DELINEATION OF LONGEVITY-REGULATING FACTORS IN *C. elegans* MITOCHONDRIAL MUTANTS

A Thesis

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ABSTRACT

The processes that govern biological aging have been so obfuscated by complexity that investigators seldom concur with one another's philosophical explanation of it. However, within the past few decades researchers have made great strides towards understanding the molecular mechanisms that govern the global organismal deterioration that plagues us all, and through this we may hope to achieve an adequate explanation of biological aging.

Mitochondria play a vital role in key physiological processes, including energy production, fat metabolism, apoptosis, and signaling (1). Their activity is paramount to maintaining cellular homeostasis, yet their activity declines with age (2). The role of mitochondria in aging has been interrogated since the 1950's, when it was suggested that the decline of mitochondrial function through reactive oxygen species (ROS) damage is what primarily leads to global organismal deterioration (3). Since then, several well-investigated longevity-modulating pathways have illustrated the importance of mitochondrial function in aging across species (4). One such modulator of longevity is the mild dysfunction of mitochondrial respiration, which can be caused by mutations in electron transport chain (ETC) components (5).

This dysfunction of the ETC can confer an extended lifespan to the organism, as in the case of an *isp-1* mutation, or it can confer a reduced lifespan, as in the case of a *mev-1* or *gas-1* mutation (6,7,8). In all three cases, the transcription factors CEP-1 and CEH-23 are each partially required for longevity modulation (9). The dichotomous mode of longevity regulation by CEP-1 and CEH-23 in response to similar mutations elicits questioning into the mechanism(s) by which ETC dysfunction can be conveyed to CEP-1 and CEH-23. I have aimed at addressing this line of

questioning by method of lifespan assessment of populations, and fluorescent imaging of a recently discovered longevity modulator.

BIOGRAPHICAL SKETCH

Steven Michael Pisano grew up in Clinton, New Jersey, where his interests in physics and music germinated. Towards the end of high school, his interest in physics morphed into a general curiosity about molecular dynamics, so much so that he felt confident in choosing biochemistry as his major. He applied only to Virginia Tech for early decision, and was accepted, silencing concerns that he should have a back up plan. There he raised his fervency for biochemistry and continued to write music as a primary hobby.

After being exposed to organic chemistry, he decided that it was time to join a lab and see what is was like to apply knowledge, rather than merely obtain it. He joined Dr. Richard Gandour's lab and began working on a nanoparticle drug delivery system. The lab environment was a comfortable one, and the thought of graduate school started to guide his thoughts about his future. After a year in this lab, Steve decided to start working in Dr. Glenda Gillaspy's plant biology lab, where he worked on alternative splicing of an inositol phosphatase gene that is imperative to energy regulation. This project was even more stimulating than the first, and Steve decided that graduate school was certainly in his future.

He graduated Virginia Tech with a B.S. in Biochemistry magna cum laude, and with this accomplishment proceeded swiftly to his next endeavor; a transition that was met with as little anxiety as the last.

ACKNOWLEDGMENTS

First and foremost I would like to acknowledge my brilliant mentor, Dr. Sylvia Lee. Her instruction was unparalleled and will certainly never be forgotten. The emphasis she placed on communication and specific use of language is something that I desperately needed, and the consistent scientific analysis she exhibited was absolutely inspiring. Through my most difficult times she never ceased to offer her time and advice and for that I am extremely thankful.

Chun Han, the other member of my special committee, was gracious enough to work with me on my NSF proposal during my first semester at Cornell. He helped me continuously along the way by recommending relevant material and offering scientific insight and critique, and now by helping me through my graduation, generously and thoughtfully.

Dr. Ella Chang developed the project of mitochondrial dysfunction-derived longevity into a wonderfully investigated form. I worked with her during my rotation and through many months after. She always took the time to introduce me to new techniques, answer my questions, or simply discuss life itself. Her contribution to my development is irreplaceable.

My other lab mates were some of the most supportive people I have had the pleasure of coming in contact with. Veerle Rottiers, Mintie Pu, Wenke Wang, Seth Sagulo, and the newest member, Amaresh Chaturbedi, were always willing to give their scientific input, and could not have been friendlier. Our lab technician and manager Rada Omanovic was a wonderful person to be able to talk to and her commitment to the lab was astounding.

My parents and my sister play an important role of course. They have always been supportive through good times and bad, and I would certainly not be here without them.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ROS - reactive oxygen species

ETC - electron transport chain

cep-1 - *C. elegans* p53-like protein

ceh-23 - *C. elegans* homeobox

isp-1 - iron-sulfur protein

mev-1 - abnormal MEthyl Viologen sensitivity

gas-1 - General Anaesthetic Sensitivity abnormal

AMPK - AMP-activated protein kinase

aak-2 - AMP-Activated Kinase

CRTC-1 - CREB-Regulated Transcriptional Coactivator homolog

NES - nuclear export signal

CRH-1 - CREB Homolog

RFP - red fluorescent protein

GFP - green fluorescent protein

ptl-1 - Protein with Tau-Like repeats

INTRODUCTION

It has been shown in diverse organisms that the mild dysfunction of the mitochondrial electron transport chain is a modulator of longevity, however the exact mechanisms are not well understood. Such dysfunction includes a point mutation in the Rieske iron-sulfur protein-encoding gene of Complex III, *isp-1*, which confers a robust extension of lifespan in *C. elegans* (6). Complex III is involved with the transfer of electrons from ubiquinol to cytochrome c through use of an iron-sulfur prosthetic group within ISP-1, and said point mutation that confers an extended lifespan codes a serine in place of a proline that is conserved from organisms ranging from yeast to humans (6). The alteration of such a conserved residue could potentially affect the structural integrity of complex III and/or the ability of the iron-sulfur prosthetic group to aid in the transfer of electrons from ubiquinol to cytochrome c. A hindrance of this nature could impede the efficiency of electron transfer of the ETC, and therefore alter the metabolic status of the organism via increased ROS generation and/or reduced energy production. Indeed, *isp-1* mutants have elevated amounts of mitochondrial superoxide, and consume less oxygen (10).

Similarly, mutations of ETC components *mev-1* and *gas-1* cause mitochondrial dysfunction, as illustrated by the increased ROS damage of proteins in these strains, however they confer a reduction of lifespan rather than an extension (6,7). *mev-1* encodes succinate dehydrogenase subunit c of complex II, and *gas-1* encodes a conserved iron protein subunit of complex I. The abrogation of either of these two components does not immediately present a logical explanation for their disparate longevity phenotypes relative to the abrogation of *isp-1*, as all three mutations affect single components required for electron transfer. Although it is evident

that the organismal responses to these individual perturbations are distinct, a clear mechanism has not been described.

