdonk and Hekimi, 2009). The extended lifespan of *sod-2*(ok1030) mutants can be suppressed by the antioxidant NAC. As the pro-longevity dose of paraquat had smaller effects on *sod-2*(ok1030) mutant lifespan than wildtype, the longer lifespan of *sod-2* mutants may depend on mitochondrial superoxide. I used *sod-2*(ok1030) mutant

animals to further test the roles of CHE-23, CEP-1, and HIF-1 in promoting longevity in response to ROS signaling. The *sod-2*(ok1030) mutant is an alternative ROS model that allows me to assess whether any of these three TFs are required for its extended lifespan. Interestingly, my results suggest that the long lifespan of the *sod-2* mutant does not require CEH-23, CEP-1, and HIF-1 (Figure AIV.3). Interestingly, CEH-23 and CEP-1 are dispensable for promoting the long life of *sod-2* mutants and for promoting lifespan extension in response to low doses of paraquat. Both *sod-2* inactivation and paraquat treatment lead to a mild elevation of ROS, which extended lifespan. The fact that CEH-23 and CEP-1 are not required for the extended lifespan in both conditions furthers the possibility that these two TFs have a role in ROS production. This hypothesis is consistent with the observation that *cep-1* mutation further extends the lifespan of the *sod-2* mutant, which suggests that lacking *cep-1* yields optimal mitochondrial ROS levelsfor promoting longevity. Although the *sod-2* mutant is long-lived, the lifespan of *sod-2* can be further extended by low dose paraquat treatment, suggesting that the pro-longevity ROS levels have not been optimized in *sod-2* mutant animals.

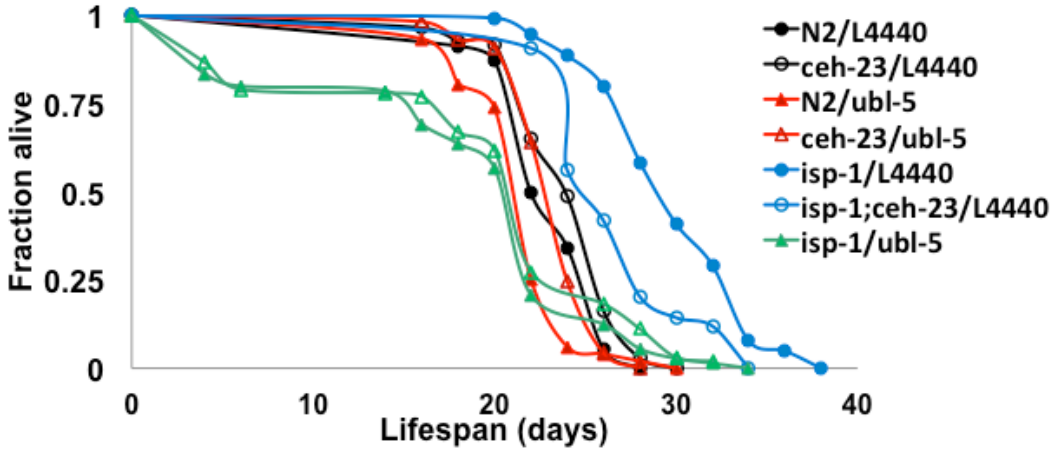
The observation that HIF-1 is dispensable for *sod-2* mutant longevity is rather surprising, as paraquat-mediated lifespan extension is partially dependent on HIF-1 (as discussed in Chapter 1). HIF-1 has been implicated in ROS-mediated lifespan determination of ETC mutants (Lee et al., 2010), and we expect that HIF-1 would have a similar effect on the *sod-2* mutant. This observation that HIF-1 is dispensable for *sod-2* mutant longevity hints at the possibility that although *sod-2* mutants have elevated

mitochondrial superoxide, it might trigger a distinct mechanism to promote lifespan that does not engage HIF-1. It is also possible that HIF-1 is only activated when the mitochondrial superoxide levels reach a certain threshold, which is not attained in the *sod-2* mutant (possibly due to the presence of another mitochondrial SOD, sod-3). How the *sod-2* mutation promotes longevity remains an interesting question, position *sod-2* as a model for studying of the effects of low-level pro-longevity ROS signaling.

