THE PENNSYLVANIA STATE UNIVERSITY
SCHREYER HONORS COLLEGE

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

# MITOCHONDRIAL SIRTUINS IN CAENORHABDITIS ELEGANS SIR-2.2 AND SIR-2.3 REGULATE LIFESPAN AND ARE NOT REDUNDANT IN FUNCTION

SARAH CHANG
SPRING 2016

A thesis
submitted in partial fulfillment
of the requirements
for a baccalaureate degree
in Biochemistry and Molecular Biology
with honors in Biochemistry and Molecular Biology

Reviewed and approved* by the following:

Wendy Hanna-Rose
Associate Professor of Biochemistry and Molecular Biology
Thesis Supervisor

Ming Tien
Professor of Biochemistry
Honors Adviser

* Signatures are on file in the Schreyer Honors College.

## ABSTRACT

Mitochondrial sirtuins are proteins that regulate metabolism and are emerging drug targets for metabolic and age-related diseases such as cancer, diabetes, and Alzheimer's disease. Although it is known that they are involved in metabolic regulation, how they carry out this regulation and many of their exact functions remain uncharacterized. In this study, I uncover a novel role for the mitochondrial sirtuins SIR-2.2 and SIR-2.3 in lifespan regulation using the *C. elegans* model. Using a genetic approach, I discovered that the mitochondrial sirtuin mutant worms surprisingly lived 25% longer than wild-type. While both overexpression of sirtuins and decreased consumption of food are known mechanisms for lifespan extension, neither decreased food intake nor upregulation of sirtuins as compensation was observed in the mitochondrial sirtuin mutants. *sir-2.2* mutants display a significant increase in mitochondrial superoxide dismutase *sod-3* expression. The increased lifespan of *sir-2.2* mutants was further extended upon knocking down pyruvate carboxylase *pyc-1* and pyruvate dehydrogenase *pdh*. LC-MS analysis revealed metabolic changes in *sir-2.2* and *sir-2.3* mutants compared to wild-type. This data suggests that SIR-2.2 and SIR-2.3 may not be redundant in function and may have different mechanisms for metabolism and lifespan regulation. These results underscore the ability of mitochondrial sirtuins to control the metabolic state of animals and reveal new metabolic pathways that can be targeted to extend lifespan.

# TABLE OF CONTENTS

## LIST OF FIGURES

# LIST OF TABLES

## ACKNOWLEDGEMENTS

I would like to thank my research advisor Dr. Wendy Hanna-Rose for her amazing support and guidance that allowed me to pursue this research project and grow as a scientist. I would also like to thank Melanie McReynolds for her assistance with the metabolomics analyses and her help in teaching me how to do many of the experiments included in this thesis. I want to thank Laura Rohan for performing the pyruvate carboxylase lifespan assays and the rest of the Hanna-Rose lab for their constant help. I would like to thank my sources of funding for this project, including the Office of the Eberly College of Science Associate Dean for Undergraduate Education, the Penn State Erickson Discovery Grant, and NIH award number GM086786 to WHR.

# Chapter 1. Introduction

## Mitochondrial Sirtuins

Sirtuins are a highly conserved family of $NAD^+$-dependent protein deacetylases (Blander and Guarente 2004) involved in regulating metabolism, stress response, and aging (Guarente 2011). The first sirtuin *Sir2* (silent information regulator 2) was discovered in the yeast *Saccharomyces cerevisiae*, where its overexpression caused an increase in lifespan (Kaeberlein, McVey, and Guarente 1999). Overexpressing the nuclear sirtuin SIR-2.1 in *C. elegans*, the homolog of yeast *Sir2*, was later shown to have a similar effect, causing a 50% increase in the animal's lifespan (Tissenbaum and Guarente 2001). Although the robustness of this phenotype has been questioned (Burnett et al. 2011), the wealth of studies on sirtuins emphasizes their consistent role in metabolic control, the progression of age-related diseases, and regulating healthspan (Aka, Kim, and Yang 2011).

Mammals have seven sirtuins (SIRT1-7), three of which (SIRT3, 4, and 5) reside in the mitochondria (Verdin et al. 2010). In *C. elegans*, there are four sirtuins, SIR-2.1 to SIR-2.4, with two of its sirtuins localized in the mitochondria: SIR-2.2 and SIR-2.3 (Wirth et al. 2013). While many studies have investigated SIR-2.1, there is little known about the functions of SIR-2.2 and SIR-2.3. SIR-2.2 and SIR-2.3 are located adjacent to each other on chromosome X and share 75.3% sequence identity, suggesting that one developed from a gene duplication event and thus, may be redundant in function (Wirth et al. 2013). These mitochondrial sirtuins are orthologs of mammalian SIRT4 (Wirth et al. 2013). SIRT4 generally lacks deacetylase activity and shows

robust ADP-ribosyltransferase activity (Shih and Donmez 2013). SIRT4 inhibits glutamate dehydrogenase by ADP-ribosylation (Haigis et al. 2006), balances lipid metabolism (Laurent et al. 2013), and regulates insulin secretion (Ahuja et al. 2007). Yet, it is the least characterized mitochondrial sirtuin and many of its functions remain unknown (Haigis et al. 2006). SIRT5 is a deacetylase that activates carbomyl phosphate synthetase 1 and regulates the urea cycle (Nakagawa et al. 2009). It also possesses lysine demalonylase and desuccinylase activities (Peng et al. 2011). SIRT3 is the most prominent deacetylase of the mitochondrial sirtuins, induced during times of limited energy (Lombard et al. 2007). It is a significant regulator of the oxidative stress response by deacetylating metabolic enzymes in the mitochondria to control reactive oxygen species production of the electron transport chain and activate antioxidant enzymes (Bause and Haigis 2013).

## Mitochondrial Sirtuins as Therapies for Age-Related Diseases

Mitochondrial sirtuins are promising targets for age-related diseases (Shih and Donmez 2013). Studies have revealed a close link between mitochondrial dysfunction and cancer (Xu et al. 1999), neurodegeneration (Lin and Beal 2006), and diabetes (Petersen et al. 2004). The mitochondria, as the organelle that houses critical metabolic pathways, is greatly affected in patients who suffer from obesity, hypertension, and cardiovascular disease, the leading cause of death in the United States according to the CDC's 2015 Heart Disease and Stroke Update. As regulators of metabolism in the mitochondria, mitochondrial sirtuins have great potential to combat metabolic imbalance and oxidative stress caused by mitochondrial dysfunction. It is

important to study the still mysterious functions of mitochondrial sirtuins to find new ways to target age-related diseases.

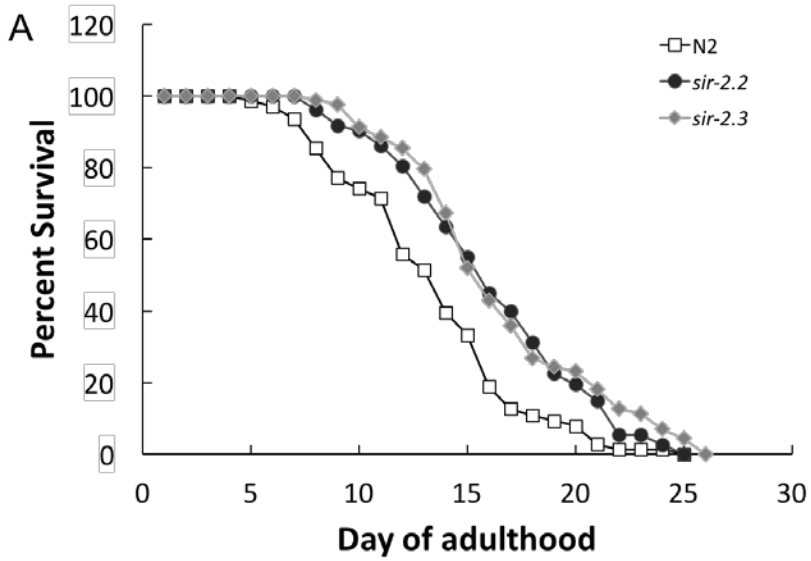
## Chapter 2. Results

### Mitochondrial sirtuin mutants live longer than wild-type when fed *ad libitum*

Because the nuclear SIR-2.1 regulates lifespan, I examined the potential role of mitochondrial sirtuins in lifespan regulation by measuring the lifespan of mitochondrial sirtuin mutants *sir-2.2(tm2673)* and *sir-2.3(ok444)*. To ensure that any changes in lifespan were solely due to a lack of mitochondrial sirtuin activity, I outcrossed the mutant strains with the N2 wild-type strain three times and used this N2 strain as a control for all experiments. Because the overexpression of *sir-2.1* can extend lifespan in *C. elegans*, it was surprising that knocking out one mitochondrial sirtuin increased lifespan by 25 to 28% when worms were fed *E. coli* OP50 *ad libitum* (Figure 1). *sir-2.2* mutants lived four days longer than N2, and *sir-2.3* mutants lived 3.6 days longer than N2. This increased lifespan was consistently observed across twelve independent experiments.

Because *sir-2.2* and *sir-2.3* may be redundant in function, I hypothesized that lifespan extension upon loss of one mitochondrial sirtuin may be due to a compensatory mechanism, where *sir-2.2* mutants upregulate *sir-2.3* and *sir-2.3* mutants upregulate *sir-2.2*. To test this hypothesis, I measured the mRNA expression of *sir-2.3* in *sir-2.2* mutants and vice versa. There was no observed significant upregulation of the functioning mitochondrial sirtuin in the

mitochondrial sirtuin mutants (Figure 2). Upon knocking down *sir-2.2* in *sir-2.3* mutants with RNAi, there was no significant change in lifespan. However, when *sir-2.3* was knocked down in *sir-2.2* mutants, there was a 24% increase in the lifespan of *sir-2.2* mutants (Figure 3A,C). These results suggest that the lifespan extension was not due to upregulation of a mitochondrial sirtuin because depleting the animals of both mitochondrial sirtuins did not result in a decrease in the extended lifespan seen in *sir-2.2* and *sir-2.3.* Instead knocking down *sir-2.3* in a *sir-2.2* mutant background caused an increase in its lifespan (Figure 3A,C,D).