The lifespan phenotypes of all three mutants are each partially dependent on two transcription factors; the p53-like protein CEP-1, and the Homeobox protein CEH-23. CEP-1 is the sole *C. elegans* ortholog of the mammalian p53 family, and it regulates DNA repair and cell cycle. CEP-1 has been observed to be a longevity modulator in response to multiple forms of mitochondrial dysfunction, including the dysfunction caused by mutations of ETC components (11). Our lab has similarly shown that CEH-23 is required for the lifespan extension of the *isp-1* mutant (12), and more recently that it is required for the lifespan reduction of the *mev-1* and *gas-1* mutants (9), however other functions of this protein have not been well characterized. The requirement of these two transcription factors on the lifespan phenotypes of the *isp-1*, *mev-1*, and *gas-1* mutants is evident based on the diminished lifespan phenotypes of these mutants following a loss of *cep-1* or *ceh-23*. Additionally, CEP-1 and CEH-23 appear to be acting in the same linear pathway, as inferred by the fact that the cumulative loss of *cep-1* and *ceh-23* does not additively suppress the lifespan phenotypes of the mitochondrial mutants (9). Although loss of *ceh-23* in an otherwise wildtype background does not affect lifespan, loss of *cep-1* slightly increases lifespan, thus its role in lifespan regulation is unclear. In addition, the precise method by which CEP-1 and CEH-23 are activated by the signal of mitochondrial dysfunction, and the precise method by which these two transcription factors permute the signal to a longevity response, is not known. Our lab has shown however, that the signal of mitochondrial dysfunction is mediated to CEP-1 and CEH-23 by the highly conserved energy-sensing kinase, AMP-activated protein kinase (AMPK) (9).

AMPK is a major regulator of energy homeostasis, and has been implicated in multiple lifespan-modulating pathways across species (4). In *C. elegans*, AMPK has been shown to be required for lifespan extension in response to ROS generation by paraquat (13), a response that is mechanistically intriguing due to the elevated mitochondrial ROS of the mitochondrial mutants. In addition, AMPK confers an extended lifespan when constitutively active in an otherwise wildtype background (14). Our lab has previously shown that the *C. elegans* ortholog of the catalytic domain of AMPK, AAK-2, is in its active state in the *isp-1* mutant and that this activation is required for lifespan extension. AAK-2 is activated in the *isp-1* mutant regardless of CEP-1 or CEH-23 presence, indicating that this activation is upstream to the activity of CEP-1 and CEH-23. The mechanism by which mitochondrial dysfunction is conveyed to AAK-2 is unknown, and it is not yet determined if ROS, low energy, or a parallel factor is responsible for this activation.

CHAPTER 1.1

The transcription factors CEP-1 and CEH-23 regulate the lifespan phenotype of the short-lived *mev-1* and *gas-1* mutants, in addition to the long-lived *isp-1* mutant. The dichotomous mode of longevity regulation by CEP-1 and CEH-23 must either be indicative of distinct upstream conditions or the variable presence of an uncharacterized factor that interacts with CEP-1 and CEH-23 or their transcriptional output. Due to the *isp-1* mutant's dependence on AAK-2 activation for lifespan extension through CEP-1 and CEH-23, I aimed to determine the dependence, if any, of *mev-1* and *gas-1* lifespan reduction on AAK-2 activation. To do this, I conducted a lifespan assay of the short-lived mutants either on *aak-2* or control RNAi and found that the reduced lifespan of *mev-1* and *gas-1* is not rescued with *aak-2* knockdown (Figure 1).

Figure 1 shows the proportion of living organisms to the sample size of a population for each genetic condition as a function of time. If the lifespan phenotypes of the *mev-1* and *gas-1* mutants were dependent on AAK-2 activity, the lifespan of the *mev-1* and *gas-1* mutants on *aak-2* RNAi would be closer to wildtype lifespan than the *mev-1* and *gas-1* mutants on control RNAi. This positive shift would indicate a rescue of lifespan in response to *aak*-2 knockdown, and therefore implicate AAK-2 as a regulator of the lifespan phenotypes of these mitochondrial mutants. Figure 2 illustrates the reciprocal of this effect in the *isp-1* mutant, in which AAK-2 is required for the extension of lifespan (9). The lifespan curve of the *isp-1* population undergoes a negative shift in the presence of *aak-2* RNAi relative to control RNAi, indicating the requirement of AAK-2 on the lifespan phenotype.

Indeed, this effect is not seen in the *mev-1* and *gas-1* mutant populations. Specifically, in the case of the *mev-1* mutant, there is no statistical difference between its mean lifespan on *aak-2*

and control RNAi, therefore, despite *aak-2* knockdown CEP-1 and CEH-23 are still able to negatively regulate lifespan. In the case of the *gas-1* mutant however, there is a negative shift from control to *aak-2* RNAi. The disparate effect of *aak-2* RNAi on the *mev-1* and *gas-1* mutants is unexpected, however it is clear that *aak-2* knockdown does not rescue lifespan in the *gas-1* mutant. The effect of *aak-2* knockdown in the wildtype strain is a minor reduction of lifespan, and this effect can be attributed to the fact that AMPK is an important regulator of homeostasis and energy production. The reduction of the amount of the catalytic domain of AMPK would certainly perturb its ability to properly regulate such important tasks.

The fact that the ability of CEP-1 and CEH-23 to regulate lifespan in the *mev-1* and *gas-1* mutants appears unperturbed implies that they are not receiving the signal of mitochondrial dysfunction from AAK-2. However, the possibility that AAK-2 activity is relevant to the lifespan phenotype of the short-lived mutants still exists. If AAK-2 were solely responsible for CEP-1 and CEH-23 activation, then the lifespan assay would have illustrated as much. The activation of CEP-1 and CEH-23 could rely on AAK-2, however in the absence of AAK-2 another factor may be able to similarly activate the transcription factors. Additionally, the remaining AAK-2 present after knockdown could be enough to activate CEP-1 and CEH-23. Finally, CEP-1 and CEH-23 might not be activated at all, which would implicate an uncharacterized downstream factor that is able to reduce the lifespan of the short-lived mutants in the absence of AAK-2. A necessary follow up experiment to the lifespan assay is to measure the activity level of AAK-2 in the short-lived mutants. This can be done by western blot using an antibody that specifically recognizes the phosphorylated residue of AAK-2 that governs its activation status. This will determine if mitochondrial dysfunction caused by *mev-1* and *gas-1* mutations leads to the activation of AAK-2, therefore elucidating the link between mitochondrial

dysfunction and CEP-1 and CEH-23 activation. If it is not activated, the tentative conclusion from figure 1 is most likely sound, in that AAK-2 is not responsible for transmitting the signal of mitochondrial dysfunction to CEP-1 and CEH-23. However if it is indeed activated, the aforementioned possibilities will have to be considered.

