Insights into the Genetics of Aging in *Drosophila melanogaster*

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Jason Wood, **Rachel Whitaker**, Stephen Helfand. Methods to Generate Sirtuin Biology Tools. Chapter In: Methods in Molecular Biology: Sirtuins. Matthew Hirschey et al Humana Press, USA

Comparative transcriptional pathway bioinformatic analysis of dietary restriction, Sir2, p53 and resveratrol life span extension in *Drosophila.* Antosh M, **WhitakerR**, Kroll A, Hosier S, Chang C, Bauer J, Cooper L, Neretti N, Helfand SL. Cell Cycle. 2011 Mar 15;10(6):904-11.

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CONFERENCE ABSTRACTS

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Abstract

Studying the biological mechanisms of the aging process in the fruit fly *Drosophila melanogaster* has been crucial to the advancement of the field. In particular, the study of the Sirtuin protein dSir2 in Drosophila has been hotly debated, as there have been several contrasting reports over whether dSir2 extends life span in the fly. In this dissertation, the measurement of life span in flies expressing many different doses of dSir2 revealed that the ability of dSir2 to extend life span is dependent on the dosage, resolving the current conflict in the field over whether dSir2 extends life span in flies. Additionally, Drosophila is a particularly good system for studying transcriptional changes that occur during dietary restriction (DR), an intervention that extends life span in many model systems. Transcriptional analysis has been important for identifying new targets of aging regulation, but has been limited in the ability to differentiate between genes that are critical to the decreased mortality and those that are simply associated with some of the other effects of DR. Through a unique time-course microarray transcriptome analysis, paired with a technique of switching the flies to a lower calorie diet, new insights into molecular pathways involved in the longevity response to DR are identified in this dissertation work. In the research presented here, problems in the study of Drosophila aging were addressed through careful and rational experimental design and analysis.

CHAPTER ONE: INTRODUCTION

Dietary Restriction Extends Life Span Across Eukaryotic Species

The process of restricting the dietary intake of an organism has been a known mechanism of extending life span for decades, having first been reported as early as 1935 (Maynakd, 1935) and confirmed in mice in 1986 (Weindruch, Walford, Fligiel, & Guthrie, 1986), when the effect of many different diets on growth, healthspan, and longevity was reported. Since then, dietary restriction (DR) has been shown to increase life span almost universally, with longevity effects seen in organisms as diverse as yeast, nematode worm, fruit flies, spiders, zebrafish, mice, and rats. The apparent universality in the relationship between longevity and diet seems to indicate that there may be a conserved genetic or biochemical mechanism responsible for extending life span when food is scarce (but not so scarce as to push the organism into starvation).

In agreement with this notion, many molecular pathways have known involvement in the caloric restriction response and perturbations in these pathways have themselves been shown to increase life span (Ribarič, 2012). Biochemical pathways involved in metabolism, especially insulin signaling, are known to mediate the response to caloric restriction (Anisimov, 2003; Tatar, Bartke, & Antebi, 2003; Wilkinson, Taylor, & Dillin, 2012). Additionally, modifications in protein translation, transcription factors, mitochondrial physiology, and epigenetic gene regulation have all been shown to mediate life span, and some (but importantly, not all) of these mechanisms have known involvement in dietary restriction(Civitarese et al., 2007; Jacinto & Hall, 2003; Kapahi et al., 2004; Mathers, Strathdee, & Relton, n.d.; McKay & Mathers, 2011; Yen & Klionsky, 2008).

Indy and DR

Indy (I'm Not Dead Yet) protein is a transmembrane transporter found at the plasma membrane, and is thought to transport Kreb's cycle intermediates (specifically, citrate and succinate) into the cell (Knauf et al., 2006). A genetic screen found several mutants with a P-element that mediated disruption of the *Indy* genetic locus extended life span (B. Rogina, 2000). Importantly, life span extension in *Indy* mutants does not appear to be additive with DR, and *Indy* mutants have many of the phenotypes and biomarkers of DR flies. For example, *Indy* mutants have decreased levels of dilp-2,3, and 5, more nuclear FOXO localization, and are sensitive to starvation, all phenotypes which are shared with DR flies. In addition, *Indy* mutants on a high calorie diet are unable to gain weight like their control counterparts. DR flies show reduced levels of *Indy* expression (Wang et al., 2009). Deletion of the mouse *Indy* homologue, *SLC13a5,* leads to a resistance to many of the pathological symptoms of a high fat diet with age, including insulin resistance, and shares many metabolic, physiological, and transcriptional characteristics with DR (Birkenfeld, Lee, & Guebre-Egziabher, 2011). Together, these data indicate that Indy-mediated life span extension, not only in flies but in mice as well, may operate through the same mechanism as DR (Shulman & Helfand, 2011).

TOR and DR

The Target of Rapamycin (TOR) complex is a protein in the phosphatidylinositol kinase related protein kinase family, and is thought to have activity as a serine/threonine

kinase and play a role in mediating cell growth as well as cell metabolism. Through various protein-protein complexes and pathways, TOR pathway activity leads to ribosome biogenesis through its activation of S6K1 and the subsequent increased translation of ribosomal proteins. Additionally, TOR pathway activity increases mRNA cap-dependent translation through its inhibition of 4E-BP, an inhibitor of eIF4E. eIF4E inhibits eIF4F's ability to bind to caps and thus increases translation. (Jacinto & Hall, 2003). Downregulation of the TOR pathway via the use of the chemical inhibitor Rapamycin, for which the complex was named, extends life span (Bjedov et al., 2010). Genetic interventions such as the over-expression of 4E-BP (Zid et al., 2009) or mutations in S6K1 (Kapahi et al., 2004) also extend life span . Additionally, 4E-BP is upregulated under DR and is required for the DR response to life span (Zid et al., 2009). Interestingly, life span extension via genetic or pharmacological reduction of the TOR signaling pathway is additive with DR by some accounts (Bjedov et al., 2010) but not by others (Hansen et al., 2007; Kaeberlein, Powers, et al., 2005). These data seem to imply that while the TOR pathway is an important life span-modulation pathway, it is unclear what aspects of the pathway intersect with DR.

Insulin/Insulin-Like Signaling and DR

The Insulin Signaling pathway is a non-autonomous nutrient sensing response that coordinates food intake with cellular functions such as glucose and lipid metabolism. In C. elegans, the insulin signaling pathway and its role in life span has been extensively studied. Briefly, Daf-2, an insulin receptor (and the only one in worms), is activated when insulin-like peptides bind at the cellular membrane. This results in the phosphorylation

and activation of Age-1 (also known as PI3K), which activates several downstream kinases, including PDK-1, SGK-1, AKT-1, and AKT-2. In turn, these kinases phosphorylate DAF-16, also known as FOXO in other organisms, and this phosphorylation prevents DAF-16 from entering the nucleus. Mutations in this pathway (most notably, mutations in daf-2, age-1, pdk-1, sgk-1, akt-1, or akt-2) result in extended life span. Additionally, reduced signaling of this pathway results in the translocation of DAF-16 from the cytoplasm into the nucleus, where it acts as a transcriptional activator, regulating transcription of genes involved in several cellular processes such as metabolism, immune system, and stress response (Wilkinson et al., 2012). However, the overlap of this pathway with dietary restriction is not entirely understood in worms.