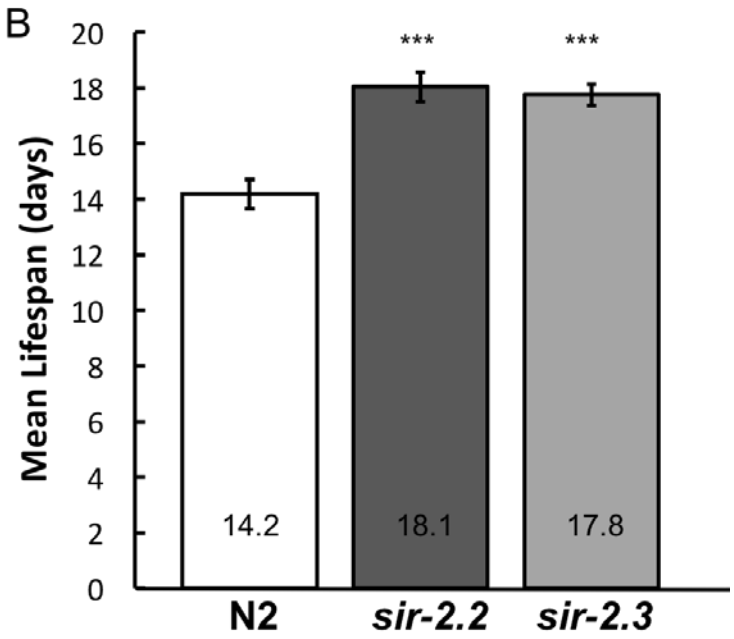



**Figure 1.** ***sir-2.2(tm2673)*** **and** ***sir-2.3(ok444)*** **live longer than N2 wild-type when fed** ***ad libitum***

**(A)** Survival curve displaying values from the average of three independent experiments, $p<0.001$, log rank t-test. **(B)** Mean lifespan values from twelve independent experiments ±SEM, ***$p<0.001$, unpaired t-test

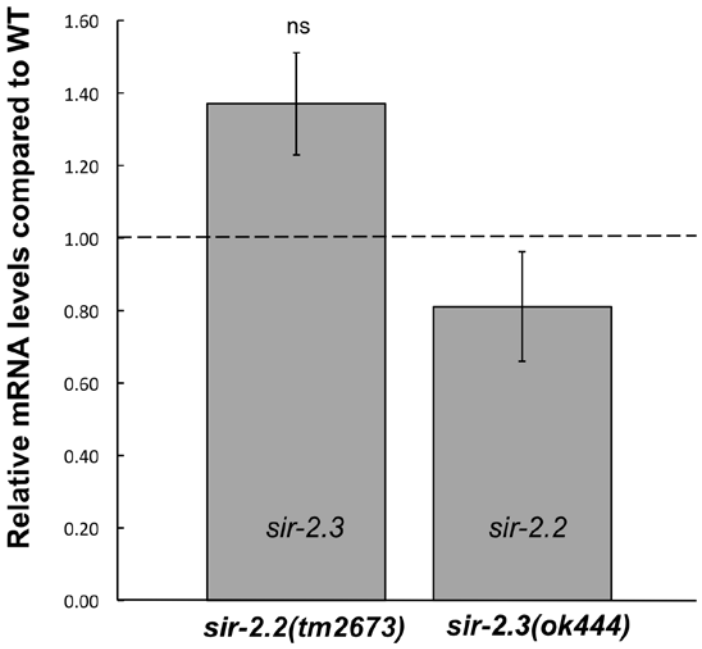



**Figure 2. Single mitochondrial sirtuin mutants do not upregulate the mRNA levels of their other mitochondrial sirtuin**

Relative expression of *sir-2.3* and *sir-2.2* was measured in the *sir-2.2* mutant and *sir-2.3* mutant, respectively, using qRT-PCR. Values are the averages of two biological replicates, each run in triplicate ±SEM. ns: p>0.5, one sample t-test

The difference in knocking down *sir-2.2* in *sir-2.3* mutants and knocking down *sir-2.3* in *sir-2.2* mutants suggests that the *sir-2.2* RNAi may not have effectively worked, as knocking down *sir-2.2* in a *sir-2.3* mutant should presumably produce a similar effect on lifespan to knocking down *sir-2.3* in a *sir-2.2* mutant. Measuring the extent of the RNAi knockdown is required before making a conclusion. It is also interesting to note that feeding *sir-2.2* and *sir-2.3* mutants on the *E. coli* strain HT115, the strain required for RNAi feeding protocols, eliminated their increased lifespans compared to N2 when fed OP50 (Figure 4). Wild-type animals were not affected by whether they were fed HT115 or OP50 (Figure 4). This suggests that the mitochondrial sirtuins may regulate the animal's lifespan in a diet-dependent manner.

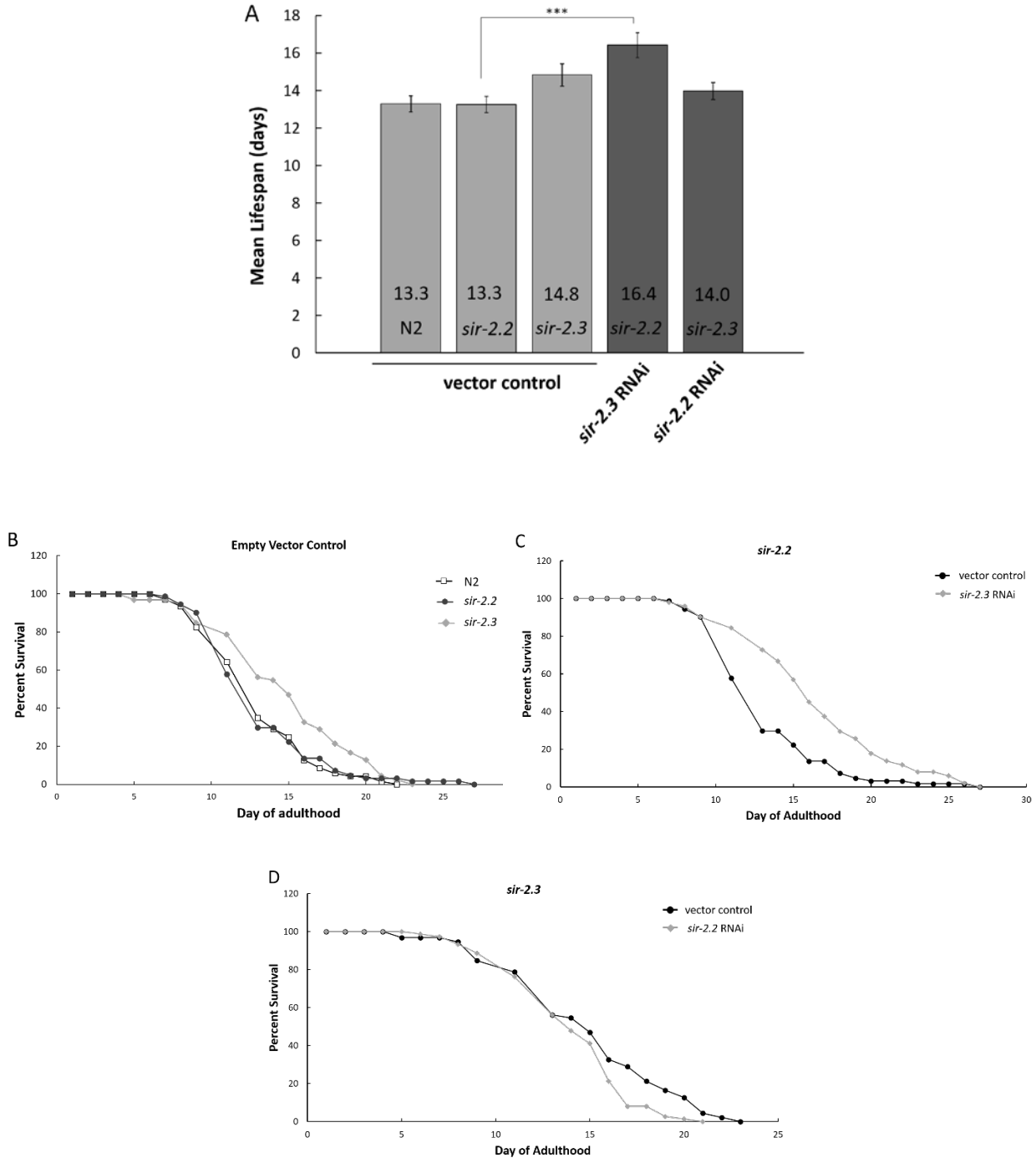



**Figure 3. RNAi knockdown of *sir-2.3* in *sir-2.2* mutants increases *sir-2.2* lifespan**

The lifespan of *sir-2.2* and *sir-2.3* worms fed RNAi knocking down the other mitochondrial sirtuin was measured. **(A)** Mean lifespan of worms on empty vector control or their respective RNAi was quantified. Values are averages of three independent experiments ±SEM, *** $p<0.001$ using an unpaired t-test. **(B,C,D)** Survival curves for worms on vector control (B), *sir-2.2* on *sir-2.3* RNAi, *** $p<0.001$, log rank t-test (C), and *sir-2.3* on *sir-2.2* RNAi (D).