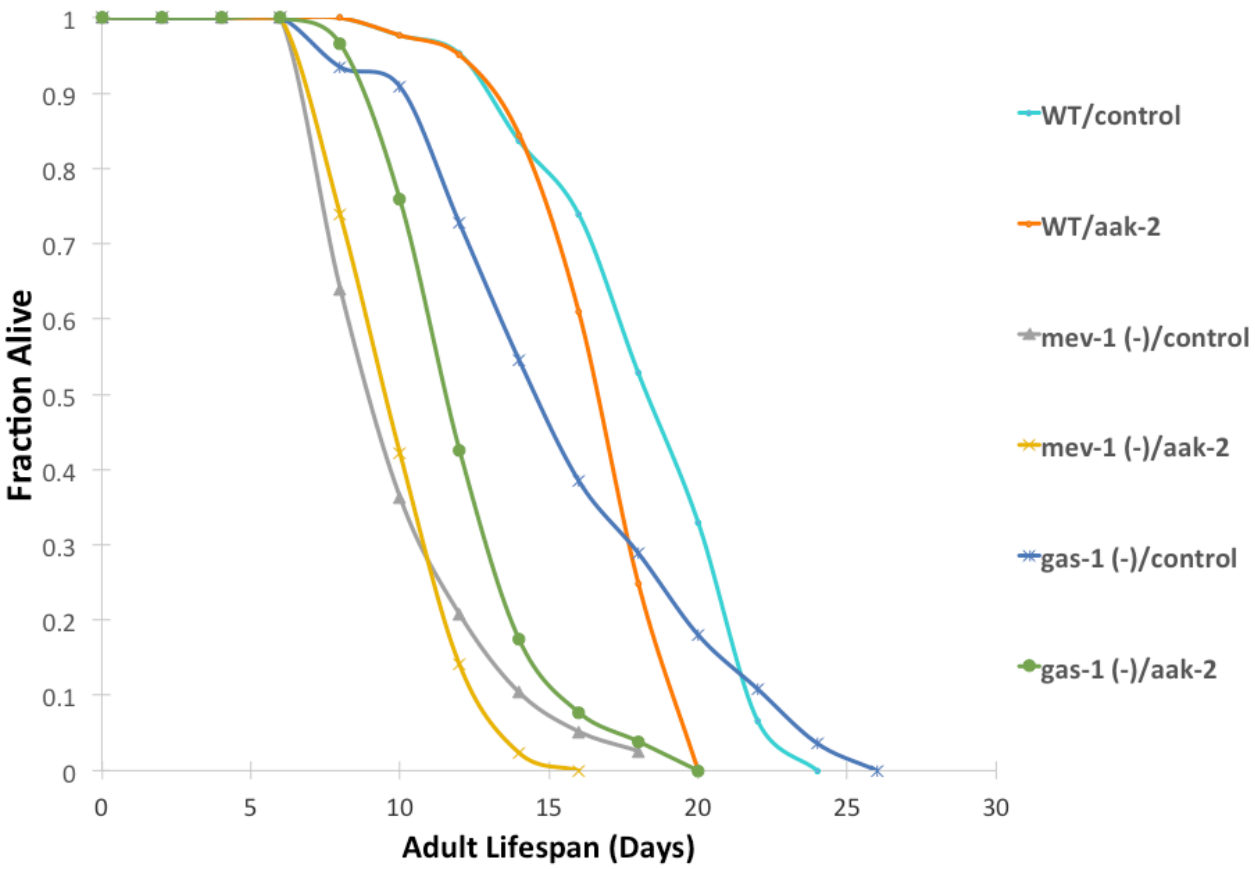

Figure 1. Knockdown of *aak-2* does not rescue the lifespan of the *mev-1* or *gas-1* mutants.

The reduced lifespan phenotypes of the short-lived mutants are not tapered in response to *aak-2* knockdown, indicating the independence of the lifespan phenotype of AAK-2 activity. The *gas-1* mutant shows a further reduction of lifespan in response to *aak-2* knockdown, in contrast to the *mev-1* mutant, which shows no change. Organisms were fed siRNA-producing bacteria throughout the entirety of their lifespan.