A


empty vector
cyc-1RNAi
isp-1mutation
hsp-6::gfp
wild type
hsp-6::gfp
ceh-23(-)

B


Fraction alive
1
0.75
0.5
0.25
0
0
10
20
30
40
Lifespan (days)
N2/L4440
ceh-23/L4440
N2/ubl-5
ceh-23/ubl-5
isp-1/L4440
isp-1;ceh-23/L4440
isp-1/ubl-5

C

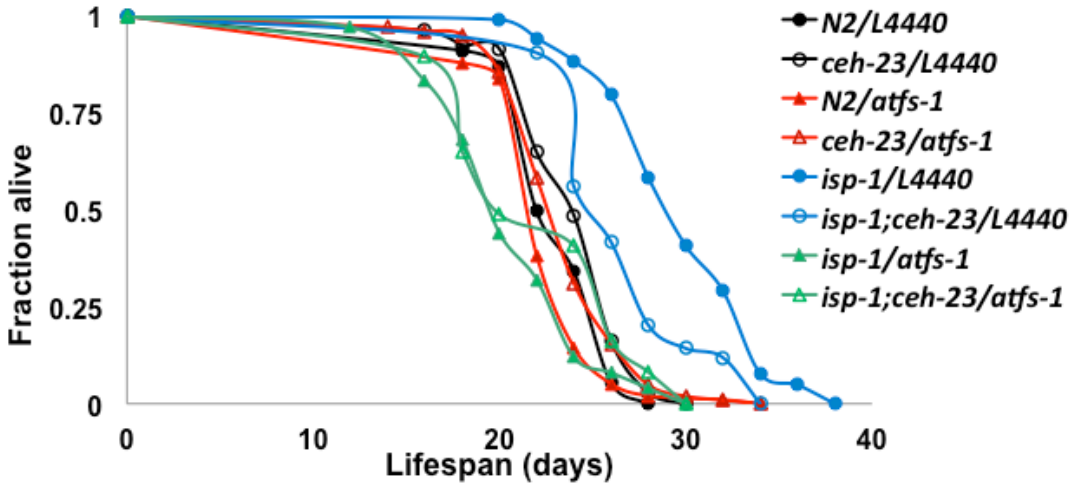
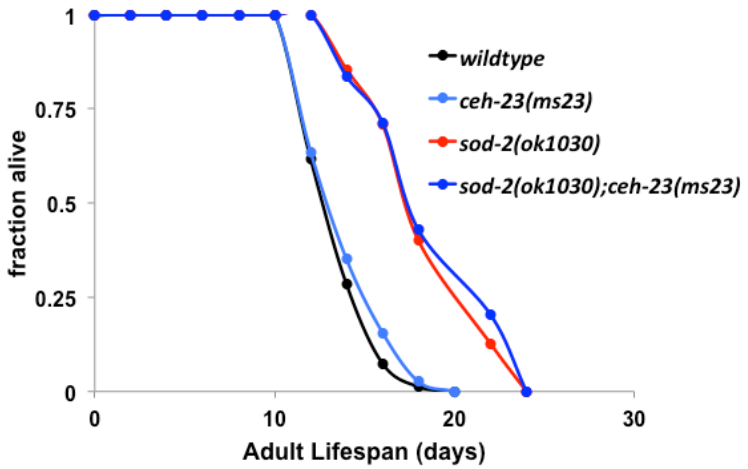
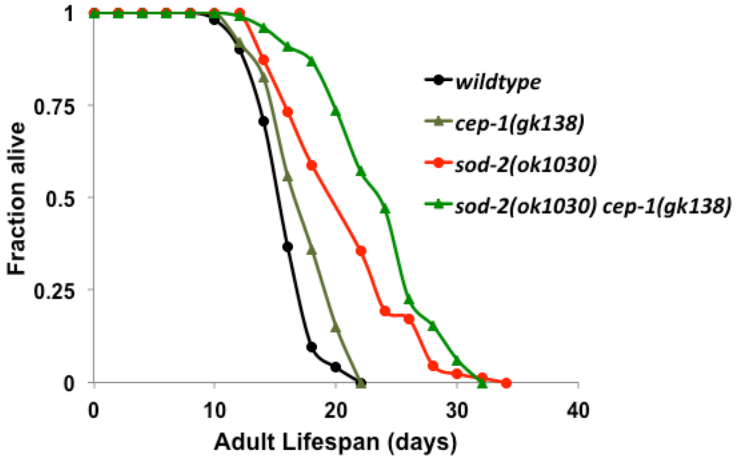