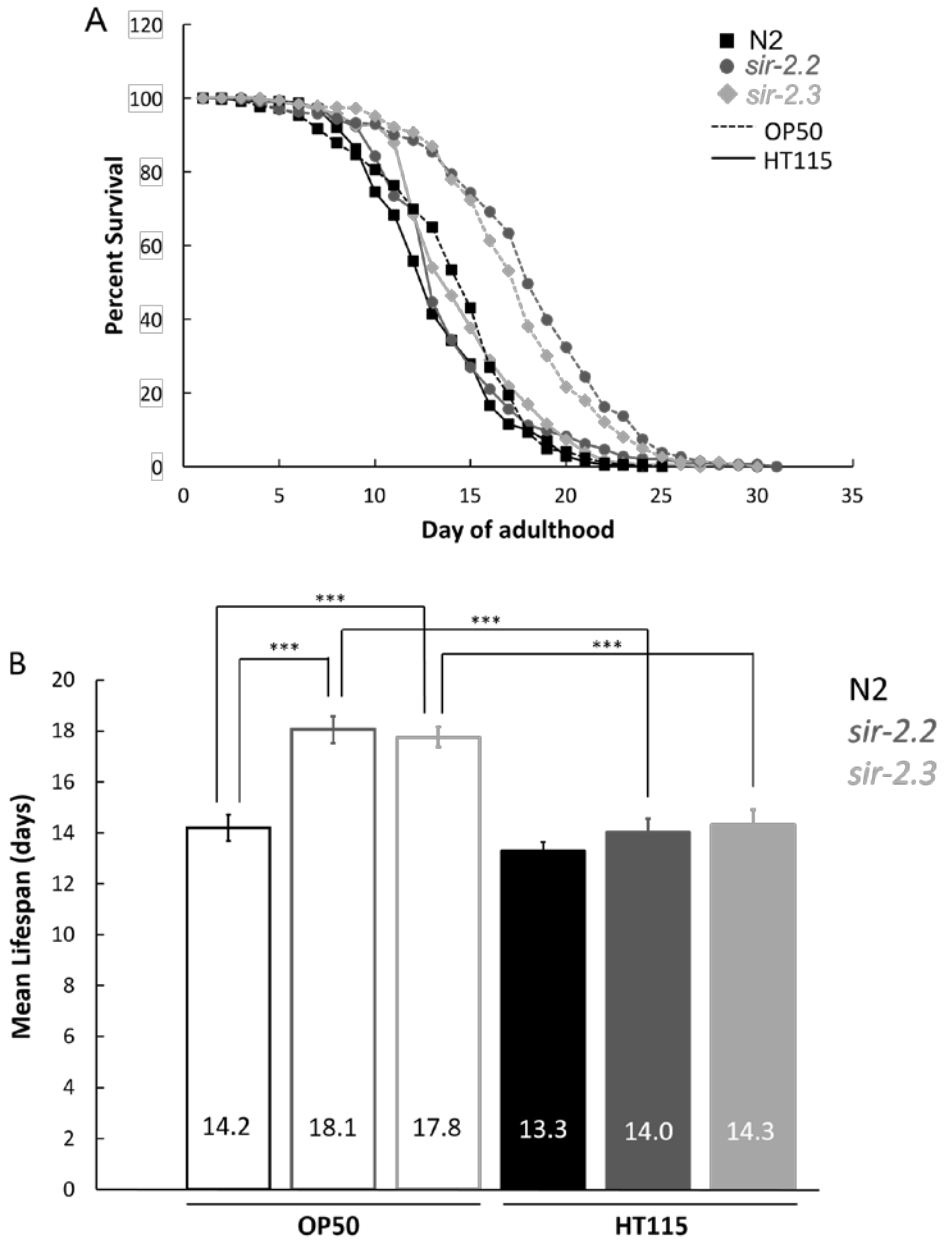



**Figure 4. *sir-2.2* and *sir-2.3* extended lifespan on OP50 is no longer observed when fed HT115**

Lifespans of N2, *sir-2.2*, and *sir-2.3* were measured on OP50 and compared with animals fed HT115. **(A)** Survival curves for N2, *sir-2.2*, and *sir-2.3* on either OP50 or HT115. OP50 values are the average of twelve independent experiments and HT115 values are the average of nine independent experiments. There is a significant difference between *sir-2.2* and *sir-2.3* fed OP50 and *sir-2.2* and *sir-2.3* fed HT115, $p<0.001$ log rank t-test. **(B)** Mean lifespan of animals on OP50 and HT115 ±SEM, *** $p<0.001$ unpaired t-test.

Because dietary restriction increases lifespan, I investigated whether the lifespan extension of the mitochondrial sirtuin mutants was due to decreased food intake by measuring their pharyngeal pumping rates. *C. elegans* ingest their food by muscular contractions of the pharynx that pump the food into the worm's intestine (Avery and You 2012). I measured the pharyngeal pumping rate of N2, *sir-2.2*, and *sir-2.3* fed OP50 *ad libitum* and fed for five minutes after a six hour fasting period. There was no observed difference between wild-type and the mitochondrial sirtuin mutants whether they were fed *ad libitum* or post-fasting, suggesting that decreased food intake does not contribute to the increase in lifespan of *sir-2.2* and *sir-2.3* (Figure 5).

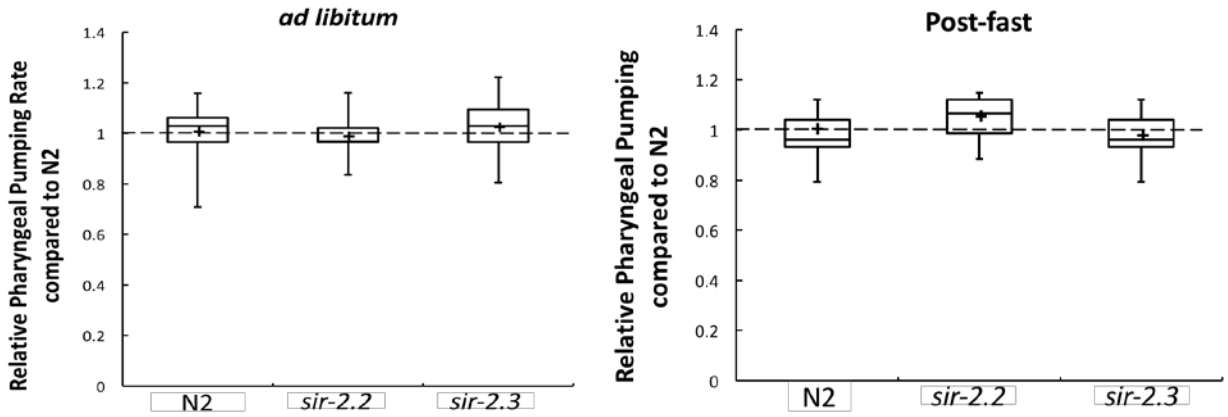


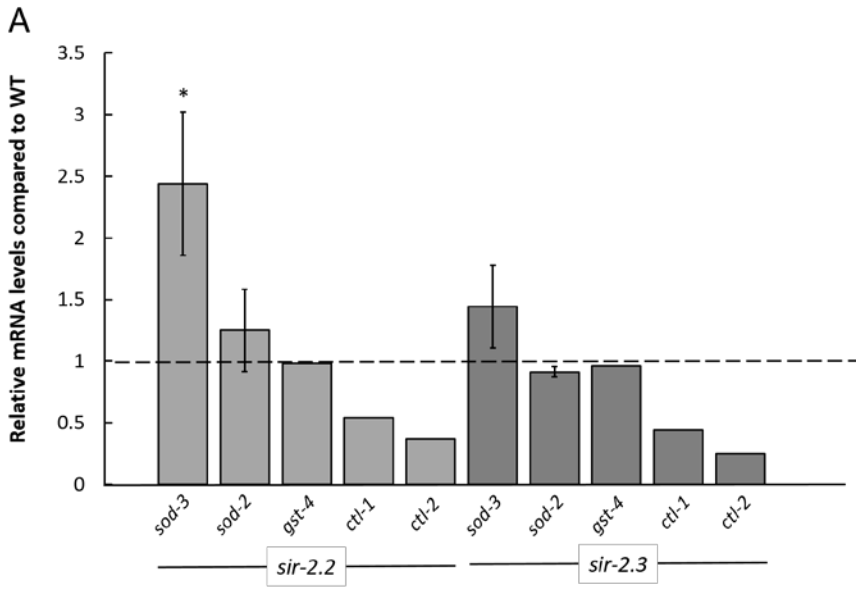

**Figure 5. Relative pharyngeal pumping rates are not different between wild-type and *sir-2.2* and *sir-2.3***

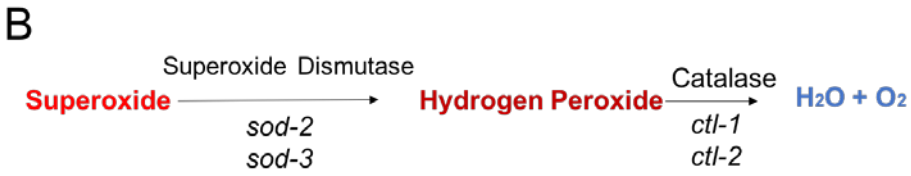
Pharyngeal pumping of N2, *sir-2.2*, and *sir-2.*3 fed *ad libitum* and five minutes post six hour fast was measured. No significant difference was detected in any comparison: $p>0.5$, unpaired t-test. Sample sizes were 10-18 animals analyzed per condition

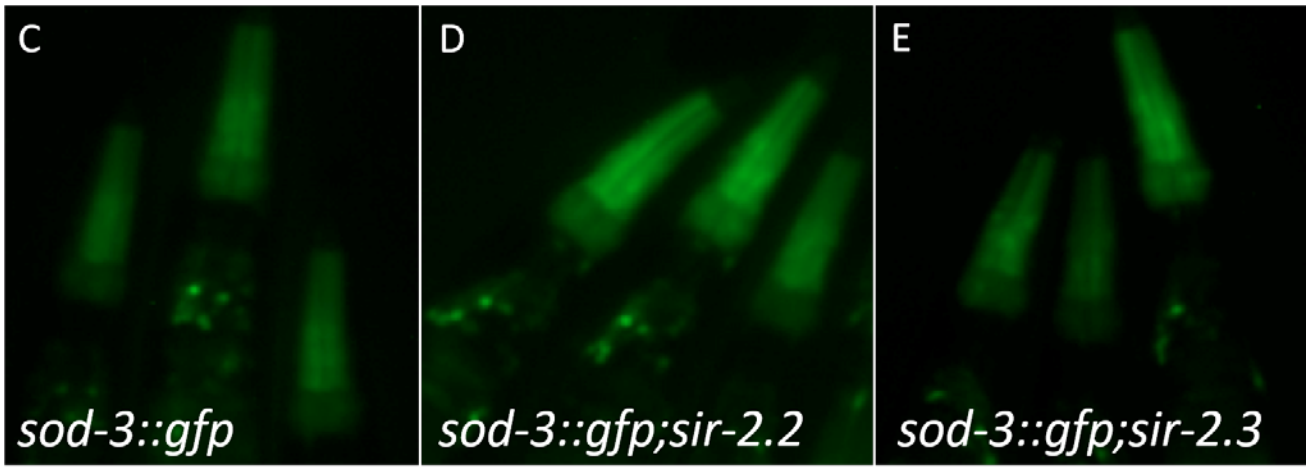
**Mitochondrial sirtuin mutants display an altered stress response compared to wild-type**

Because sirtuins respond to oxidative stress and can confer resistance to oxidative stress, I hypothesized that the extended lifespans of *sir-2.2* and *sir-2.3* may be due to a hormetic response. Hormesis occurs when a low level of a stress provides beneficial effects to a cell or an organism but is toxic at higher doses (Mattson 2008). Hormesis has long been described in *C. elegans* (Cypser and Johnson 2002), *Drosophila* (Hercus, Loeschcke, and Rattan 2003), and mice (Wang and Cai 2000) where it can heighten stress resistance and trigger an overcompensation mechanism that leads to cell survival and lifespan extension (Heidler et al. 2010). To test the hypothesis that *sir-2.2* and *sir-2.3* are undergoing hormesis, I measured the expression of key stress response genes: the mitochondrial superoxide dismutases *sod-2* and *sod-3*, glutathione-S-transferase *gst-4*, cytoplasmic catalase *ctl-1*, and peroxisomal catalase *ctl-2*. There was an observed significant 2.44-fold increase in *sod-3* expression only in *sir-2.2*. Although there seems to be a mild 1.44-fold upregulation of *sod-3* in *sir-2.3*, this was not considered statistically significant. Interestingly, there was around a two-fold decrease in both *ctl-1* and *ctl-2* expression in both *sir-2.2* and *sir-2.3* compared to N2, and no change in either *sod-2* or *gst-4* was observed (Figure 6A). The analysis of *gst-4*, *ctl-1*, and *ctl-2* has not been confirmed with biological replicates. Thus, while this data is suggestive, I cannot yet conclude that the catalase genes are indeed downregulated. The increase in *sod*-3 expression in *sir-2*.2 indicates that it may be under more oxidative stress and may be upregulating *sod-3* to combat this stress. To seek independent evidence for this regulation, I crossed *psod-3::gfp* with either *sir-2.2* or *sir-2.3* mutants and measured *sod-3* expression levels via examination of GFP intensity. There was a significant increase in GFP intensity in *sod-3::gfp;sir-2.2* day one adults

compared to *sod-3::gfp* (Figure 6C,D,F). *sod-3::gfp;sir-2.3* did not show a significant change in GFP intensity (Figure 6C,E,F).