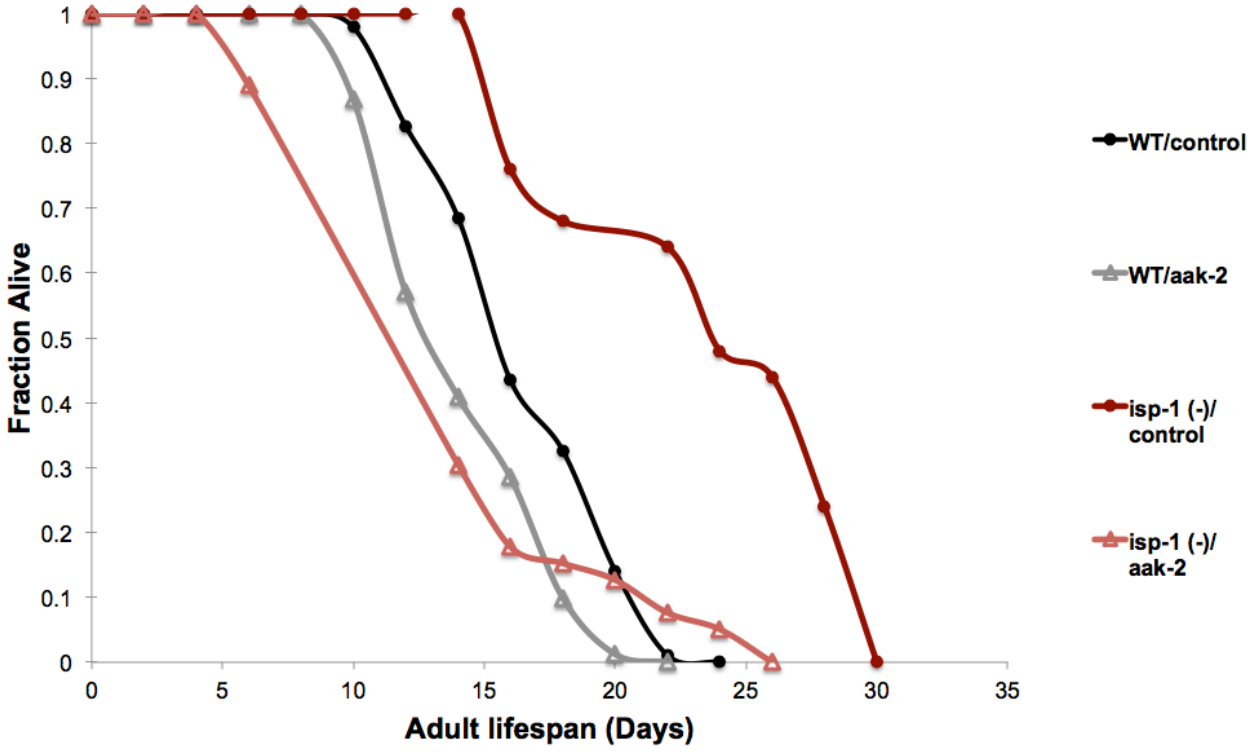

Figure 2. Knockdown of *aak-2* suppresses lifespan extension of the *isp-1* mutant. The full lifespan extension of the *isp-1* mutant is suppressed by *aak-2* knockdown. This indicates that there are no factors parallel to *aak-2*. The wildtype lifespan is modestly affected by *aak-2* knockdown, a phenotype that is indicative of AAK-2 regulation of homeostasis.

CHAPTER 1.2

Recently, CREB-regulated transcription coactivator (CRTC-1), which is a direct target of AMPK and whose phosphorylation leads to inactivation by nuclear expulsion, has been characterized as a negative regulator of constitutively active AAK-2-mediated longevity (14). The phosphorylation of CRTC-1 by AMPK occurs at two conserved residues, that when phosphorylated expose a nuclear export signal (NES) that lead to nuclear expulsion. This regulation remains fluid by action of the corresponding phosphatase, calcineurin. CRTC-1 is required for CREB-homologue-1 (CRH-1) transcriptional activity and a reduction of CRTC-1 shows a transcriptional output that is similar to *crh-1* null mutants, thus nuclear exclusion of CRTC-1 is essentially equivalent to reducing CRH-1 activity. The relationship between AAK-2 and CRTC-1 certainly has relevance to longevity determination, and AAK-2 is activated by mitochondrial dysfunction caused by the *isp-1* mutation, therefore it is reasonable to suggest that CRTC-1 exclusion by AAK-2 is a requirement of longevity in the *isp-1* mutant. To begin the investigation into this, I have examined the sub-cellular localization of CRTC-1 in the *isp-1* mutant background with and without CEP-1 or CEH-23 (Figure 3).

CRTC-1 is expressed throughout the intestine and also in some head and tail neurons, however the relationship between AAK-2 and CRTC-1 is most aptly illustrated in the intestinal cells due to their immense size. Figure 3 shows CRTC-1 expression in wildtype intestinal cells and in *isp-1* mutant intestinal cells by utilizing a fused red fluorescent protein (RFP) reporter. In the wildtype, CRTC-1 is diffused throughout the cell. It is evenly distributed throughout the cytosol and nucleus and often exhibits strong localization to the nucleolus, represented by the bright dot in the center. This pattern indicates the basal level of AMPK activity, as the

localization of CRTC-1 is dependent upon its phosphorylation status. In the *isp-1* mutant however, localization of CRTC-1 is limited to the cytosol, indicating strong regulation by AMPK leading to nuclear exclusion. This is expected of course because AAK-2 has been shown to be activated in the *isp-1* mutant. In conclusion, it is confirmed that the previously characterized relationship between AMPK and CRTC-1 is maintained in the *isp-1* mutant, and it is therefore appropriate to hypothesize that their relationship plays a significant role in the longevity phenotype.

To further delineate the relationship between CRTC-1 and known regulators of *isp-1*-mediated longevity, I examined the sub-cellular localization of CRTC-1 in the *isp-1* mutant in the absence of CEP-1 and CEH-23. If localization of CRTC-1 were perturbed by the loss of CEP-1 or CEH-23, then that transcription factor would be implicated as an upstream factor with regards to CRTC-1 regulation. Figure 4 shows the percentage of organisms that have CRTC-1 excluded from their intestinal nuclei in each genetic condition. The fourth larval stage and young adult stage were assayed in order to account for any discrepancy in energy status due to the generation of embryos, although there are no statistical differences between stages. As expected, CRTC-1 is rarely excluded from the nucleus in the wildtype, but nearly always excluded in the *isp-1* mutant. Knockout of *cep-1* or *ceh-23* does not perturb the localization of CRTC-1 in the wildtype or the *isp-1* mutant. As such, the regulation of CRTC-1 by AMPK does not appear to rely on CEP-1 or CEH-23 in the *isp-1* mutant.

In addition, the results of figure 4 are complemented by the use of RNAi methodology pursuing the same experimental goals in figure 5. Wildtype and *isp-1* mutant egg lays were performed on bacteria producing siRNA to target *cep-1*, *ceh-23*, or *aak-2*, as well as a negative control. At the fourth larval stage, they were either assayed for CRTC-1 localization or

transferred in order to perform a second egg lay in order to account for the maternal effect. The knockdown of *cep-1* and *ceh-23* in the *isp-1* mutant for both generations yielded results similar to their respective knockouts; the sub-cellular localization of CRTC-1 remains cytosolic in the absence of CEP-1 or CEH-23. In both generations, the *isp-1* mutant treated with either control, *cep-1* or *ceh-23*-targeting siRNA showed a similar frequency of CRTC-1 nuclear exclusion. This indicates that CRTC-1 localization is not dependent on CEP-1 or CEH-23. The apparent reduction of percent exclusion in the *cep-1* siRNA treated sample in generation 1 is most likely due to experimental error, as the percent exclusion of that condition is significantly greater than that of the *aak-2*-treated positive control, and the reduction is not validated in the generation 2 data or the knockout data. *aak-2*-targeting siRNA results in a lower percentage of nuclear exclusion, supporting the relationship that a reduction in AAK-2 activity results in a reduction in CRTC-1 phosphorylation, and therefore cytosolic sequestration.

From this compiled imaging assessment, it is evident that AMPK is free to phosphorylate and therefore cytosolically sequester CRTC-1 in the *isp-1* mutant, and that this process does not require CEP-1 or CEH-23. Although this imaging assessment does not implicate CRTC-1 as a longevity regulator in the *isp-1* mutant, it does tentatively place regulation of CRTC-1 upstream of CEP-1 and CEH-23, provided the data to support CRTC-1 as a longevity modulator. Fortunately, a former lab member interrogated the role of CRTC-1 as a longevity modulator by utilizing a constitutively nuclear CRTC-1, which cannot be phosphorylated by AMPK due to AMPK target serines on CRTC-1 being mutated to alanine. The result is that the lifespan extension caused by the *isp-1* mutation is inhibited by constitutively nuclear CRTC-1, regardless of CEP-1 or CEH-23 function. This result implicates CRTC-1 as a negative regulator of longevity upstream of CEP-1 and CEH-23. Thus, from this data we can now conclude that AAK-

2 activation and subsequent phosphorylation of CRTC-1 is a requirement of *isp-1*-mediated longevity. By inactivating CRTC-1, AAK-2 is functionally inactivating CRH-1, however the relationship between CRH-1, CEP-1, and CEH-23, is unknown.