Fraction alive
1
0.75
0.5
0.25
0
0
10
20
30
40
Lifespan (days)
N2/L4440
ceh-23/L4440
N2/atfs-1
ceh-23/atfs-1
isp-1/L4440
isp-1;ceh-23/L4440
isp-1/atfs-1
isp-1;ceh-23/atfs-1

Figure AIV.1 CEH-23 is dispensable for activation of the mitoUPR.
(A) The mitoUPR can be activated by cyc-1 RNAi and *isp-1* mutation, and the degree of mitoUPR activation is comparable between wildtype and *ceh-23* mutant animals. (B) *ceh-23* and ubl-5 exhibit an epistatic relationship. *ceh-23* mutation does not further suppress *isp-1*mutant lifespan when ubl-5 is knocked down by RNAi. (C) Similarly, *ceh-23* mutation does not further suppress *isp-1* mutant lifespan when atfs-1 is knocked down.

A


1
0.75
0.5
0.25
0
fraction alive
0
10
20
30
Adult Lifespan (days)
wildtype
ceh-23(ms23)
sod-2(ok1030)
sod-2(ok1030);ceh-23(ms23)

B


1
0.75
0.5
0.25
0
Fraction alive
0
10
20
30
40
Adult Lifespan (days)
wildtype
cep-1(gk138)
sod-2(ok1030)
sod-2(ok1030) cep-1(gk138)

C

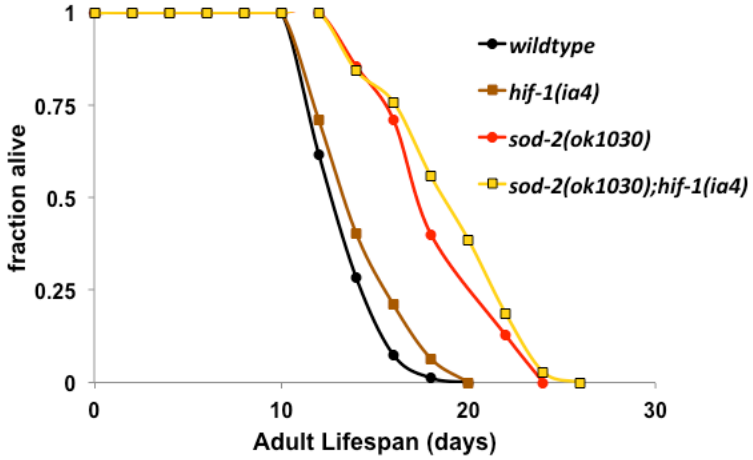

1
0.75
0.5
0.25
0
fraction alive
0
10
20
30
Adult Lifespan (days)
wildtype
hif-1(ia4)
sod-2(ok1030)
sod-2(ok1030);hif-1(ia4)

Figure AIV.2 CEH-23, CEP-1, and HIF-1 are not required for *sod-2* mutant longevity.
sod-2(ok1030) exhibits a longer lifespan compared to wildtype animals, and deficiency for *ceh-23* (A), *cep-1*(B), or *hif-1*(C) does not suppress the extended lifespan

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