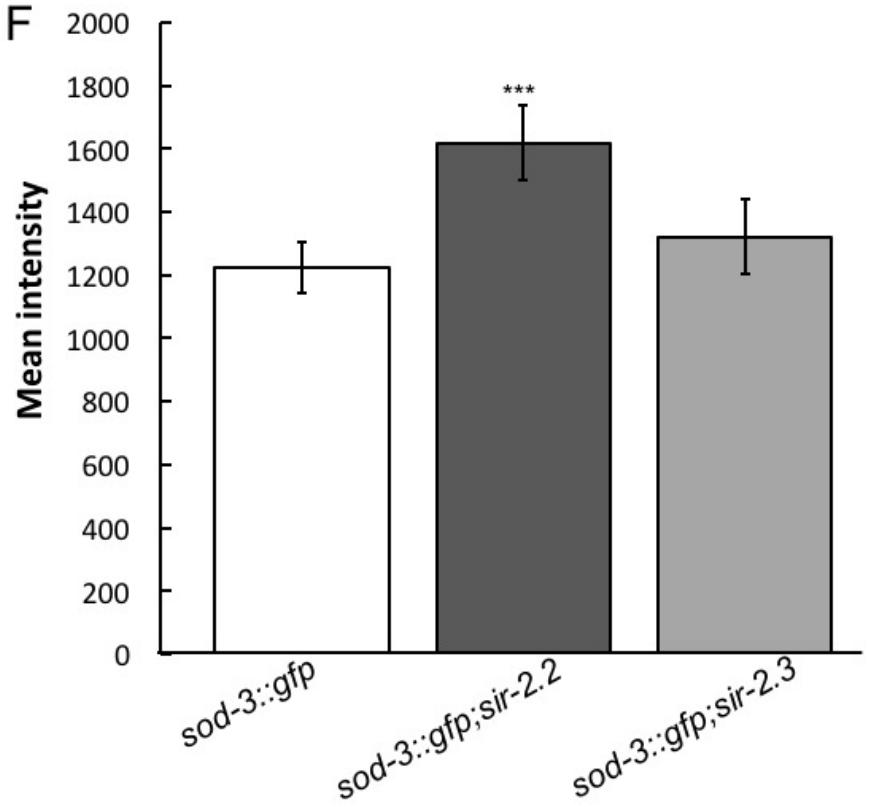
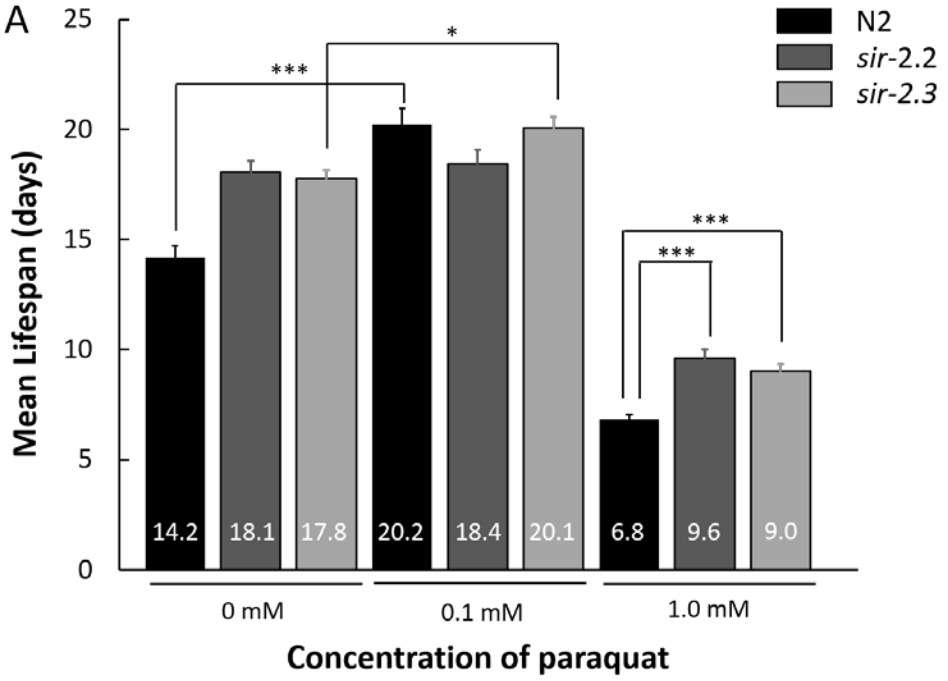


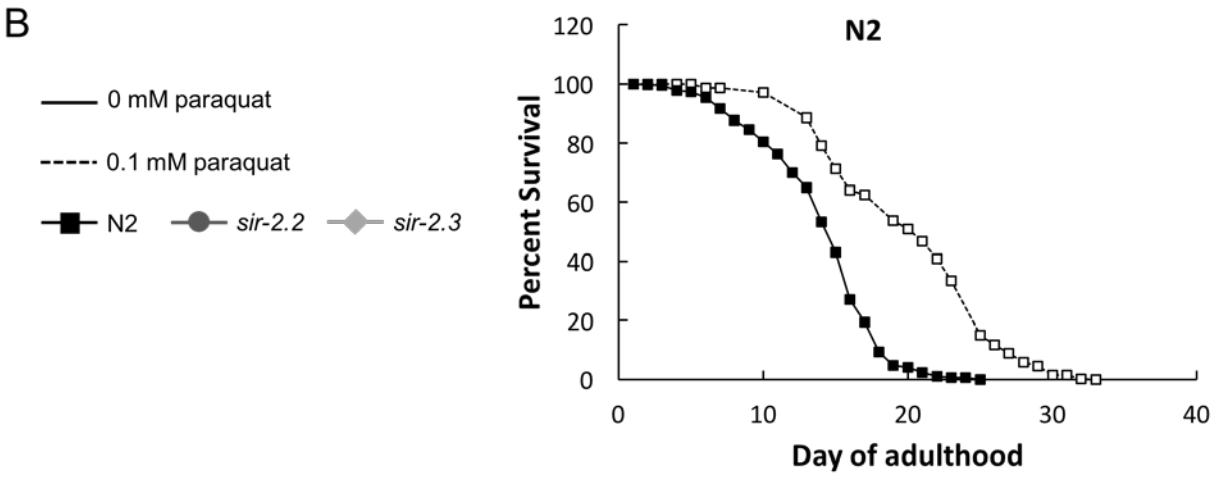

**Figure 6. Expression of stress response genes in *sir-2.2* and *sir-2.3* mutants**

**(A**) Values are relative fold change compared to N2 wild-type, which is represented by the dotted line at 1. Values of *sod-2* and *sod-3* expression in *sir-2.2* and *sir-2.3* mutants are the average of six technical replicates across two biological replicates. *gst-4*, *ctl-1*, and *ctl-2* expression values are preliminary and represent three technical replicates from one biological replicate, * denotes $p<0.5$ using one sample t-test comparing to 1. **(B)** Superoxide detoxifying pathway. Superoxide gets converted to hydrogen peroxide through superoxide dismutase. Hydrogen peroxide can in turn be detoxified by catalase. **(C,D,E)** Images representative of the relative GFP levels in the pharynx of *sod-3::gfp* (C), *sod-3::gfp;sir-2.2* (D), and *sod-3::gfp;sir-2.3* (E). **(F)** Quantification of the mean GFP intensity in the pharynx of *sod-3::gfp*, *sod-3::gfp;sir-2.2*, and *sod-3::gfp;sir-2.3* animals. Sample sizes were 33 to 35 animals, *** $p<0.001$ unpaired t-test.

To continue testing the hypothesis that a hormetic response contributes to the extended lifespan of the mitochondrial sirtuin mutants, I treated the worms with varying concentrations of paraquat, a chemical that induces the formation of superoxide radicals. Low levels of superoxide generators such as 0.1 mM of paraquat extend the lifespan of wild-type worms (Yang and Hekimi 2010). If *sir-2.2* and *sir-2.3* are under more stress than wild-type, I suspected that 0.1

mM of paraquat would not extend their lifespans further. At 0.1 mM of paraquat, N2 and *sir-2.3* mutant worms lived longer than when untreated. N2 worms treated with 0.1 mM paraquat lived 42% longer, extending their lifespan to 20 days from 14 days. *sir-2.3* mutant worms lived 13% longer when treated with 0.1 mM paraquat, extending their lifespan to 20 days from 17.8 days (Figure 7A,B). This lifespan extension was not observed in *sir-2.2* mutants (Figure 7A,B). This supports the hypothesis that *sir-2.2* is more stressed than wild-type and undergoes hormesis, causing a lifespan extension. Although the lifespan of *sir-2.3* was significantly extended, it was extended to a lesser degree than N2 (Figure 7A,B). If the extended lifespan of *sir-2.3* was completely independent of a hormetic mechanism, I would expect the increase in lifespan due to the treatment of low oxidative stress to be similar to that of N2, which was not observed. This suggests that *sir-2.3* is also under more oxidative stress but to a lesser degree than *sir-2.2.* When treated with 1.0 mM of paraquat, *sir-2.2* and *sir-2.3* seemed more resistant than wild-type to this amount of stress, living on average two to three days longer than wild-type (Figure 7A, C) where *sir-2.2* appears to have greater resistance (Figure 7C). Perhaps this resistance is due to increases in oxidative stress response such as the *sod*-3 upregulation in *sir-2.2* (Figure 5A).