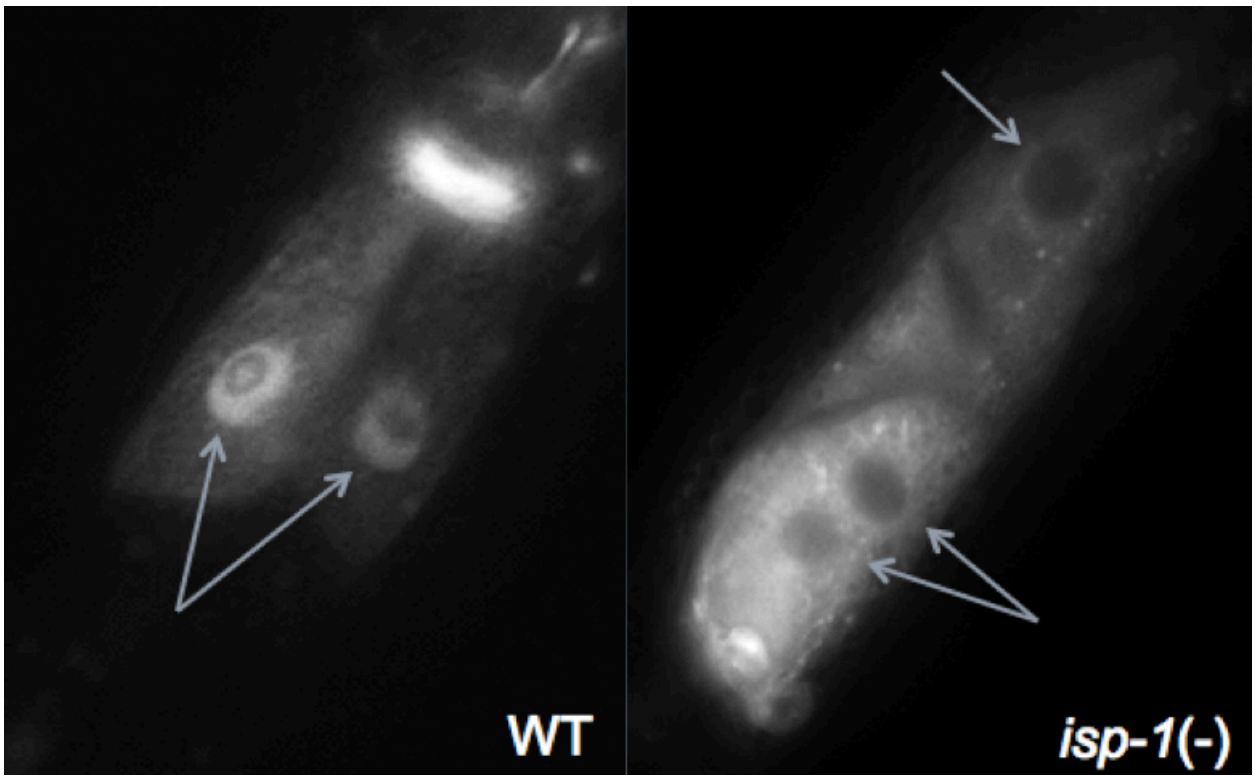

Figure 3. CRTC-1 is excluded from intestinal nuclei in the *isp-1* mutant. CRTC-1 is excluded from the nucleus via AMPK phosphorylation, thus the activation of AAK-2 in response to mitochondrial dysfunction caused by the *isp-1* mutation results in increased nuclear exclusion of CRTC-1 relative to wildtype, where basal levels of AAK-2 activation allow CRTC-1 to localize to the nucleus as well as the cytosol.