The mechanism of lifespan extension via reduced insulin-like signaling (ILS) is still an important topic for further research, and in flies, the insulin signaling pathway is similar to that in worms. Drosophila has seven insulin-like peptides (dilp), and they have differing tissue/developmental expression patterns. Several of the dilps are known to be involved in the longevity response to CR and/or ILS (Bai, Kang, & Tatar, 2012). While it was thought that reducing the ILS pathway would be a likely mechanism for dietary restriction's effect on life span, life span extension by DR has been shown to be independent of ILS in Drosophila, both at the level of insulin-like peptide secretion (Min, Yamamoto, Buch, Pankratz, & Tatar, 2008) and FOXO upregulation (Giannakou, Goss, & Partridge, 2008). Therefore, while DR is known to affect the ILS pathway in Drosophila, it is not necessary for the DR response.

Sir2 and DR

Another molecular pathway that has the potential to link DR with life span extension is that of Sir2, or Silent Information Regulator 2. While the function and importance of this protein will be explored in more detail later in this chapter, it is important to note as a potential link between the mechanisms of dietary restriction and life span, especially in Drosophila. Clearly, Insulin signaling, TOR, and Sir2 are all genetic pathways with the potential to connect the mechanisms that DR may use to extend life span, but it is crucial to note that these pathways are not distinct from one another, and have many cross-roads amongst them. It is primarily due to the very complicated interaction network of these pathways that the exact mechanisms by which DR extends life span have been so difficult to identify. Classical genetic screens and mutational analysis have enabled the study of these many pathways, but a more multi-dimensional approach to studying dietary restriction is likely to be more informative about the multiple pathways that are changed with DR.

New Insights into Dietary Restriction: The Advantages of Transcriptomic Approaches

Where the mechanisms into DR are still unclear, a relatively recent technology has allowed a new system for uncovering genetic pathways that may be key players in the process. While the molecular study of an individual gene and its effect on DR and life span remains the only way to definitively determine whether it is involved in the DR response, studying the changes in gene expression between DR and control conditions can reveal new candidate genes and pathways that might be involved.

An example of the power of this approach can be seen in the identification of the gene *takeout* as a regulator of life span in *Drosophila*. In this study, microarray analysis was used to compare genome-wide expression profiles of three conditions: DR, increased dSir2 expression, and a life span extending Dmp53 mutation. By narrowing the field of interest to only the genes that were significantly altered in all three conditions, a new gene, *takeout*, was identified as a possible target for further study into its role in life span regulation. When *takeout* expression was experimentally increased, life span was extended, confirming the power of using comparative transcriptomics to identify potential genetic regulators of life span (J. Bauer et al., 2010).

Importantly, changes in mRNA expression do not necessarily reflect changes in protein translation or activity. However, the ability to compare genome-wide changes in gene expression during an aging intervention such as DR provides a powerful tool for understanding the cellular changes that occur during these interventions. Another caveat to the use of microarrays to understand changes that occur with DR is that not all of the changes observed will correlate with longevity. Some of the transcriptional changes will be responsive to the diet without having any role in determining life span, but the method used in (J. Bauer et al., 2010) shows the importance and potential of identifying common transcriptional signatures of longevity.

In order to identify some of the common transcriptional changes that occur with various longevity-conferring interventions, Antosh et al compared microarray expression datasets between DR, dSir2 over-expression, dmp53 mutation, and resveratrol treatment (Antosh et al., 2011). Additionally, Antosh et al compared DR signatures between two different laboratory strains of Drosophila as an added method of reducing the “noise”

associated with strain-specific responses to DR. The Kyoto Encyclopedia of Genes and Genomes (KEGG) contains a useful collection of gene lists arranged by their molecular interactions within the cell, allowing analysis of the change in entire molecular pathways in transcriptome analyses. Once the number of KEGG pathways changed with DR was recalculated using the overlap of both *Drosophila* strains, a total of 54 KEGG pathways were identified as changed with DR. Most of these pathways were shared with the dSir2 over expressing flies and with DN-Dmp53 expressing flies. Pathways in oxidative phosphorylation, metabolism, circadian rhythm, fatty acid biosynthesis, folate biosynthesis, proteasome, and several nucleotide repair pathways were changed in DR conditions.

The next step in narrowing the field of pathways significantly altered in DR was a comparison between whole-body transcription changes and changes in the head/thorax only. Interestingly, after comparison of whole body with head/thorax, many of the nucleotide repair, proteasome, and fatty acid elongation pathways were found to be specific to whole-animal studies. Some pathways, such as oxidative phosphorylation, actually changed the direction of their enrichment with DR between whole body and head/thorax DR flies.

Finally, transcription profiles of DR flies were compared with flies fed resveratrol (Antosh et al., 2011), an intervention known to extend life span, likely through its activation of the Sir2 protein (J. G. Wood et al., 2004). Again, a significant overlap of individual changed genes and KEGG gene pathways was observed. However, the number of individual genes that were changed with DR and with resveratrol treatment was relatively low, compared with the number of enriched KEGG pathways obtained using

GSEA analysis, an approach whereby user-defined sets of genes (rather than those in the KEGG Pathway database) can be analyzed. This result indicates that a pathway-centric analysis approach may be more useful than looking at the individual changes in gene expression. This is likely due to an enhanced ability to detect smaller differences in expression over a large number of genes, rather than the gene-centric analysis that looks for large differences in expression, resulting in a smaller subset of genes.

The power of using these combined transcriptomic approaches lies in the fact that they allow for the identification of a common longevity "signature" between those conditions. However, these techniques still do not rule out the possibility that some of the gene expression pathways that change in response to DR or other life span extending conditions may not be involved in the increase in life span and decrease in mortality seen with DR. In order to identify pathways that are important for the changes in life span and mortality specifically, other technical solutions are necessary.

Sir2 and Life Span

Sir2 as a histone deacetylase, and interactions with Rpd3

Sirtuins are a class of proteins originally discovered in the yeast S. cerevisiae as being involved in yeast mating type determination. Yeast mating types (a or α) are determined by the transcriptional regulation of a particular genetic locus, and four genes called Silent Information Regulator (SIR) 1-4 are necessary for repression of these sites (Ivy, Klar, & Hicks, 1986; Rine & Herskowitz, 1987; Shore, Squire, Nasmyth, Smith, & Limited, 1984). Yeast ribosomal RNA genes are known to recombine in

extrachromosomal circles as the mother cell ages, and rDNA circle accumulation was found to be a cause of aging in yeast (Defossez, Park, & Guarente, 1998; Sinclair & Guarente, 1997). It was hypothesized that as rDNA circles accumulate with age, SIR2 was necessary to repress their further replication, resulting in a titration of SIR2 in the nucleus away from other important loci where it was required for gene repression, and ultimately contributing to aging in the mother cell. In agreement with this hypothesis, increased dosage of SIR2 in extends yeast replicative life span, or the number of times a mother cell can produce a daughter cell before death (Kaeberlein, Mcvey, & Guarente, 1999).