A
Mean Lifespan (days)
25
20
15
10
5
0
N2
sir-2.2
sir-2.3
***
*
***
***
14.2
18.1
17.8
20.2
18.4
20.1
6.8
9.6
9.0
0 mM
0.1 mM
1.0 mM
Concentration of paraquat


B
0 mM paraquat
0.1 mM paraquat
N2
sir-2.2
sir-2.3
N2
Percent Survival
120
100
80
60
40
20
0
0
10
20
30
40
Day of adulthood

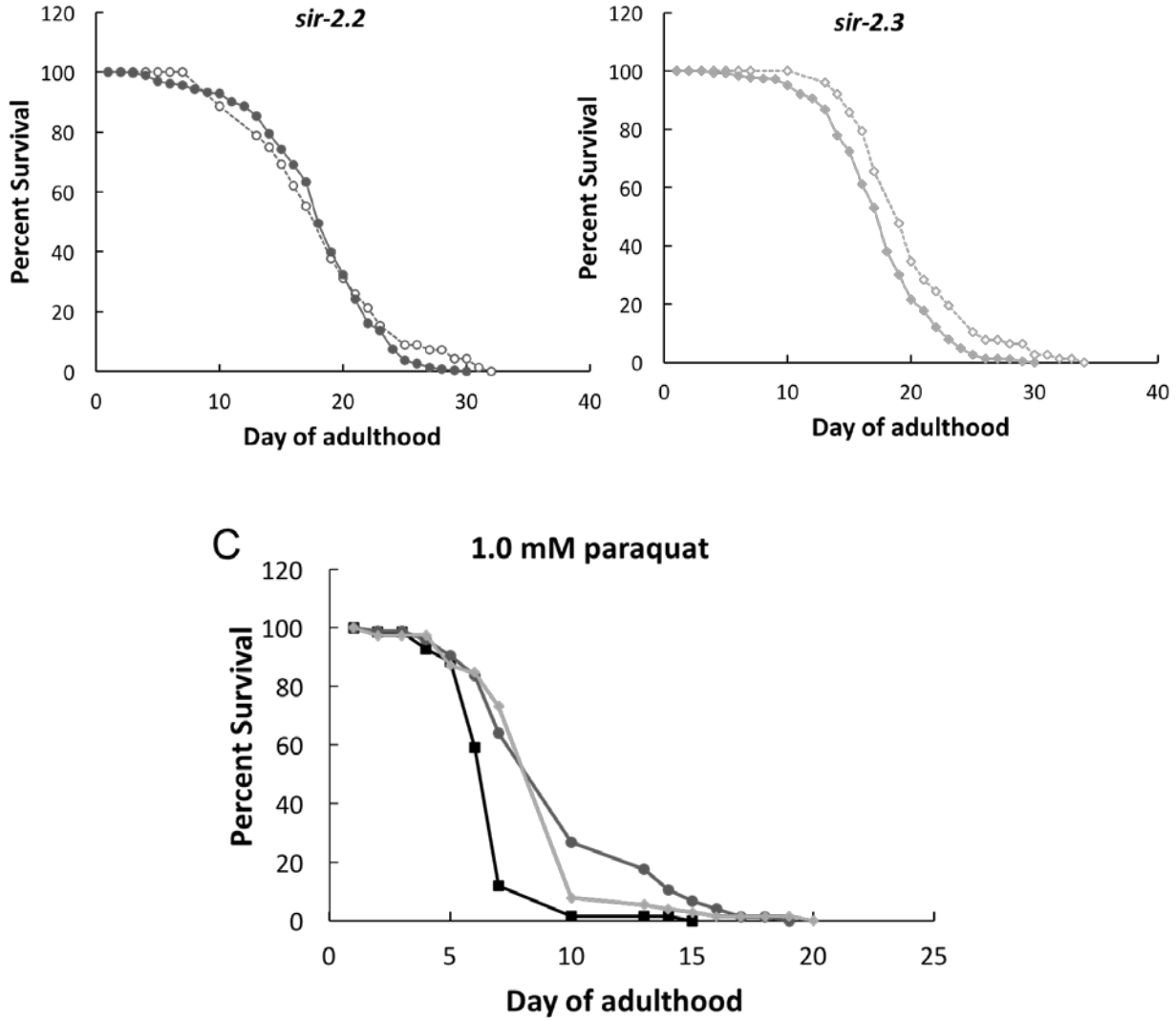
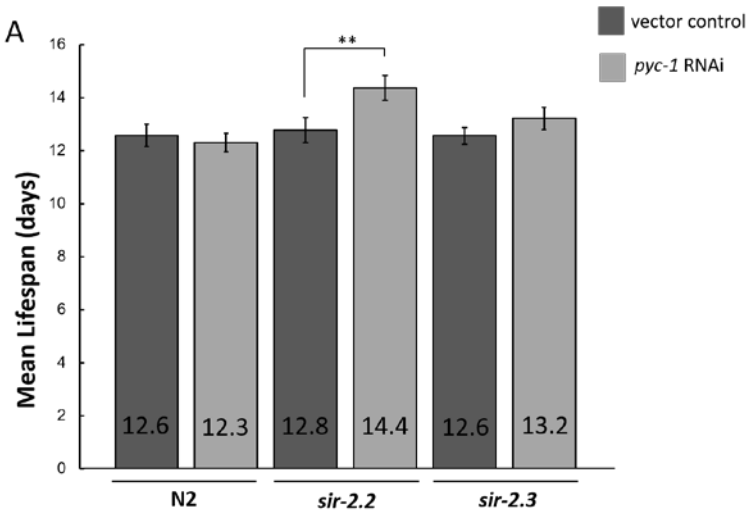


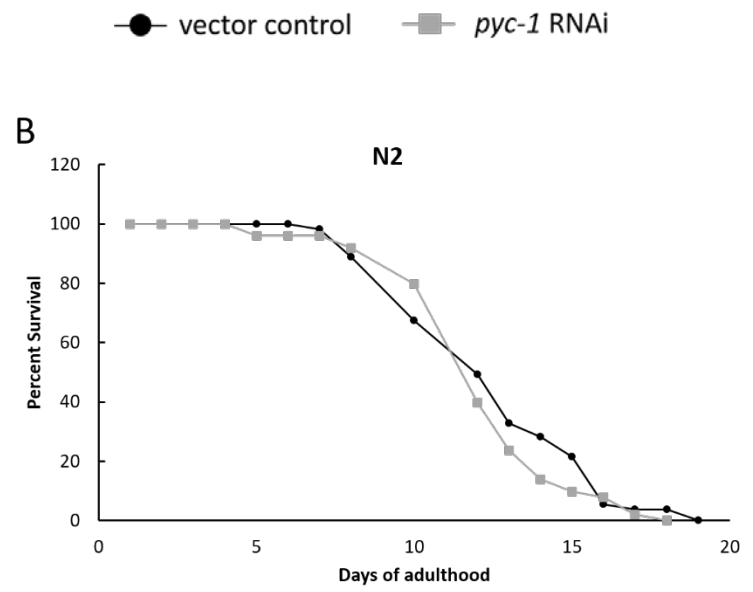

**Figure 7. 0.1 mM paraquat extends lifespans of N2 and *sir-2.3* but not that of *sir-2.2***

Survival of worms was quantified when treated with 1.0, 0.1, and 0 mM of paraquat. **(A)** Mean lifespan of worms treated with 1.0, 0.1 mM or no paraquat. Values are representative of three independent experiments ±SEM, * $p<0.05$, *** $p< 0.001$ unpaired t-test. **(B)** 0.1 mM paraquat extends lifespan in N2 ($p<0.001$, log rank t-test) and *sir-2.3* ($p<0.001$, log rank t-test) and has no effect on *sir-2.2.* **(C)** Survival curve of N2, *sir-2.2,* and *sir-2.3* treated with 1.0 mM paraquat. Both *sir-2.2* and *sir-2.3* show more resistance to this level of oxidative stress compared to N2, $p<0.001$ log rank t-test.

**Knockdown of pyruvate carboxylase *pyc-1* in *sir-2.2* mutants extends lifespan**

SIR-2.2 and SIR-2.3 physically interact with mitochondrial biotin-dependent carboxylases, including pyruvate carboxylase, propionyl-CoA carboxylase, and methylcrotonyl-CoA carboxylase (Wirth et al. 2013). These physical interactions are also conserved in SIRT4, the mammalian ortholog of *sir-2.2* and *sir-2.3* (Wirth et al. 2013). To study the functional contribution of these metabolic enzymes to lifespan extension in *sir-2.2* and *sir-2.3* mutants, we knocked down pyruvate carboxylase *pyc-1* in a *sir-2.2* or *sir-2.3* mutant background and measured lifespan. Pyruvate carboxylase converts pyruvate to oxaloacetate, which replenishes the TCA cycle and acts as the first step in gluconeogenesis. The RNAi knockdown of *pyc-1* did not significantly affect the mean lifespan of N2 or *sir-2.3* but extended the lifespan of *sir-2.2* from 12.8 days to 14.4 days, a 12.5% increase (Figure 8A,C). Although the mean lifespan of *sir-2.3* on vector control and *pyc-1* RNAi was not significantly different, the mean lifespan of *sir-2.3* is increased from 12.6 days to 13.2 days upon *pyc-1* knockdown (Figure 8A), and the survival curve demonstrates some lifespan extending effects (Figure 8D). This suggests that in *sir-2.2* and perhaps *sir-2.3* mutants, *pyc-1* has different negative effects on lifespan and may reveal a regulatory role for SIR-2.2 and SIR-2.3 on PYC-1.


A
vector control
pyc-1 RNAi
**
Mean Lifespan (days)
16
14
12
10
8
6
4
2
0
12.6
12.3
12.8
14.4
12.6
13.2
N2
sir-2.2
sir-2.3


vector control
pyc-1 RNAi
B
N2
Percent Survival
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100
80
60
40
20
0
0
5
10
15
20
Days of adulthood