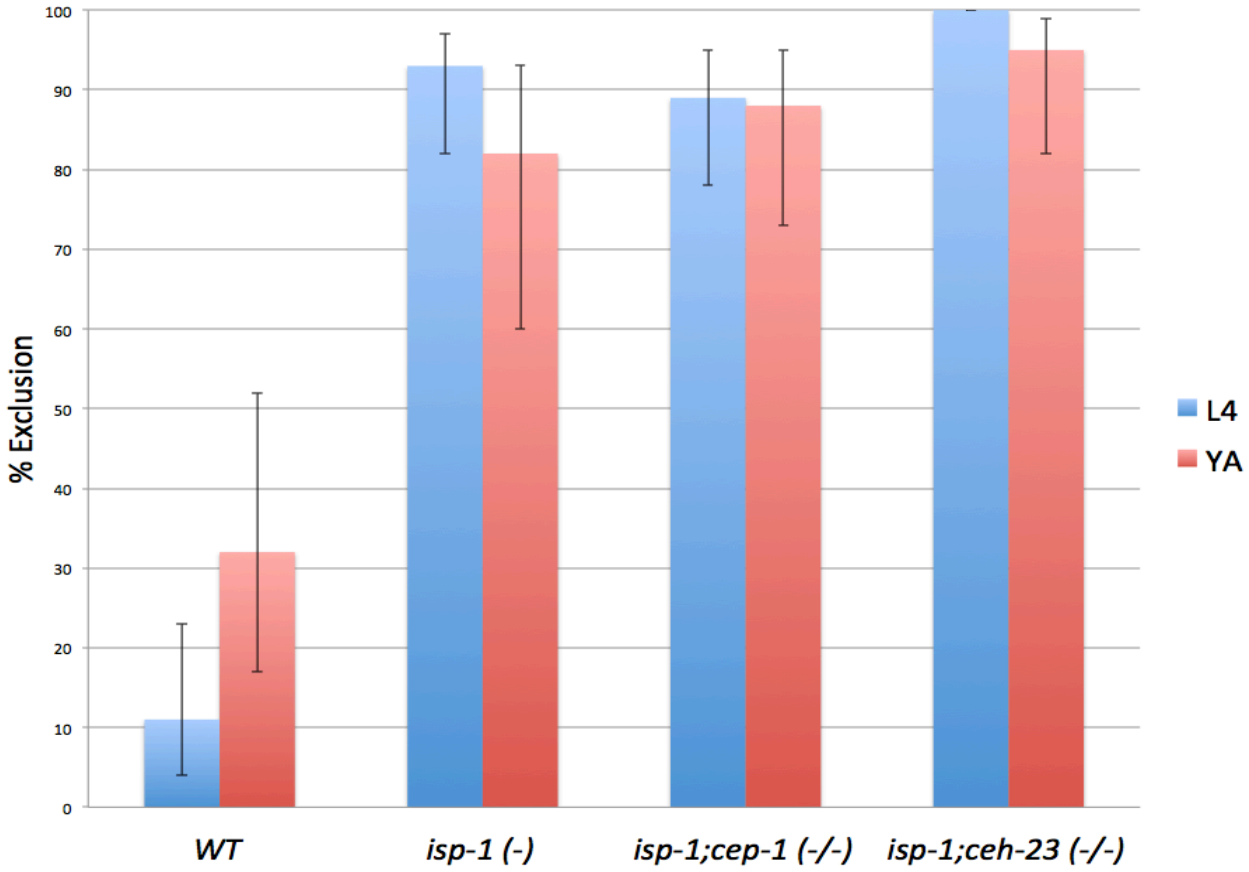

Figure 4. The loss of CEP-1 or CEH-23 does not affect regulation of CRTC-1 by AMPK.

CRTC-1 is excluded from intestinal nuclei in the *isp-1* mutant, regardless of CEP-1 or CEH-23 activity. This indicates that CEP-1 and CEH-23 do not alter the ability of AMPK to regulate CRTC-1 localization in the *isp-1* mutant. Stage four larvae and young adults were assayed for CRTC-1 sub-cellular localization. 30 organisms were assayed per condition.

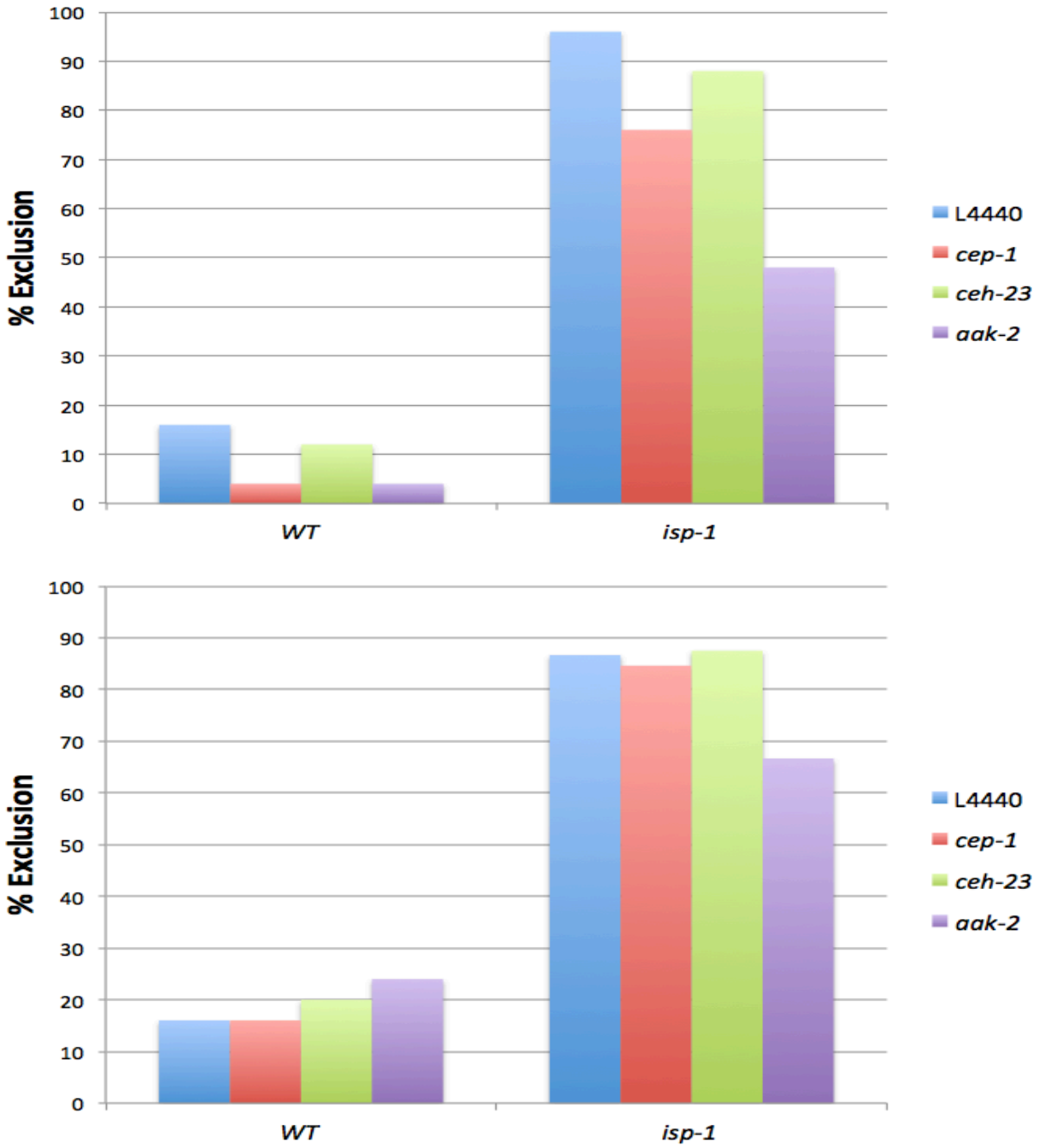

Figure 5. Knockdown of *cep-1* or *ceh-23* does not affect regulation of CRTC-1 by AMPK.

The ability of AMPK to regulate CRTC-1 localization is unaffected by *cep-1* or *ceh-23*-siRNA treatment of the *isp-1* mutant. This complements the mutant data to support the hypothesis that CEP-1 and CEH-23 do not regulate the localization of CRTC-1. Organisms were assayed for CRTC-1 localization as the first generation (top) or the second generation (bottom) to be fed siRNA. 25 organisms were assayed per condition.

DISCUSSION

The roles of CEP-1 and CEH-23 appear confounding, as they positively and negatively regulate lifespan in response to similar degrees of mitochondrial dysfunction. AAK-2 is responsible for CEP-1 and CEH-23 activation in the *isp-1* mutant, however in the *mev-1* and *gas-1* mutants, the pathway is more complex. Even if AAK-2 is activated in the short-lived mutants, the pathway appears to be novel based off of the inability to rescue wildtype lifespan with *aak-2* knockdown, in contrast to the long-lived *isp-1* mutant. In addition, even among the short-lived mutants we see potential discrepancies in their modes of longevity regulation. The further reduction of *gas-1* mutant lifespan following *aak-2* knockdown indicates a distinction between the longevity-modulating pathways of the *mev-1* and *gas-1* mutants. It is possible however, that complex I and complex II each initiate their own compensatory machinery, and that this machinery perturbs the measured lifespan of the mitochondrial mutants to an extent that is limited to effects from factors that are parallel to CEP-1 and CEH-23.