Since then, SIR2 and its homologues in worms, flies, fish, and mammals has been extensively studied, and has involvement in many cellular processes. The first known function of Sir2 was its activity in repressing chromatin structure so that gene expression (specifically, at telomeres, rDNA circles, and mating type loci in yeast) was silenced (Defossez et al., 1998; Fritze, Verschueren, Strich, & Easton Esposito, 1997; Kaeberlein et al., 1999). Chemical modifications on chromatin impact the density of nucleosomes and ultimately, the transcriptional accesibility of the genes in the area being modified (Larson et al., 2012). Some modifications result in a more densely packed chromatin state, and along with other cofactors and modifications, can repress the transcriptional activity of a genomic location. Other modifications, such as acetylation of some histone lysine residues, cause a euchromatic chromatin state, leading to enhanced accessibility of the DNA to transcriptional machinery and increased transcription. Histone deacetylating enzymes, or HDACs, are responsible for removing these acetylation marks from histones, and therefore keep the chromatin in a more packed heterochromatic state. There are

several main classes of HDACs, and each class has a different set of characteristic inhibitors and activators that affect their activity. Sirtuins are known to be Class III HDACs, and are characterized by their dependence on the metabolic cofactor NAD+ as a substrate for their enzymatic activity (Avalos, Boeke, & Wolberger, 2004; Chang et al., 2002). That these HDACs, and especially Sir2, require the metabolite NAD+ for their mechanism of action is of critical importance for their impact on the aging process, as the dependence on NAD+ links their activity to the metabolic state of the cell.

It has long been theorized that altered gene expression through age-induced changes in chromatin states may be a contributing factor in the aging process (Villeponteau, 1997). In Drosophila, it has been shown that enrichment of repressive marks in heterochromatic regions of the genome is lost in aged flies (J. Wood, Hillenmeyer, & Lawrence, 2010). Sir2, through its deacetylation of histon H4 lysine 16, helps maintain transcriptional silence in subtelomeric regions, and is lost at these locations with age (replicative life span) in yeast (Dang et al., 2009). Additionally, Sir2 and Rpd3, both histone deacetylase proteins, have been shown to affect heterochromatic gene silencing and life span. Deletion of Rpd3, a class I HDAC, in yeast extended life span (Kim, Benguria, Lai, & Jazwinski, 1999), and the life span extension was not additive with dietary restriction (Jiang, Wawryn, Shantha Kumara, & Jazwinski, 2002). This indicates that, at least in yeast, DR and Rpd3-mediated life span extension may be operating through the same pathway. While a strong hypomorphic Rpd3 allele caused lethality in Drosophila (Mannervik & Levine, 1999), a weaker mutant showed life span extension and confirmed the results of the yeast study by showing that there was no

additional life span benefit when long-lived Rpd3 mutants were diet-restricted (Blanka Rogina, Helfand, & Frankel, 2002).

The relationship between Rpd3 and Sir2 was probed in Drosophila in a study that showed that the life span extension seen in Rpd3 mutants was dependent upon Sir2. In combination with the previous observation that Sir2 expression was increased in long-lived Rpd3 mutants and with CR, a model was proposed whereby caloric restriction inhibited the activity of Rpd3, while Rpd3 activity repressed Sir2. In this way, the model proposes that CR would lead to reduced activity of Rpd3, lessening the inhibition on Sir2 and allowing for increased Sir2 activity and life span extension (Blanka Rogina et al., 2002).

The role that Sir2 and Rpd3 play in heterochromatin maintenance and gene silencing was more recently investigated in yeast. Yeast that are Rpd3 deficient show increased repression at boundary regions between heterochromatic and euchromatic genomic locations (Zhou, Zhou, Lenzmeier, & Zhou, 2009). Interestingly, this result was dependent upon Sir2, again indicating that Rpd3 and Sir2 act together to regulate heterochromatic states. Another study also showed that mutation of Rpd3 leads to an irregular localization of Sir2 at telomeres and a spreading of heterochromatin, as measured with chip-qPCR (Ehrentraut, Weber, Dybowski, Hoffmann, & Ehrenhofer-Murray, 2010). The newer model proposed in this study is that Sir2 and Rpd3 may compete for binding on acetylated lysines on histone tails, at least in telomeric heterochromatin regions. In yeast, the formation of the SIR silencing complex (which contains Sir2, Sir3, and Sir4 in a complex) propagates repressive heterochromatic states along the telomeres. A possible model for the relationship between Rpd3 and Sir2 is that

Sir2 may need to bind to acetylated lysine groups in order for the SIR complex to form. This would mean that a disruption of these acetyl groups (or their removal via Rpd3 or another HDAC) may prevent Sir2 binding and the ability of SIR complexes to bind and repress chromatin. Given that enrichment for repressive chromatin marks is lost with age in *Drosophila*'s heterochromatic regions, it is possible that the maintenance of a more heterochromatic state may abrogate the aging process, and that interventions such as Sir2 and Rpd3 may extend life span in this way.

Known Sir2 targets and protein relationships

Sir2, in addition to its function as a class III HDAC, has also been shown to target many other proteins, many of which are also implicated in the aging process. Some of Sir2's targets are transcription factors, such as FOXO and p53, and genetic manipulation of both FOXO and p53 can increase life span. Other targets are involved in cellular processes such as mitochondrial biogenesis and DNA repair mechanisms. The number of identified Sir2 targets is ever increasing, and it is becoming increasingly clear that Sir2 plays a role in multiple cellular processes and is a nexus point for age-related genes. Therefore, Sir2 is also a natural drug target, and much work has been done to examine the effects of pharmacological activators of Sir2 such as resveratrol.

The Forkhead bOX-containing protein, O subfamily (FOXO) transcription factor activates transcription of a large number of genes upon its activation during dietary restriction. It is regulated (in part) by phosphorylation by Akt, a serine threonine kinase that is itself activated by the insulin receptor pathway. When phosphorylated by Akt, FOXO is sequestered out of the nucleus, where it is unable to activate gene transcription

(Accili & Arden, 2004). Upon DR, FOXO is translocated into the nucleus, where it is responsible for activating as much as 28% of the genes whose transcriptional activity is changed upon DR (Gershman et al., 2007). When FOXO is over expressed in Drosophila fat body, life span is extended, however FOXO's relationship to DR is not entirely understood, as FOXO null flies are still responsive to dietary restriction (Min et al., 2008). However, FOXO is required for a long-lived InR mutant (Slack, Giannakou, Foley, Goss, & Partridge, 2011) and for chico mutants (Yamamoto & Tatar, 2011) to extend life span. Importantly, FOXO has been shown to be deacetylated by mammalian Sirt1, and this deacetylation enhances its activity as a transcription factor (Brunet et al., 2004). Finally, C. elegans with increased Sir2.1 do not extend life span in the absence of FOXO (Tissenbaum & Guarente, 2001). All of this suggests that FOXO may be downstream of insulin signaling, DR, and Sir2.

Another protein that Sir2 is known to modify is p53, a gene mostly known for its role as a tumor suppressor and its involvement in the DNA damage response. Sir2 deacetylation of p53 inhibits its activity (Langley et al., 2002; Luo et al., 2001; Vaziri et al., 2001). In flies, the expression of a dominant negative p53 mutant extends life span in a manner that is not additive with DR, requires endogenous p53 to be present, but does not require FOXO (J. H. Bauer et al., 2007; J. H. Bauer, Chang, Bae, Morris, & Helfand, 2010; Shen & Tower, 2010). The absence of an additive effect on life span between DR and expression of the dominant negative p53 suggests that the two mechanisms use overlapping pathways to extend life span. Additionally, *dilp2* and 4E-BP are both required for p53-mediated life span extension. However, it is so far unclear how these

results pertain to mammalian systems, where the decrease or disruption in p53 leads to increases in cancer (Ryan, Phillips, & Vousden, 2001).