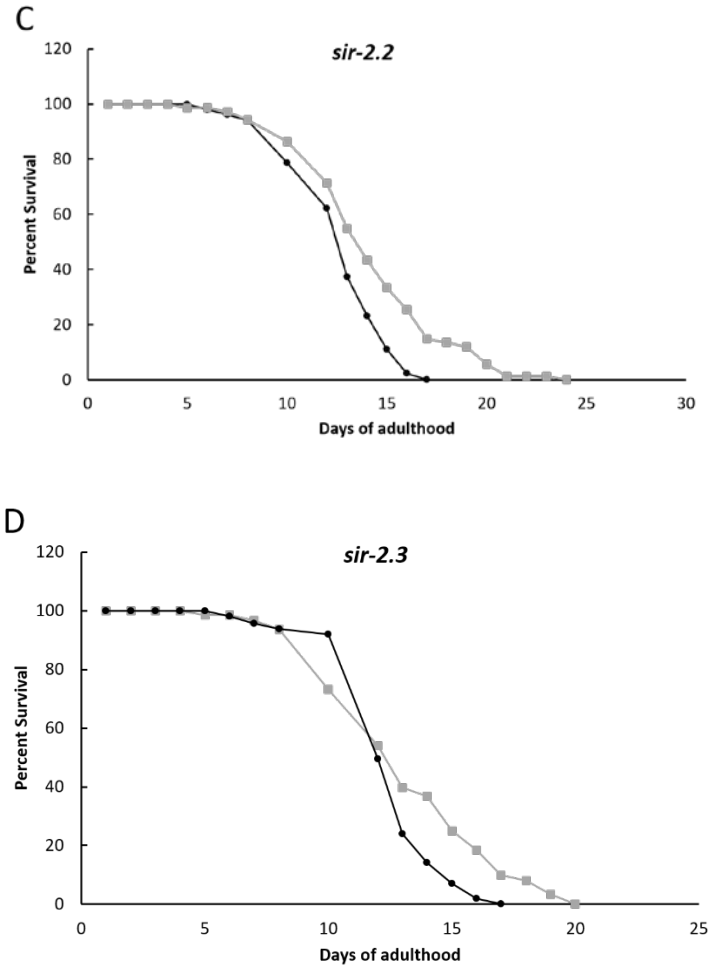
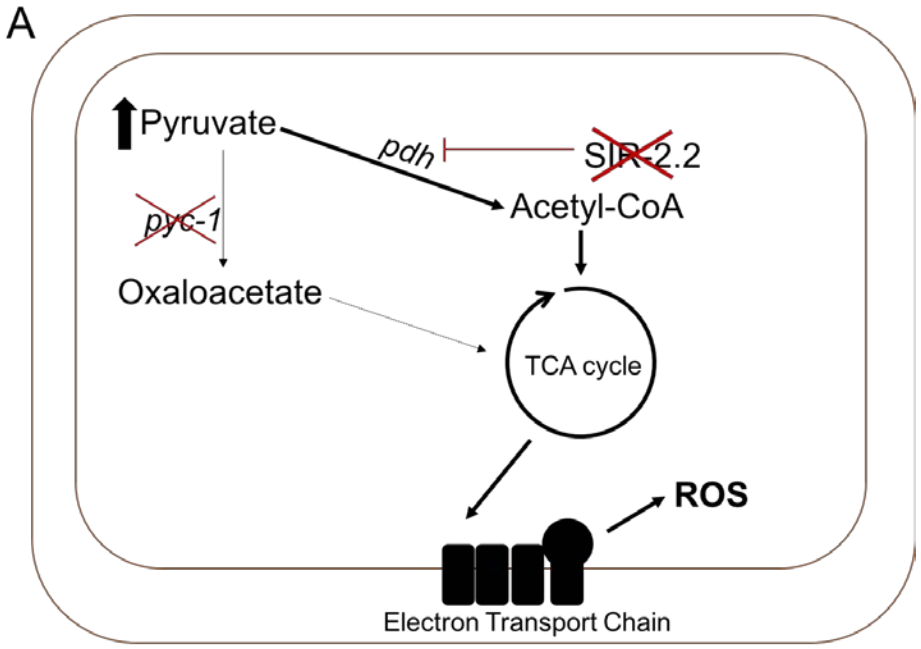


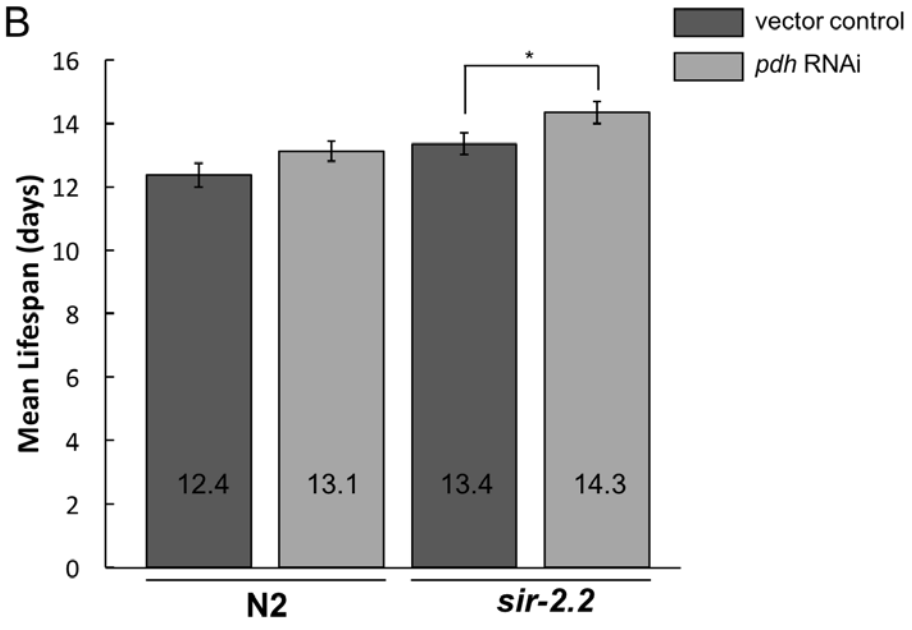

**Figure 8. RNAi knockdown of pyruvate carboxylase *pyc-1* extends lifespan of *sir-2.2***

The lifespans of N2, *sir-2.2*, and *sir-2.3* worms were quantified when fed either empty vector control or *pyc-1* RNAi. **(A)** *pyc-1* knockdown in *sir-2.2* mutants caused a significant increase in lifespan. Mean lifespan values are an average of three independent experiments ±SEM, ** $p<0.1$ using an unpaired t-test. **(B,C,D)** Survival curves showing percent alive per day of adulthood. (B) *pyc-1* knockdown has no effect on N2 lifespan, $p>0.5$, log rank t-test (C) *pyc-1* knockdown appears to have a significant positive effect on lifespan in *sir-2.2*, *** $p<0.001$, log rank t-test (D) Although the p value in the statistical test does not meet the level of significance ($p=0.051$, log rank t-test), the trend in the *pyc-1* knockdown is towards a positive effect on lifespan in *sir-2.3*. This effect is specifically evident in the survival curve.

**Knockdown of pyruvate dehydrogenase extends lifespan of *sir-2.2* mutants**

Mammalian SIRT4 inhibits pyruvate dehydrogenase (PDH), the enzyme that catalyzes the conversion of pyruvate to acetyl-CoA, by hydrolyzing the necessary lipoamide cofactors from the E2 component of the enzyme complex (Mathias et al. 2014). I hypothesized that SIR-2.2 also inhibits PDH. If this were the case, *sir-2.2* mutants would have increased PDH activity, resulting in increased acetyl-CoA levels to drive the TCA cycle and electron transport chain. This may explain the increased *sod-3* expression in *sir-2.2* mutants and their extended lifespans, as increased oxidative phosphorylation extends the lifespan of *C. elegans* by hormesis (Schulz et al. 2007). I also hypothesized that the extended *sir-2.2* lifespan due to knocking down pyruvate carboxylase *pyc-1* was dependent on PDH. Knocking down *pyc-1* would increase pyruvate levels, making more pyruvate available to be converted to acetyl-CoA by PDH (Figure 9A). To test this hypothesis, I knocked down the E2 component of pyruvate dehydrogenase using RNAi and measured lifespan. Unexpectedly, knocking down *pdh* resulted in a 6.7% increase in *sir-2.2* lifespan while having no significant effect on the lifespan of N2 (Figure 9B,C).


A
Pyruvate
pdh
SIR-2.2
pyc-1
Acetyl-CoA
Oxaloacetate
TCA cycle
ROS
Electron Transport Chain


B
vector control
pdh RNAi
*
Mean Lifespan (days)
16
14
12
10
8
6
4
2
0
12.4
13.1
13.4
14.3
N2
sir-2.2

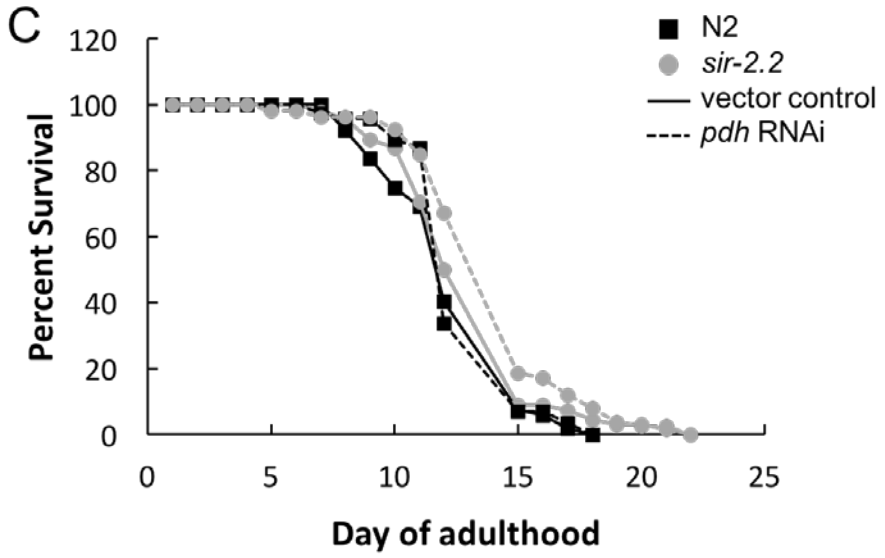



**Figure 9. RNAi knockdown of pyruvate dehydrogenase extends *sir-2.2* lifespan by 6.7%**

**(A)** Hypothesis that SIR-2.2 inhibits pyruvate dehydrogenase and *sir-2.2* mutants would thus have increased oxidative phosphorylation. This could explain why knocking down pyruvate carboxylase increases *sir-2.2* lifespan and why *sir-2.2* mutants upregulate *sod-3*. **(B)** Mean lifespan of N2 wild-type and *sir-2.2* mutants either on vector control of pyruvate dehydrogenase *pdh* RNAi. Values are means of three independent experiments, ±SEM, * $p<0.05$, unpaired t-test. **(C)** Survival curves of N2 and *sir-2.2* on vector control and *pdh* RNAi. *pdh* RNAi significantly extends the lifespan of *sir-2.2* mutants, $p<0.05$, log rank t-test and has no effect on N2 lifespan.

## Metabolic changes are detectable in mitochondrial sirtuin mutants compared to wild type

Because mitochondrial sirtuins regulate metabolism, I used LC-MS to quantify differences in metabolite levels in *sir-2.2* and *sir-2.3* to discover their functions. Because SIR-2.2 and SIR-2.3 interact with mitochondrial biotin-dependent carboxylases that catalyze anaplerotic reactions or synthesize products that, through other reactions, eventually replenish the TCA cycle, I measured key TCA metabolites and biotin levels. I also measured metabolites that are precursors to TCA metabolites and metabolites involved in the malate-aspartate shuttle. I observed that both *sir-2.2* and *sir-2.3* have decreased levels of biotin relative to N2 (Table 1, Figure 10). However, the degree of reduction was significantly different between the

mitochondrial sirtuin mutants where *sir-2.2* showed 85% of wild-type levels and *sir-2.3* showed 71% of wild-type levels (Table 1, Figure 10).