The role of CRTC-1 in *isp-1*-mediated longevity is curious, in that its nuclear exclusion is required for CEP-1 and CEH-23 activity, although in the short-lived mutants CEP-1 and CEH-23 do not appear to be dependent upon AAK-2 and therefore CRTC-1 localization. If it is shown that AAK-2 is indeed activated in the short-lived mutants, the role of CRTC-1 must be interrogated as a potential longevity modulator in those mutants.

The distinct lifespan phenotypes of different mitochondrial mutants provide us with closely related longevity-modulating pathways. The long-lived *isp-1* mutant utilizes a pathway by which AAK-2 mediates a transcriptional reprogramming response to mitochondrial dysfunction. The lifespan extension that results from this may be a primary effect, guarded by

evolution with purposeful intent, or it may simply be a consequence of slowing metabolism in response to perturbed mitochondrial function. If we aim to extend human lifespan by investigating these longevity-modulating pathways, we must be able to separate the lifespan phenotype from other factors, such as the requisite of mitochondrial dysfunction.

APPENDIX I.

THE ROLE OF CEH-23 IN LONGEVITY MODULATION

CEH-23 transcription factor is required for longevity in the long-lived *isp-1*, and short-lived *mev-1* and *gas-1* mitochondrial mutants. Loss of CEH-23 does not affect lifespan, however the overexpression of *ceh-23* confers an extended lifespan in an otherwise wildtype background (12). The extension of lifespan was first shown with *ceh-23* transgenes that include the *ceh-23* promoter and 3' UTR, and this extension was maintained following the random integration of one of the transgenes, which created a mutant line.

CEH-23 has been shown to act downstream of AAK-2 activity in the *isp-1* mutant to increase lifespan, and it shows increased expression in the dual complex III mitochondrial mutant, *isp-1;ctb-1* (12). One may hypothesize given this information that CEH-23 expression is increased by AAK-2 in response to mitochondrial dysfunction, however AAK-2 is required for lifespan extension in the overexpressed *ceh-23* line (figure 6). This presents an apparent paradox, however as previously stated, CRTC-1 nuclear exclusion by AAK-2 is required for the lifespan extension of the *isp-1* mutant. AAK-2 may therefore be required to fulfill its role in CRTC-1 phosphorylation. In order to examine this, I crossed the *ceh-23* overexpressed line with a constitutively nuclear CRTC-1 as well as constitutively active AAK-2 line (table 1). If the requirement of *ceh-23* overexpression-mediated longevity on AAK-2 is due to its regulation of CRTC-1, then constitutively nuclear CRTC-1 will suppress the lifespan extension of the *ceh-23* overexpressed line. Also, constitutively active AAK-2 should not further increase the lifespan of the *ceh-23* overexpressed line, as AAK-2 acts through *ceh-23* to confer lifespan extension (9). If constitutively nuclear CRTC-1 does not suppress the lifespan of the *ceh-23* overexpressed line,

then the overexpression of *ceh-23* may confer longevity through mechanisms that are distinct from its role in *isp-1*-mediated longevity.

In addition, the requirement of *isp-1*-mediated longevity on CRTC-1 nuclear exclusion should be investigated on another level; in order to root out uncertainty between mitochondrial dysfunction and AAK-2 activation, the longevity of constitutively active AAK-2 must be assayed in the presence of constitutively nuclear CRTC-1 as well as *cep-1* or *ceh-23* mutant alleles, to determine if constitutively nuclear CRTC-1 suppresses the lifespan extension of constitutively active AAK-2 regardless of CEP-1 or CEH-23. To date, the *cep-1* triple mutant has been generated, but the *ceh-23* triple mutant has not. The lifespan assay of these genetic conditions will detail the role of CRTC-1 further, as the possibility of a parallel factor to AAK-2 may play a role with regards to CRTC-1 regulation of CEP-1 and CEH-23 activity.