Another interaction of Sir2 that stresses that DNA damage may be a key factor in the aging process is its deacetylation activity on the KU-70 protein. KU-70's primary role is in non-homologous end joining (NHEJ) to repair double-stranded DNA breaks (Pawelczak, Bennett, & Turchi, 2011). KU-70 binds to the protein Bax to regulate cellular apoptosis by inhibiting it from activating the apoptotic machinery. When two of KU-70's lysine residues are acetylated, Bax is released from KU-70, and apoptosis is initiated. However, SIRT1 deacetylates these lysine residues (Cohen et al., 2004), presumably allowing KU-70 to sequester Bax.

Sir2 also has connections to other cellular processes such as mitochondrial biogenesis. SIRT1 deacetylates PGC1-alpha, a protein that is responsible for activating several transcription factors and therefore controlling the expression of genes that are involved in mitochondrial and metabolic processes (Sepharose et al., 2005). Increased expression of PGC1-alpha in mouse skeletal muscle promotes healthy aging (Wenz, Rossi, Rotundo, Spiegelman, & Moraes, 2009).

Clearly, the interactive network of Sir2 is vast, and this list only covers some of the known targets of Sir2 that are also known to impact aging and extend life span. While the details of how these processes occur or what role Sir2's interaction with them plays in the aging process may still be unclear, what is clear is that Sir2 is a central node for the regulation of multiple aging-related targets, and in all of the cases shown here, the direction of Sir2's interaction or activity with these proteins is correlative with the effect those proteins have on life span (for example, Sir2's deacetylation of FOXO leads to an

increase in its activity (Brunet et al., 2004), and FOXO increase in the fat body extends life span (Hwangbo, Gersham, Tu, Palmer, & Tatar, 2004). Given that Sir2 has effects on so many variable cellular processes, it is an ideal target for pharmacological activation, as a drug with the ability to increase the activity or expression of Sir2 would likely be able to affect all or many of these interacting pathways at once. As such, much work has been done to identify and characterize the effect of pharmacologically activating Sir2.

Resveratrol and other Sir2 Activators

In 2003 it was reported that several small molecules that were capable of increasing the activity of human SIRT1 were able to extend life span in yeast (Howitz, Bitterman, & Cohen, 2003). This was done using a fluorescence-based assay that measured the ability of human SIRT1 to deacetylate a synthetic p53 peptide that was attached to a fluorophore. Resveratrol, a polyphenol most commonly known for being a component of red wine, was seen as the most potent of these activators, followed by two other molecules, butein and fistein. Resveratrol also extends life span when fed to worms, flies, and fish (Valenzano et al., 2006; J. G. Wood et al., 2004), and fistein extends fly life span as well. In yeast, worms, and flies, resveratrol-induced life span extension is Sir2-dependent, as resveratrol administration does not extend life span in Sir2 mutants (J. G. Wood et al., 2004).

Resveratrol, when fed to mice on a high-calorie diet, increases early survival, improves insulin sensitivity, increases mitochondrial number, and improves liver pathology when compared to animals fed a high calorie diet without resveratrol. In fact, almost all of the experiments measured indicated that resveratrol fed mice more closely

resembled mice fed a lower-fat diet than their high-fat diet control counterparts. Finally, gene expression as measured by microarray analysis in resveratrol-fed mice more closely resembled the expression patterns in mice on a standard, lower-fat diet (Baur et al., 2006) and showed an increase in gene pathways involved in mitochondrial biogenesis and mitochondrial function (Lagouge et al., 2006).

There has been notable debate, however, about the ability of resveratrol to activate Sir2 *in vivo*, due mostly to a study that reported that resveratrol was able to induce SIRT1-mediated deacetylation of p53 peptides only when they were attached to the fluorophores used for assay measurement in the previous studies (Beher et al., 2009). Additionally, life spans were reported in several different strains of yeast without any life span extension when given resveratrol (Kaeberlein, McDonagh, et al., 2005). However, a recent study observed resveratrol-dependent activation of dSir2 in an assay that did not use fluorophores for detection, showing that resveratrol does activate dSir2 in the absence of the fluorophore (Hubbard et al., 2013). Very recently, it was reported that the contradictory results on whether resveratrol activates SIRT1 in vivo are likely due to their finding that SIRT1 is activated by resveratrol only in the presence of Lamin A, a protein that partially comprises the nuclear matrix (Liu et al., 2012). This result is of critical importance, because it accounts for the apparent difference in the ability of resveratrol to activate SIRT1 in vitro vs in vivo.

Importance of *Drosophila melanogaster* in the Study of Sir2

Much of the work cited in the above sections was performed in worms or flies, or was confirmed in those organisms before work was completed in higher eukaryotes such as mice. Due to their short life spans and wide array of genomic toolsets, invertebrate systems are an integral aspect of understanding the aging process and making basic discoveries. Therefore, it is also of critical importance to fully understand the genetics and phenotypes associated with the experiments being conducted. Recently, some controversy has fallen on the *D. melanogaster* model organism with respect to whether increased Sir2 expression extends life span as reported.

Increased expression of the Drosophila homologue of Sir2, dSir2, has been shown to increase life span using a system that drives over expression from the endogenous genetic locus of dSir2 via the P-element mediated insertion of an Upstream Activating Sequence (UAS) upstream of dSir2 (Rørth, 1996). The most commonly used of these lines is referred to as EP2300, although several other EP insertions in that region have also been shown to increase dSir2 expression and extend life span (Blanka Rogina & Helfand, 2004). When expressed along with the Gal4 protein, increased expression of the gene is induced. This system can be controlled in both a tissue-specific manner by the use of tissue-specific Gal4 promoters, and temporally controlled via the use of the Gene-Switch system. The Gene-Switch system only permits increased expression of the gene of interest when an activating drug, RU-486, is present in the fly's food, allowing researchers to control the expression of the protein of interest by controlling when the RU486 drug is given. This is achieved via a fusion of the Gal4 protein to a receptor for RU-486, which is a progesterone not endogenously present in flies. Upon receptor binding to the RU-486 ligand, the Gal4 protein is translocated to the nucleus, where it is

able to induce expression of the gene under UAS control. There are two primary advantages to the Gene-Switch system: the expression levels of the protein of interest remain the same during development of the fly, allowing researchers to rule out developmental effects of the protein's over expression, and this system eliminates problems of appropriate genetic controls, as the control animals are those which are not given the RU486 drug and are therefore genetically identical to the treatment animals.