**Table 1. Relative measured metabolite levels in *sir-2.2* and *sir-2.3* mutants are different**

| | *sir-2.2* | *sir-2.3* |
|---|---|---|
| NAD+ | 0.93 | 0.92 |
| Phosphoenolpyruvate | 0.88 | 0.69** |
| Pyruvate | 0.82 | 0.73 |
| α-ketoglutarate | 0.73* | 0.95 |
| Glutamate | 0.92 | 0.74 |
| Malate | 0.78 | 0.74 |
| Oxaloacetate | 1.13 | 0.93 |
| Fumarate | 0.95 | 0.84 |
| Glutamine | 1.03 | 0.73 |
| Citrate/Isocitrate | 0.95 | 1.02 |
| Succinate | 1.25 | 0.85 |
| Biotin | 0.85* | 0.71* |
| Glutathione | 1.31 | 0.96 |
| Aspartate | 0.72 | 0.64* |

Values are the average of three biological replicates which were each run in triplicate and represent relative levels compared to N2. Light red denotes significant decrease in metabolite levels, * $p<0.5$, ** $p<0.1$ using a one sample t-test.

Phosphoenolpyruvate (PEP) and aspartate levels are significantly decreased in *sir-2.3*, 69% and 64% of wild-type, respectively (Table 1, Figure 10). This change was not observed in *sir-2.2* mutant worms. Instead, *sir-2.2* showed a 27% decrease in α-ketoglutarate levels (73% of wild-type) (Table 1, Figure 9). These metabolites whose levels are altered in either *sir-2.2* or *sir-2.3* are involved in glycolysis, the malate-aspartate shuttle, or the TCA cycle (Figure 11). Because sirtuins use NAD+ as a cofactor, I expected NAD+ levels to be higher in the mutants. However, NAD+ levels were the same in the mitochondrial sirtuin mutants and wild-type (Table 1), suggesting that the excess NAD+ that results from loss of *sir-2.2* or *sir-2.3* may be used

elsewhere in the cell or that NAD+ homeostasis is actively controlled in the mutants. Because *sir-2.2* and *sir-2.3* have different variations in metabolite levels compared to wild-type, they may possess different metabolic profiles, suggesting that each have independent functions and may not be redundant in function despite their sequence homology.

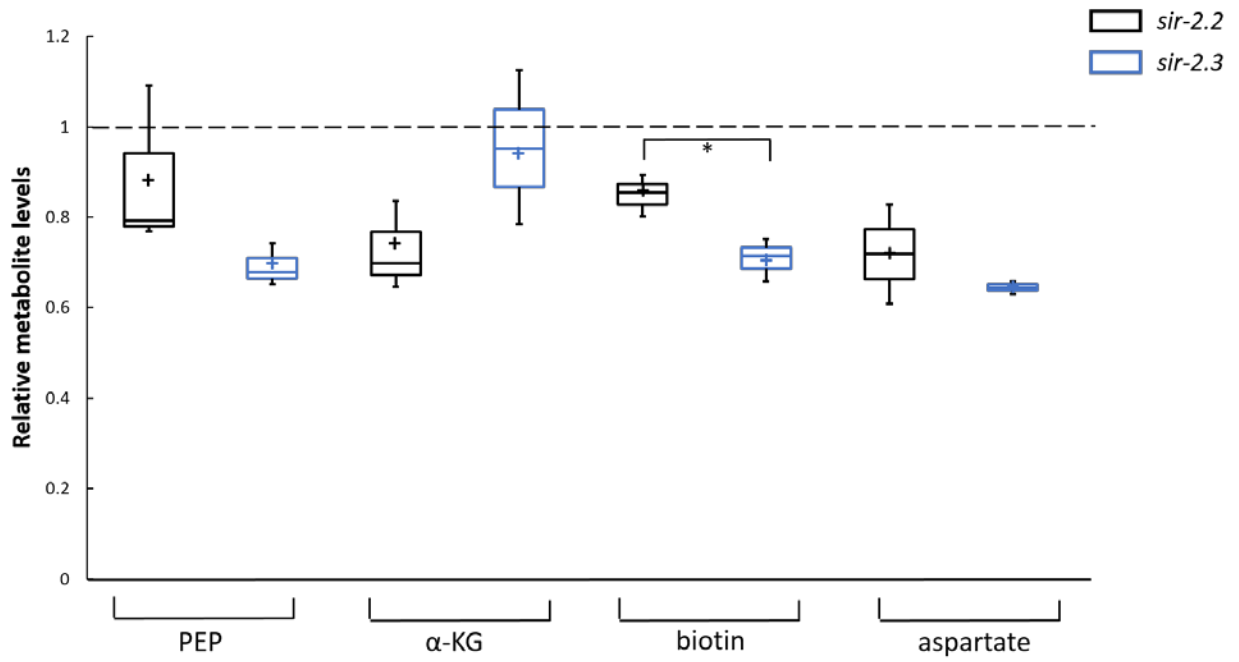



**Figure 10. *sir-2.2* and *sir-2.3* mutants have different metabolite levels compared to wild-type**

Phosphoenolpyruvate (PEP), α-ketoglutarate (α-KG), biotin, and aspartate were significantly different in either *sir-2.2, sir-2.3,* or both. Their relative levels are shown for both mutants. Relative metabolite levels were measured in *sir-2.2* and *sir-2.3* using LC-MS. Values are the means of three biological replicates, each run in triplicate, * $p<0.5$, unpaired t-test

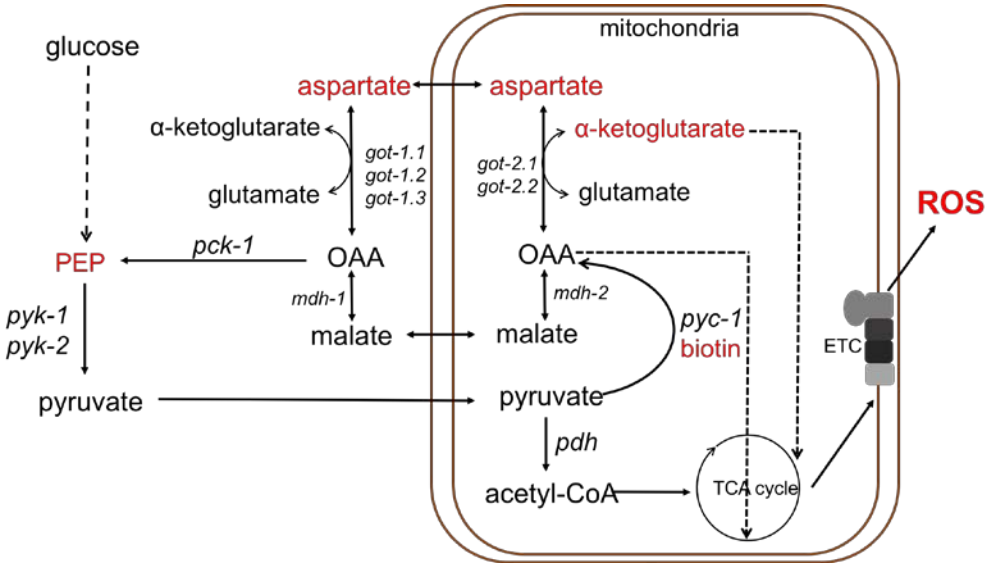



**Figure 11. Metabolites decreased in the mitochondrial sirtuin mutants are involved in glycolysis, the malate-aspartate shuttle, and the TCA cycle**

Red denotes metabolites that are decreased in either *sir-2.2* or *sir-2.3* or in both

## Chapter 3. Discussion

In this study, I uncovered a novel role of the mitochondrial sirtuins *sir-2.2* and *sir-2.3* in lifespan regulation, where removing either *sir-2.2* or *sir-2.3* increases lifespan. Interestingly, *sir-2.1* mutants have also been reported to have an increased lifespan compared to wild-type when fed OP50 *ad libitum* (Moroz et al. 2014). My data emphasizes the importance of the sirtuin family as modulators of metabolism and lifespan and broadens our ability to target them for new therapies, potentially in ways that can improve healthspan.

To discover the function of SIR-2.2 and SIR-2.3, I investigated why their mutants had an increased lifespan by examining changes in food intake, oxidative stress response, and metabolism. *sir-2.2* but not *sir-2.3* had a significant increase in the mitochondrial superoxide dismutase *sod-3*. *sir-2.2* was unresponsive to low levels of oxidative stress, which extends

lifespan in wild-type and extended lifespan in *sir-2.3* by 12.9%. Both *sir-2.2* and *sir-2.3* were more stress resistant than wild-type when treated with 1.0 mM paraquat. It would be necessary to remove *sod-3* in *sir-2.2* mutants and measure lifespan to test whether the upregulation of *sod-3* is required for their lifespan extension. Curiously, preliminary data shows a two-fold decrease in both cytoplasmic and peroxisomal catalase in *sir-2.2* and *sir-2.3*. Superoxide dismutases convert superoxide to hydrogen peroxide which in turn gets detoxified to water by catalase. It would be interesting to see if hydrogen peroxide accumulates in the mitochondrial sirtuin mutants because of their decreased catalase expression. More likely, *sir-2.2* and *sir-2.3* use another pathway to remove hydrogen peroxide if they have less catalase expression. Glutathione peroxidase converts hydrogen peroxide to water by oxidizing reduced glutathione (GSH) to oxidized glutathione (GSSG). To test whether *sir-2.2* and *sir-2.3* use this pathway to remove hydrogen peroxide, I could measure the expression of *gpx-4*, the glutathione peroxidase in *C. elegans*. If this is the case, it would be worth investigating why the mitochondrial sirtuin mutants show a bias towards a certain detoxification pathway.

Oxidative stress and metabolism are linked through the electron transport chain. A shift towards oxidative metabolism leads to increased output of reactive oxygen species, primarily superoxide, at the electron transport chain (Murphy 2009). Increase *sod-3* in *sir-2.2* mutants may in part be due to an increased oxidative metabolism, suggesting that SIR-2.2 is a negative regulator of oxidative phosphorylation. Interestingly, *sir-2.3* mutants do not display as high of a response to superoxide.

While performing RNAi lifespan analyses, worms were fed *E. coli* strain HT115 rather than OP50. On HT115, *sir-2.2* and *sir-2.3* mutants no longer had the increased lifespan they had on OP50. The lifespan of *C. elegans* is regulated by their ability to respond to changes in diet

(Pang and Curran 2014). When fed either OP50 or HT115, wild-type has similar lifespans, observed in this study and published elsewhere (Brooks, Liang, and Watts 2009). This ability to adapt to different diets seems to involve *sir-2.2* and *sir-2.3*, as *sir-2.2* and *sir-2.3* mutant worms do not have the same lifespan on either type of food, unlike wild-type.