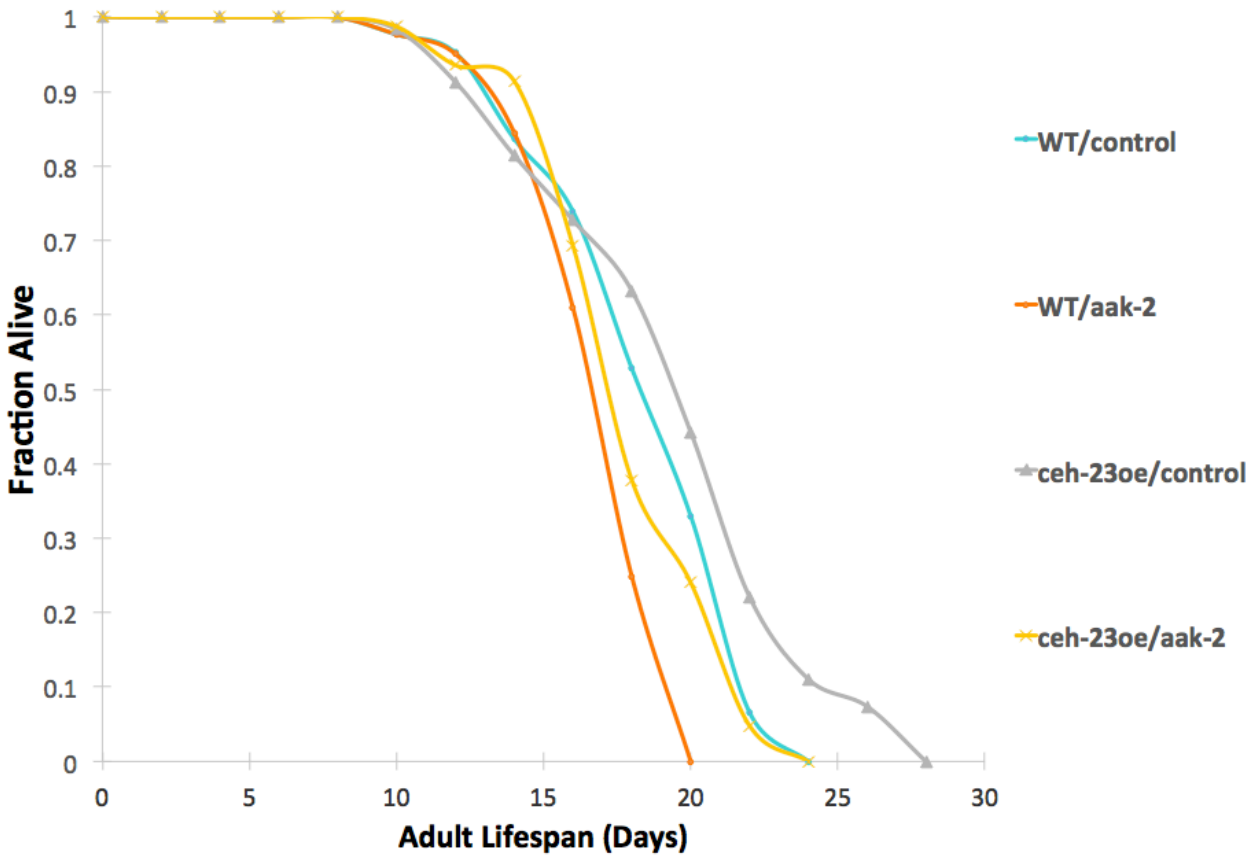

Figure 6. *ceh-23* overexpression confers an extended lifespan dependent upon AAK-2. The ability of CEH-23 to positively regulate lifespan requires AAK-2 activity. This implies that although *ceh-23* expression is increased in the *isp-1;ctb-1* double mutant, lifespan regulation by *ceh-23* is not simply a function of active CEH-23, but of specific AAK-2 activity unrelated its activation of CEH-23.

IU Number	Strain
547.1-3	isp-1(qm150);cep-1(gk138);uthIs226[crtc-1p::crtc-1(S76A, S179A)::tdTOMATO::unc-54 3'UTR + rol-6(su1006)]
548	isp-1(qm150);ceh-23(ms23);uthIs226[crtc-1p::crtc-1(S76A, S179A)::tdTOMATO::unc-54 3'UTR + rol-6(su1006)]
549.1-3	isp-1(qm150);uthIs226[crtc-1p::crtc-1(S76A, S179A)::tdTOMATO::unc-54 3'UTR + rol-6(su1006)]
550.1-3	rsks-1(sv31);cep-1(gk138)
551.1-3	rsks-1(sv31);cep-1(gk138);ceh-23(ms23)
553	isp-1(qm150);cep-1(gk138);CRTC-1::RFP
554.1-2	isp-1(qm150);ceh-23(ms23);CRTC-1::RFP
555.1-3	[aak-2p::aak-2(genomic aa1-321)::GFP::unc-54 3'UTR + myo-2p::tdTOMATO];[crtc-1p::crtc-1(S76A, S179A)::tdTOMATO::unc-54 3'UTR + rol-6(su1006)]; cep-1 (gk138)
In progress	ceh-23; mec-7::RFP,pBSK crossed with WBM55 uthIs226 [crtc-1p::crtc-1(S76A, S179A)::tdTOMATO::unc-54 3'UTR + rol-6(su1006)].X
In progress	ceh-23; mec-7::RFP,pBSK crossed with WBM60 uthIs248 [aak-2p::aak-2(genomic aa1-321)::GFP::unc-54 3'UTR + myo-2p::tdTOMATO].

Table 1. Strains generated in the Lee Lab.

APPENDIX II.

GFP-TAGGING OF ENDOGENOUS CEH-23 THROUGH CRISPR METHODOLOGY

Microarray analysis of CEH-23 target genes in the *isp-1* mutant background has been compared with analysis of AAK-2 target genes in the *isp-1* mutant background, and most interestingly, the genes that are similarly regulated by CEH-23 and AAK-2 show an overrepresentation of kinases of the Tau-tubulin kinase like family, and phosphatases that show sequence similarity to protein phosphatase I catalytic subunit, as determined by GO analysis (9). The significance of these two gene sets is their relation to phosphate metabolism surrounding tau protein (PTL-1 in *C. elegans)*. Tau phosphorylation is studied greatly in the context of Alzheimer's disease, and the notion that a long-lived mitochondrial mutant is up-regulating genes required for phosphorylation and de-phosphorylation of tau is thoroughly intriguing.

In order to confirm that these genes are relevant to *isp-1*-mediated longevity, the gene targets of CEH-23 in the *isp-1* mutant must be directly analyzed using ChIP-seq. In addition, CEH-23 has been shown to be expressed in a select number of neuron subtypes, therefore if we are to dissect the longevity pathway beyond CEH-23, we must determine which cells are expressing CEH-23 in the *isp-1* mutant to properly assess the CEH-23-regulated genes.

To do this, I have generated sgRNA to target *ceh-23* using a U6 pol III promoter-containing backbone to drive expression, as described by Friedland et al. (15). Two guides were constructed per *ceh-23* terminus to account for variability in efficiency of homologous recombination. In addition, I have designed repair templates using a self-excising drug selection cassette method (16). This method allows for screening of progeny post-injection without the need for numerous trials of PCR. Instead, the homologous recombination event inserts a fluorescent marker (GFP) and a flag tag adjacent to the gene of interest along with a selection marker, that after screening for positive events, can be excised by Cre recombinase, which is expressed following heat shock. This method will quickly allow for construction of a GFP-

tagged *ceh-23* allele that can be assayed using ChIP analysis to determine CEH-23-regulated genes.

METHODS

Chapter 1.1 & Appendix I.

Lifespan experiment was performed at 20°C on Nematode Growth Media plates seeded with E. coli HT115 siRNA-producing bacteria. Prior to seeding, bacteria were concentrated to OD600=4. 4mM IPTG was added to seeded plates the night before use in order to induce siRNA production. Well-fed gavid adults were allowed to egg lay and six groups of 18 progeny per condition were assessed for lifespan on separate plates, except for the wildtype, whose lifespan was measured with 9 groups. Survival was scored every other day followed by a transfer to a fresh plate. Survival curves were estimated using the Kaplan-Meier method, and a log-rank test was performed for statistical analysis.

Chapter 1.2

For mutant imaging, well-fed L4s and young adults grown on E. coli OP50 in 20°C were immobilized with levamisole on a 2% agarose pad and assayed immediately for CRTC-1 localization. 5 worms were imaged per pad. 3 experiments were performed, two with a sample size of 30 and one with a sample size of 10 per condition. Logistic regression was used in SPSS to conduct statistical analysis.

For RNAi imaging, well-fed L4s and young adults grown on E. coli HT115 siRNA-producing bacteria in 20°C were immobilized with levamisole on a 2% agarose pad and assayed immediately for CRTC-1 localization. 5 worms were imaged per pad. 1 experiment was performed per generation. The first generation hatched on HT115 plates and were imaged after development proceeded to the appropriate stage. Worms that were not assayed were transferred to fresh plates where their progeny were subsequently assayed for the second experiment. Both experiments used a sample size of 25 per condition, with the exception of the *isp-1* mutant in the second experiment which used 30, 13, 8, and 18, respective of the RNAi condition.

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