Increased expression of dSir2 in Drosophila has been shown to increase life span when over expressed from the endogenous locus ubiquitously, using the Tubulin-Gal4 driver, neuronally, using the Elav-Gal4 and Elav-Gene Switch drivers, and in the fat body using the S_{106} switch driver (Banerjee et al., 2012; J. H. Bauer et al., 2009; Blanka Rogina & Helfand, 2004). Importantly, over expression of dSir2 increases life span in Gene-Switch systems where the control flies are genetically identical to the over expressing flies, eliminating concerns of genetic background effects on life span in these flies. These studies have over expressed dSir2 from its endogenous locus via the insertion of an UAS upstream of dSir2. This UAS insertion was generated along with many other similar "EP" lines when flies were subjected to a mobile P-element that contained a UAS sequence, and the element was allowed to move and mutant lines generated. This created a library of fly lines with P-elements that could be located within a gene, creating a mutant, or upstream of a gene, resulting in the ability to induce over expression of this gene in the presence of Gal4 (Rørth, 1996). While the most commonly used of these lines to increase Sir2 expression is called EP2300, it is important to note that several other EP lines with UAS insertions upstream of dSir2 have also been shown to extend life span in Drosophila (Blanka Rogina & Helfand, 2004).

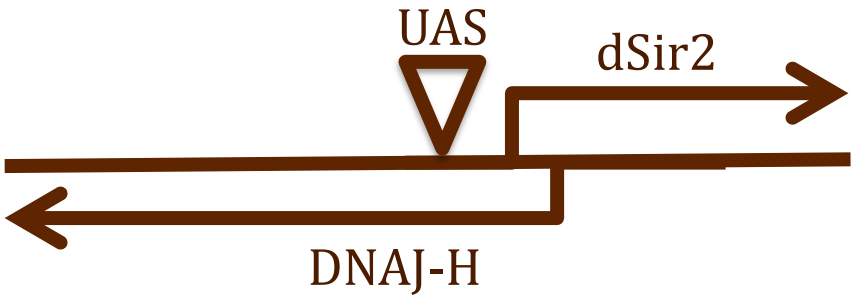

Figure 1-1. The *dSir2/dnaJ-H* genomic locus in Drosophila melanogaster and the position of the p-element UAS insertion in the EP2300 line. The transcriptional start site of dSir2 overlaps with that of DNAJ-H by 83 base pairs. The position of the UAS insertion in the EP2300 line is upstream of the *dSir2* locus, but located within the 5' UTR of *dnaJ-H*.

In Drosophila, the Sir2 genomic locus is on the second chromosome, and is located near a gene that is transcribed head-to-head in the opposite direction as Sir2 (Figure 1-1). While the function of DNAJ-H in flies is largely unknown, it has homology to the Heat Shock Protein 40 family of proteins, which are known to be involved in protein folding, translation, and degradation. A 2008 publication reported that when dSir2 was over expressed from a transgene that was not located near its endogenous genetic locus using the eye-specific GMR-Gal4 driver, lethality phenotypes were observed, inducing JNK-signaling and apoptosis (Griswold, Chang, Runko, Knight, & Min, 2008). This report also observed that when dSir2 was over expressed in the eye using the EP2300 line, dnaJ-H expression was 2-fold increased. Griswold et al hypothesized that dSir2 over expression was lethal, but that the EP2300 was able to extend life span due to the co-over expression of both dSir2 and DNAJ-H in that line. However, it has since been reported in two different studies, using gene-switch drivers with genetically identical

controls, that over expression of the EP2300 line in life span extending conditions does not induce DNAJ-H over expression (Banerjee et al., 2012; J. H. Bauer et al., 2009). It is unclear why one study would find that eye-specific expression of dSir2 would lead to lethality and induce dnaJ-H, while other studies reporting life span did not. However, it is important to note that the Griswold study did not test over expression conditions that extend life span, and using the GMR-Gal4 driver induced expression at more than double the levels found to extend life span.

A more recent study over expressed dSir2 and measured life span from other transgenic lines where dSir2 was over expressed under the control of a UAS-Gal4 system in an ectopic genetic location, and measured life span in flies over expressing dSir2 from the EP2300 line, all of which were driven using the constitutive ubiquitous Tubulin-Gal4 promoter (Burnett et al., 2011). This study reported that there was no life span extension when over-expressing dSir2 from these constructs when compared to a Tubulin-Gal4-containing control. The authors attribute the opposing results on whether dSir2 over expression extends life span to the use of appropriate genetic background controls in their studies. However, over expression of dSir2 using the EP2300 line extends life span when driven by the Gene-Switch system (J. H. Bauer et al., 2009; Blanka Rogina & Helfand, 2004), where the treatment (dSir2 over expressing) group is genetically identical to the control flies. Additionally, this recent study successfully over expressed dSir2 from a transgene, a condition that had been reported to result in lethality in the Griswold study. The three over expression conditions represented two different doses of Sir2 over expression as measured by qPCR of transcript levels, however in order to properly assess whether there is no effect, more than two Sir2 doses need to be tested. Just as more than

two different caloric levels need to be tested when evaluating whether a gene is involved in dietary restriction (Tatar, 2007), more than two levels of overexpression need to be examined before determining the effect of the gene on lifespan. Finally, the Tubulin-Gal4 "control" flies in their experiments were robustly long-lived compared to the other control lines in their study, making it difficult to fully assess the proper controls and interpretations of their results.

Clearly, the abundance of confusing and conflicting reports on whether dSir2 over expression extends life span in Drosophila melanogaster has prevented a clear consensus on whether dSir2 over expression extends life span. This is critically important, because ambiguity about the effects of increased dSir2 expression in flies will limit its ability to be used as a model system for aging and sirtuin research. Therefore, a thorough understanding of whether increased dSir2 expression extends life span, and the best conditions to test this, is desperately needed.

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CHAPTER TWO:
INCREASED EXPRESSION OF DSIR2 EXTENDS LIFE SPAN IN A DOSE-DEPENDENT MANNER IN *DROSOPHILA*

Abstract:
Sir2, a member of the histone deacetylase protein family, deacetylates lysine residues within many proteins, and is associated with lifespan extension in a variety of model organisms. Recent studies have questioned the positive effects of dSir2 on organismal lifespan in *Drosophila*. Several studies have shown that increased expression of the *Drosophila* Sir2 homolog (dSir2) extends life span(Kushal Kr Banerjee, Ayyub, Ali, et al., 2012; Bauer et al., 2009; Rogina & Helfand, 2004) while other studies have reported no effect on life span (Burnett et al., 2011) or suggested that increased dSir2 expression was cytotoxic(Griswold, Chang, Runko, Knight, & Min, 2008). To attempt to reconcile the differences in these observed effects of dSir2 on *Drosophila* life span, we hypothesized that a critical level of dSir2 may be necessary to mediate life span extension. Using approaches that allow us to titrate dSir2 expression, we describe here a strong dose-dependent effect of dSir2 on life span. Using the two transgenic dSir2 lines that were reported not to extend life span(Burnett et al., 2011), we are able to show significant life span extension when dSir2 is overexpressed by 2 to 5-fold. However, higher levels decrease life span, and can induce cellular toxicity, manifested by increased expression of the JNK-signaling molecule *Puc* phosphatase and induction of *dnaJ-H*. Our results help to resolve the apparently conflicting reports by demonstrating that the effects of increased dSir2 expression on life span in *Drosophila* are dependent upon dSir2 dosage.