*pyc-1* knockdown extends *sir-2.2* lifespan and shows a similar trend in *sir-2.3* mutants. Knocking down pyruvate carboxylase should result in increased pyruvate levels. Previous studies have shown that pyruvate supplementation increases lifespan in wild-type, an effect dependent on pyruvate dehydrogenase (Mouchiroud et al. 2011). However, initial experiments do not support this in *sir-2.2* mutants. Knocking down pyruvate dehydrogenase in *sir-2.2* mutants do not abrogate their extended lifespan and instead increases it further. It would be worth looking at other pathways by which pyruvate is processed and their roles in lifespan.

Interestingly, both *sir-2.2* and *sir-2.3* mutants shared a decrease in biotin, a required cofactor for pyruvate carboxylase. SIR-2.2 and SIR-2.3 physically interact with mitochondrial biotin-dependent carboxylases like pyruvate carboxylase (Wirth et al. 2013). If these mutants have decreased biotin levels, the activity of these biotin-dependent carboxylases could be suppressed. It would be necessary to study the relationship between the other biotin-dependent carboxylases propionyl-CoA carboxylase and methylcrotonyl-CoA carboxylase with the mitochondrial sirtuins to reveal how SIR-2.2 and SIR-2.3 may regulate these enzymes to affect lifespan.

Although the exact mechanisms for the lifespan extension seen in *sir-2.2* and *sir-2.3* worms still require investigation, our data suggests that the mitochondrial sirtuin mutants use distinct mechanisms for their lifespan extension despite having 75.3% sequence homology and being adjacent on the same chromosome (Wirth et al. 2013). Although sharing some similarities,

our data suggest that SIR-2.2 and SIR-2.3 are not redundant in function. Although both had decreased biotin levels, the amount of change in biotin is significantly different between the two mutants. Only *sir-2.2* shows a significant upregulation of the superoxide dismutase *sod-3*. Although both are resistant to 1.0 mM paraquat, *sir-2.2* seems more resistant. LC-MS analysis reveals different variations in metabolite levels, where *sir-2.2* shows decreased α-ketoglutarate levels whereas *sir-2.3* shows decreased phosphoenolpyruvate and aspartate levels. The metabolomics data is most telling of the distinct metabolic functions each may possess. A principle component analysis for each mutant should be done to test whether *sir-2.2* and *sir-2.3* have different metabolic profiles.

## Chapter 4. Materials and Methods

### *Nematode strains and maintenance*

*C. elegans* strains were maintained using standard methods at 20°C on *E. coli* OP50 (Brenner 1974). The following strains were used: Bristol N2, wild type; RB654 *sir-2.3(ok444)*; CF1553 *muIs84*[(*pAD476*) *sod-3p::*GFP + *rol-6*]; CF1038 *daf-16(mu86)* were provided by *Caenorhabditis* Genetics Center (CGC). *sir-2.2(tm2673)* was provided by the Mitani lab through the National Bio-Resource Project of the MEXT, Japan. *sir-2.2(tm2673)* and *sir-2.3(ok444)* were each outcrossed three times into the wild-type N2 background that was used for all experiments.

***Lifespan Analysis***

Lifespan assays were conducted at 20°C on standard NGM plates with 400 µl of *E. coli* OP50 and were replicated in at least three independent experiments. Animals were synchronized using a timed egg lay or an egg preparation. 30 L4 animals were picked onto each OP50 plates at the start of the assay and moved to new plates every day. To assess survival, worms were prodded with a platinum wire every day and scored as dead if non-responsive. Worms with internal hatching or an "exploded vulva" phenotype were censored.

For paraquat lifespan analysis, 200 µl of paraquat (methyl viologen dichloride hydrate 98% Sigma) diluted in water was added to NGM plates spotted with 400 µl OP50 for a final concentration of either 1.0 mM or 0.1 mM paraquat. 200 µl of water was added to control plates. 30 synchronized L4 worms were picked onto plates at the start of the assay and transferred to new plates every day. Results represent an average of three independent experiments.

For RNAi lifespan analysis, worms were synchronized using an egg preparation, which was placed on RNAi agar plates (NGM agar with 1 mM IPTG, 25 µg carbenicillin) spotted with 500 µl of RNAi culture grown overnight in LB with 50 µg/ml ampicillin and 12 µg/ml tetracycline. Egg preparation was added so that each plate contained around thirty worms. Survival was quantified starting at L4 stage, where worms were transferred to fresh plates every day. Results represent an average of three independent experiments.

***Pharyngeal Pumping***

Pharyngeal pumping was measured in day one adults fed *ad libitum* or five minutes post six hour fasting period on OP50 as described (Lemieux et al. 2015). All worms were grown on OP50 at 20°C. Pumping rates were measured in ten second intervals on a Nikon SMZ1500 stereoscope.

***Quantitative RT-PCR***

RNA was isolated from mixed stage worms using TRIZOL reagent (Invitrogen). 1 µg of RNA was converted to cDNA using the qScript cDNA Synthesis Kit (Quanta Biosciences). cDNA was diluted 1:10 and used for quantitative PCR using SYBR Green and Applied Biosciences RT-PCR machine. A combination of three control primer sets (*cdc-42, tba-1, and pmp-3*) were used. Results represent the average of two independent biological samples unless otherwise denoted, each of which was amplified in triplicate.

**sod-3::GFP *expression quantification***

Day 1 adult animals were placed on unspotted NGM plates and imaged on a Nikon SMZ1500 stereoscope. Images were collected and analyzed using ImageJ to measure mean GFP intensity in the pharynx of each animal.

***Metabolomics***

LC-MS metabolomics analysis was done with the Metabolomics Core Facility at Penn State. ~50 µL of worms were collected in dd$H_2O$, flash frozen in liquid nitrogen and stored at -80°C. 15 µL samples were extracted in 1 mL of 3:3:2 acetonitrile:isopropanol:H2O with 1 µM chlorpropamide as internal standard. Samples were homogenized using a Precellys™ 24 homogenizer. Extracts from samples were dried under vacuum, resuspended in HPLC Optima Water (Thermo Scientific) and divided into two fractions, one for LC-MS and one for BCA protein analysis. Samples were analyzed by LC-MS using a modified version of an ion pairing reversed phase negative ion electrospray ionization method(Lu et al. 2010). Samples were separated on a Supelco (Bellefonte, PA) Titan C18 column (100 x 2.1 mm 1.9 µm particle size) using a water-methanol gradient with tributylamine added to the aqueous mobile phase. The LC-MS platform consisted of Dionex Ultimate 3000 quaternary HPLC pump, 3000 column compartment, 3000

autosampler, and an Exactive plus orbitrap mass spectrometer controlled by Xcalibur 2.2 software (all from ThermoFisher Scientific, San Jose, CA). The HPLC column was maintained at 30°C and a flow rate of 200 uL/min. Solvent A was 3% aqueous methanol with 10 mM tributylamine and 15 mM acetic acid; solvent B was methanol. The gradient was 0 min., 0% B; 5 min., 20% B; 7.5 min., 20% B; 13 min., 55% B; 15.5 min., 95% B, 18.5 min., 95% B; 19 min., 0% B; 25 min 0% B. The orbitrap was operated in negative ion mode at maximum resolution (140,000) and scanned from m/z 85 to m/z 1000. Metabolite levels were corrected to protein concentrations determined by BCA assay (Thermo Fisher).

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# ACADEMIC VITA

SARAH CHANG
sqc5459@psu.edu | 814-321-1678

## EDUCATION

**Pennsylvania State University, Schreyer Honors College**
*2012 – 2016*
B.S. and Honors in Biochemistry and Molecular Biology

## RESEARCH EXPERIENCE

**Undergraduate Researcher, Penn State University** *Sept. 2012 - Present*
Advisor: Dr. Wendy Hanna-Rose

- Investigating the role of mitochondrial sirtuins in regulating metabolic processes and aging using *C. elegans* genetic and metabolomic approaches

**Research Intern, Icahn School of Medicine at Mount Sinai** *June 2014 – Aug. 2014*
Advisor: Dr. Ross Cagan

- Conducted high-throughput drug screens and cell signaling studies to identify potential therapeutics for Ret-fusion dependent papillary thyroid cancer using *Drosophila melanogaster*

**Research Intern, CHOP Research Institute** *June 2013 – Aug. 2013*
Advisor: Dr. Adam Resnick

- Developed an analogue sensitive inhibitor approach for pharmacological targeting of Inositol Polyphosphate Multikinase to uncover its role in regulating HDAC activity and reveal novel opportunities for pediatric cancer therapies

## PRESENTATIONS

**San Antonio Nathan Shock Center Symposium on Metabolism and Aging** *Oct. 2015*

- One of six travel award recipients among 45 applicants

**Effective Teaching and Learning in Life Sciences Higher Education Pedagogy** *July 2015*

"Learning Assistants: a tool for improved student learning"

- Selected speaker for the workshop at Fudan University

**20th International *C. elegans* Meeting** *June 2015*

"Mitochondrial Sirtuins and Oxidative Stress Response in *C. elegans*" Poster

- GSA Undergraduate Travel Award Recipient

**Mid-Atlantic Society of Developmental Biology Regional Meeting** *March 2015*

"Mitochondrial Sirtuins and Oxidative Stress Response in *C. elegans*" Poster

- Award for Presentation Excellence

## LEADERSHIP EXPERIENCE

**Learning Assistant, Penn State University**
*Aug. 2013 – Present*

- Initiating peer-facilitated active learning in science courses to improve science education

**Penn State Science LionPride Ambassador**
*Sept. 2012 – Present* | Vice President, Recruitment Director

- Forming the main liaison between the students of Penn State's Eberly College of Science with prospective students, community, and alumni
- Leading a team of student ambassadors to give tours and presentations on behalf of the college of science

**Mentor for Penn State Science Outreach Programs**
*Sept. 2012 – Present*

- Serving as a frequent mentor for Penn State Science's various community outreach programs, including STEM Career Days for Young Women, Exploration-U, and THON

**Training Volunteer Leader, Mount Nittany Medical Center Emergency Department**
*July 2012 – Sept. 2013*

- Volunteered 135 hours in the E.R. and Ambulatory Services
- Training leader for new E.D. volunteers

**Piano Teacher**
*Sept. 2012 – Present*

- Started own piano studio to teach and share piano music

## HONORS AND AWARDS

**Ronald Venezie Scholarship in Science for Honors Education,** 2015

**Diana M. Hutchings Science Scholarship,** 2015

**Erickson Discovery Grant Recipient,** 2015

**Braddock Scholarship,** 2012

**George S. Wykoff Scholarship,** 2012

**Academic Excellence Scholarship,** 2012