Introduction:

Silent Information Regulator 2 (Sir2) is an NAD^+ -dependent deacetylase protein that is highly conserved from bacteria to mammalian systems. While originally reported to de-acetylate histones in yeast, Sir2 has since been shown to deacetylate a wide variety of other proteins including p53, Foxo, PGC-1α, KU-70 and NF- **κ**B (Haigis & Guarente,

2006). Increased Sir2 expression has been found to increase replicative life span in yeast (Kaeberlein, Mcvey, & Guarente, 1999), and to increase organismal life span in *C. elegans* (Tissenbaum & Guarente, 2001; Viswanathan & Guarente, 2011) and *Drosophila melanogaster* (Kushal Kr Banerjee, Ayyub, Ali, et al., 2012; Bauer et al., 2009; Rogina & Helfand, 2004). Additionally, many of the identified targets of Sir2 have been shown to have significant effects on metabolism and longevity (Haigis & Guarente, 2006).

Increasing the expression of *dSir2* increases fly life span when expression is induced from the endogenous locus by either the ubiquitous Tubulin-Gal4 driver or the neuron-specific ELAV-Gal4 driver (Rogina & Helfand, 2004). Importantly, life span is also increased when increased expression of *dSir2* is temporally restricted to adulthood, and spatially to either neurons (Bauer et al., 2009) or abdominal fat body (Kushal Kr Banerjee, Ayyub, Ali, et al., 2012) by use of the Gene Switch system. The Gene Switch system has the advantage of allowing post-eclosion induction of expression via the presence of the inducing agent, RU-486, in the fly food. Therefore, genetically identical siblings from the same cohort as the experimental flies that are not induced with RU-486 can serve as controls, thereby eliminating any potential concern regarding genetic background effects on life span. In these studies (Kushal Kr Banerjee, Ayyub, Ali, et al., 2012; Bauer et al., 2009; Rogina & Helfand, 2004), increased *dSir2* expression was mediated from the endogenous *dSir2* locus through use of an insertion of the UAS sequence upstream of *dSir2* (Rørth, 1996). While the most commonly used such line is the $dSir2^{EP2300}$ line, other similarly constructed fly lines have also been shown to extend life span when *dSir2* expression is increased using some of the same drivers (Rogina & Helfand, 2004).

A study in 2011 (Burnett et al., 2011) reported finding no life span extension when *dSir2* was over expressed by using the constitutive, ubiquitously expressing Tubulin-Gal4 driver, either with the dSir2^{EP2300} line, or with either of two newly constructed lines containing an inducible UAS-dSir2 transgene in addition to the endogenous copy of *dSir2*. The authors attribute these results to their use of "more appropriate" genetic background controls than those used in the initial study reporting extended life span in flies (Rogina & Helfand, 2004). However, increased expression of *dSir2* in the dSir2^{EP2300} line had also been shown to extend life span when driven by the inducible neuron-specific ELAV-Gene Switch driver (Bauer et al., 2009; Rogina & Helfand, 2004) and more recently by the inducible abdominal fat-body specific S_1-106-Gene Switch driver (Kushal Kr Banerjee, Ayyub, Ali, et al., 2012), where the controls are genetically identical flies from the same cohort, ruling out genetic background as the cause for life span extension.

The genetic locus of *dSir2* partially overlaps with that of a gene that is transcribed in the opposite direction, the *dnaJ-h*omologue (*dnaJ-H*) gene. While the specific function of DNAJ-H has not been shown, it has homology to the Heat Shock Protein 40 family of proteins, which are known to be involved in protein folding, translation, and degradation(Chan, Warrick, Gray-Board, Paulson, & Bonini, 2000). The importance of *dnaJ-H* to the life span-extending effect of *dSir2* was suggested in (Griswold et al., 2008), where either ubiquitous or neuronal expression from a UAS transgene containing a UAS-dSir2 transgene, not located near the endogenous genetic locus of *dSir2*, was developmentally lethal. Furthermore, expression of this UAS-dSir2 gene under control of the strong eye-specific GMR-Gal4 driver caused developmental apoptosis of cells of

the eye, while simultaneously inducing JNK-signaling (Griswold et al., 2008). This study (Griswold et al., 2008) further observed that expression of *dSir2* from the native *dSir2* locus in the eye using the same robust GMR-Gal4 driver with the dSir2^{EP2300} line, resulted in a greater than 12-fold increase in *dSir2* mRNA and a 2-fold induction of *dnaJ-H* mRNA expression. From these results, the authors concluded that increased *dSir2* expression is lethal and that *dSir2* normally regulates cell survival and death in *Drosophila.* They hypothesized that the life span extension seen in the dSir2^{EP2300} line was likely a result of the simultaneous over- expression of both *dSir2* and *dnaJ-H*. In contrast, subsequent reports demonstrated that 2.5-fold, 3-fold, and 5-fold increased expression of *dSir2* from the native *dSir2* locus results in life span extension without an increase in *dnaJ-H* mRNA expression(Kushal Kr Banerjee, Ayyub, Ali, et al., 2012; Bauer et al., 2009).

The relationship between *dSir2* and Dietary Restriction has also been a point of some literary contradiction, likely (in part) due to the genetic nature of these studies. In 2002, Newman et al (Newman, Lundblad, Chen, & Smolik, 2002) reported that two *dSir2* hypomorphs, which were homozygous lethal but which, when combined in a transheterozygote, created a viable fly with disrupted *dSir2* expression, and did not affect the life span of the organisms. Rogina et al (Rogina, Helfand, & Frankel, 2002) in 2002 also provided the first hint that dSir2 function may be related to DR by showing that dSir2 expression was increased under DR conditions. Interestingly, a 2003 study showed that yet another dSir2 hypomorphic mutant decreased life span, but this was shown in a fly with a different non-lethal disruption of the *dSir2* locus. The overlap of the dSir2 mechanism of life span extension with DR was shown in 2004, in the first publication to

show increased life span with dSir2 over expression in flies (Rogina & Helfand, 2004). This paper showed that while dSir2 over expression increased life span, this life span was not further increased by DR, indicating that the two operate through a common mechanism. Additionally, the same transheterozygous mutant lines used in (Newman et al., 2002) and in (Åström, Cline, & Rine, 2003) were subjected to DR, and did not respond with an increase in life span, again showing that DR requires functional dSir2 to extend life span (Rogina & Helfand, 2004). The increase in locomotor activity that is observed when flies are DR is also dependent on the presence of an in-tact dSir2 gene (Parashar & Rogina, 2009). Most recently, RNAi was used in combination with the gene-switch system to knock down (KD) dSir2 expression only in adulthood. dSir2 KD flies showed physiological and metabolic qualities that support the evidence that dSir2 is involved in the DR response, since many of the the parameters measured in dSir2 KD flies reflected the effect of a high-calorie or high-fat diet in flies (Kushal K Banerjee, Ayyub, Sengupta, & Kolthur-Seetharam, 2013a, 2013b). Together, these data point towards at the least, an intersection of the DR and dSir2 pathways in flies, and a requirement for dSir2 in the DR response. However, the 2011 Nature publication (Burnett et al., 2011) that reported the absence of an effect of dSir2 over expression on life span also reported that the two original hypomorphic lines from (Newman et al., 2002), when outcrossed to yet a different strain and re-homozygosed (removing the lethality-causing second-site mutations from these lines), did not show further life span extension under DR.

The conflicting results of these studies, demonstrating in one case that increased expression of *dSir2* is lethal (Griswold et al., 2008), in a second that there is no effect on

life span (Burnett et al., 2011), and in third, fourth and fifth cases, that there is significant life span extension (Kushal Kr Banerjee, Ayyub, Ali, et al., 2012; Bauer et al., 2009; Rogina & Helfand, 2004), in addition to the confusion over *dSir2's* role in DR, have been a barrier to understanding the role of *dSir2* in *Drosophila* aging. In the present study, we present data showing that when dose-dependent effects of *dSir2* are taken into account, these many discrepancies can be resolved. Through a detailed series of studies in which we examine the effects of expression of *dSir2* at low, moderate and high levels on life span, we demonstrate that modest levels of *dSir2* are indeed effective at extending life span, and that high levels of expression likely induce a cellular stress response that is reflected by induction of *dnaJ-H*. Thus, our studies show that moderately increased levels of *dSir2* can extend life span in *Drosophila melanogaster*.

Results:

dSir2 expression from UAS-dSir2 transgenes can increase or decrease life span.

To examine the effect of increasing dSir2 expression on longevity, we examined the life span of flies carrying either of two newly generated UAS-dSir2 transgenes (Sir2-1 and Sir2-3) driven by the ubiquitous Tubulin-Gal4 driver. The life span of these flies was compared to Tubulin-Gal4 flies crossed to wild type flies from the same genetic background as the UAS-dSir2 transgenes. Under these conditions, Tubulin-Gal4>Sir2-3 flies showed life span extension, while Tubulin-Gal4>Sir2-1 flies had a shortened life span (Fig. 2-2-1A). The discrepancy of finding of a decrease in life span with the Sir2-1 transgene and an increase with the Sir2-3 transgene, led us to measure the levels of *dSir2* expression in these flies. We found that flies containing the Sir2-1 transgene over

expressed *dSir2* very highly (~ 45-fold increased), compared to the more moderate induction from Sir2-3 (~ 11-fold increased *dSir2*) (Fig. 2-1A, inset).

To eliminate the possibility that over-expression of dSir2 during development in the presence of the constitutively expressed Tubulin-Gal4 driver might contribute to the deleterious effect we see on adult life span in the Sir2-1 line, we utilized the RU-486 inducible Gene Switch system in which control and experimental flies are genetically identical offspring from the same cohort to examine the life span of flies over-expressing dSir2 from the Sir2-1 transgene driven by the conditional Tubulin Gene Switch driver (Tubulin-Gene Switch). When expression of the Sir2-1 construct was induced using RU-486 in the food of adult flies, the life span of these flies was shortened, as seen for the Tubulin-Gal4>Sir2-1 life span (Fig. 2-1B). We measured the level of dSir2 mRNA in these flies,and found an approximately 50-fold induction of dSir2, consistent with the high level of induction seen with the constitutive Tubulin-Gal4 driver (Fig. 2-1B, inset). Additionally, we crossed another UAS-dSir2 line, Sir2-4, to the Tubulin Gene Switch driver, and observed an increase of ~15-fold in dSir2 levels for RU-486-induced flies compared to EtOH controls, without any observable change in median life span.

Given that the level of dSir2 induction using both the Sir2-1 and Sir2-4 transgenes in these studies was high, we hypothesized that these levels of dSir2 might be detrimental to life span. In order to better test the effects of dSir2 over-expression at more moderate levels, we performed life span and mRNA expression experiments using the Sir2-3 transgene, which induced dSir2 expression more moderately than Sir2-1. We also included an additional genetic background control in this experiment, where we crossed w^{1118} flies (injection stock for the transgene line) to the Tubulin-Gene Switch driver, and

measured life span in these flies with and without RU-486 induction. Life spans of control w^{1118} flies carrying the Tubulin-Gene Switch driver was the same with or without RU-486, demonstrating that RU-486 treatment does not directly affect life span in our w^{1118} strain.

When the Sir2-3 transgene was expressed ubiquitously using the Tubulin-Gene Switch driver in the presence of RU-486, we observed no life span extension compared with genetically identical uninduced (ethanol–fed) flies. However, inclusion of the additional w^{1118} genetic background control in this study,allowed us to compare the life span of the uninduced ethanol "control" flies (Tubulin-Gene Switch> Sir2-3) with the w^{1118} controls. Surprisingly, we found that the Tubulin-Gene Switch>Sir2-3 flies that were fed ethanol exhibited an extended life span compared with the Tubulin-Gene Switch>w^{1118} controls (Fig. 2-1C). This result was unexpected, given that the absence of RU-486 in the food should have meant that there was no induction of dSir2. In order to clarify this result, we also examined the levels of dSir2 mRNA in all of these flies, and found that the ethanol-fed control Tubulin-Gene Switch>Sir2-3 flies had a mild but significant elevation in dSir2 levels (~1.5-fold induction) when compared with the Tubulin-Gene Switch>w^{1118} controls. The Tubulin-Gene Switch>Sir2-3 flies that were induced with RU-486 in the food showed an ~ 11-fold increase in dSir2 levels (Fig. 2-1C, inset). This finding suggests that in these conditions, modest elevation of dSir2 was observed in the absence of RU-486 in the food, and was sufficient to extend life span, but that the higher level of dSir2 expression induced in the RU-486-fed flies does not lead to a further increase in life span.

Finally, we crossed the Sir2-3 transgenic flies to the neuron-specific ELAV-Gene Switch driver and evaluated life span extension and dSir2 mRNA levels. Again, we included the ELAV-Gene Switch>w^{1118} genetic background control to allow for comparison with the uninduced ELAV-Gene Switch>Sir2-3 ethanol controls. In this experiment, the uninduced ELAV-Gene Switch>Sir2-3 flies did not have significantly elevated dSir2 levels compared to the ELAV-Gene Switch>w^{1118} genetic control. Consistent with this finding, we saw no life span extension for the uninduced ELAV-GS>Sir2-3 flies with this comparison. However, when RU-486 was added to the fly food, we measured a 5-fold increase in dSir2 mRNA levels in the ELAV-Gene Switch>Sir2-3 flies, as well as a significantly extended life span, compared to uninduced controls (Fig. 2-1D).

Moderate increases in dSir2 expression from UAS-dSir2 transgenes extend life span

The ability of the Sir2-3 line to induce dSir2 expression in the ethanol-only condition of the Tubulin-Gene Switch life span led us to question whether there might be some spontaneous ("leaky") induction of dSir2 expression from some or all of these UAS-dSir2 constructs in the absence of Gal4. To directly test whether the presence of one copy of the UAS-dSir2 transgene could lead to over-expression of *dSir2* without the presence of a Gal4 driver, we crossed three different homozygous UAS-dSir2 transgenic lines (Sir2-1, Sir2-3, and Sir2-4) to their genetic background control (w^{1118}) and measured *dSir2* mRNA levels in the F1 heterozygotes (Fig. 2-1E, inset). One copy of the Sir2-1 insertion (Sir2-1 / +) did not result in significantly elevated *dSir2* levels, but both Sir2-3 / + and

Sir2-4 / + exhibited significantly increased *dSir2* mRNA levels (~2-fold increase over w^{1118} dSir2 levels). This result suggests that the life span extension observed.
in the control condition of the Tubulin-Gene Switch>Sir2-3 and Sir2-4 life spans may be due to leaky expression of the *dSir2* construct under some conditions.

In flies from the same cohort as those used for qPCR, we tested whether leaky *dSir2* expression in the absence of a Gal4 driver was sufficient to extend life span (Fig. 2-1E). A single copy of Sir2-1 that did *not* show elevated dSir2 mRNA levels did not extend lifespan, whereas one copy of either Sir2-3 or Sir2-4 did increase lifespan, compared to w^{1118} controls. These results suggest that modest *dSir2* over-expression, here from un-driven UAS-dSir2 constructs, extends fly lifespan.

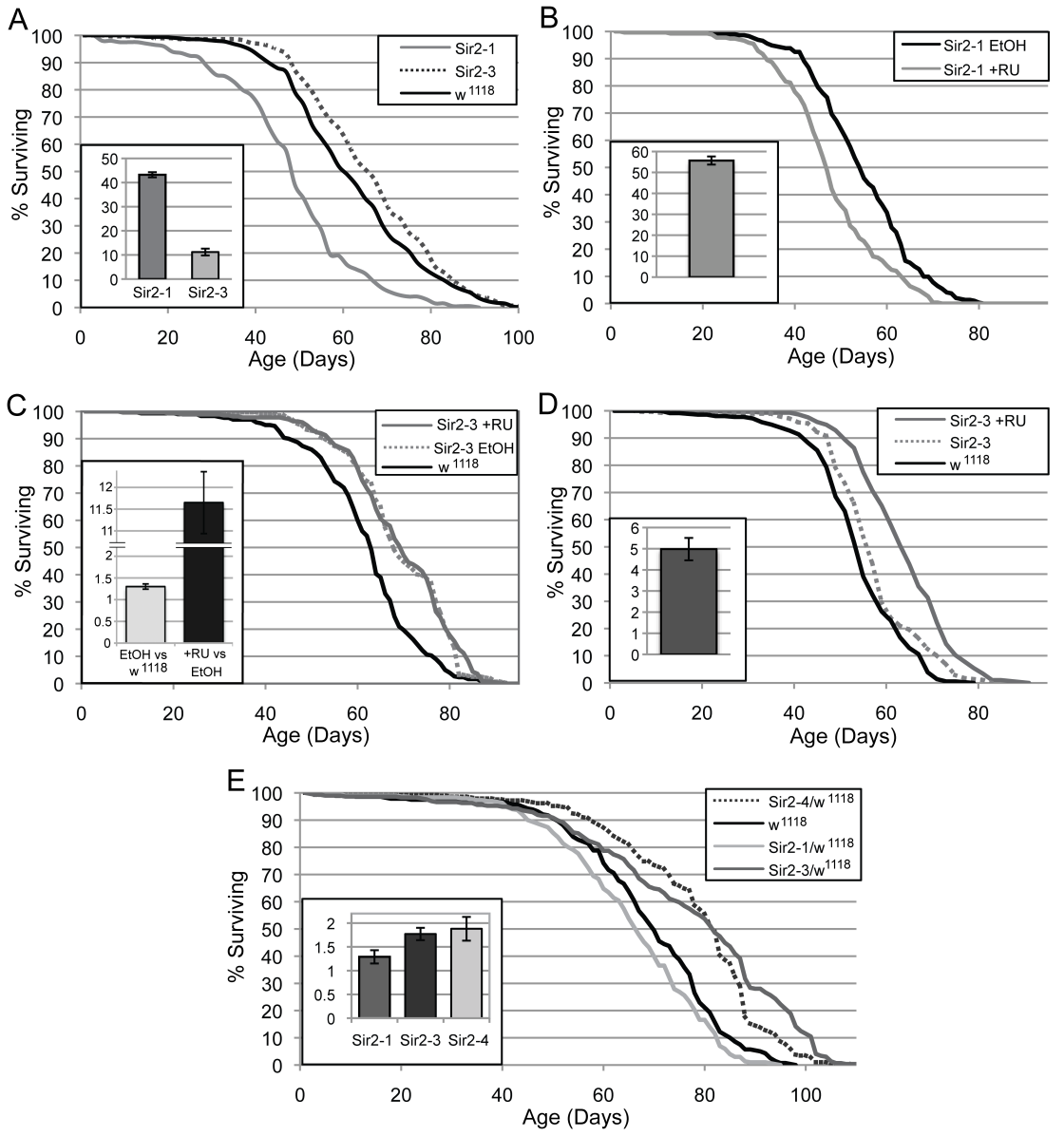

Figure 2-1.
Effects of *dSir2* over-expression on life span are variable under different conditions.
When two different UAS-dSir2 over-expressing lines are driven by the Tubulin-Gal4 driver, one line extends life span (Sir2-3), while the other is detrimental (Sir2-1) , compared to the control (Tubulin-Gal4>w^{1118}) (A). Sir2-1 displays very high *dSir2* over-expression, while Sir2-3 over-expresses *dSir2* more moderately (A, inset). When over-expressed from the Sir2-1 transgene by the Tubulin Gene Switch driver, *dSir2* is expressed at greater than 50-fold and decreases life span (B and B, inset). In the Tubulin-Gene Switch>Sir2-3 flies, *dSir2* is mildly elevated and life span extended when comparing the ethanol-only condition (no RU-486) to the genetic background control. The induction of higher levels of *dSir2* using RU-486 does not further increase life span (C and C, inset). When the Sir2-3 transgene is expressed using the neuron-specific ELAV Gene Switch driver, life span is extended when RU-486 is used to induce 5-fold increased *dSir2* expression (D and D, inset). Some UAS-dSir2 lines show elevated *dSir2* levels in a heterozygous state without the presence of a driver (E, inset). Lines that show an increase in *dSir2* levels also extend life span in the absence of a driver (E). Error bars represent SD of 3 biological replicates.

dSir2-mediated life span extension is dose-dependent

The extension of life span seen in the moderately over expressing *dSir2* lines above led us to the hypothesis that there may be a dose-dependent element to the effect of *dSir2* induction on organismal life span. Previous studies showing that *dSir2* over-expression extends life span used fly lines that expressed *dSir2* from its endogenous locus at relatively modest levels (~3-5 fold increase)(Bauer et al., 2009; Rogina & Helfand, 2004). We noted that the flies over-expressing dSir2 from the Sir2-3 and Sir2-4 transgenes express *dSir2* to a similar extent when life span is extended, but some of the conditions that we had tested had much higher levels of *dSir2, and rarely extended life span*. It is interesting that the study reporting no effect of UAS-dSir2 on life span also showed that *dSir2* levels were at either very low levels (~1.5-fold increase) or higher levels (~8-fold increase) (Burnett et al., 2011). In order to see whether *dSir2* over-expression might have a dose-dependent effect on life span, we plotted *dSir2* over-expression levels and life span extension from both our studies and those of published life spans (Fig. 2-2A and B).

As can be seen in Fig. 2-2, *dSir2* over-expression is associated with life span extension when mRNA levels are elevated approximately 2 to 5-fold compared to controls. In addition, *very* high *dSir2* expression (greater than 30-fold increase) correlates with shortened life span, suggesting that such high levels of *dSir2* may have a toxic effect. Interestingly, over-expression of *dSir2* in the dSir2^{EP2300} line extends life span more reliably and to a greater extent than induction at similar levels from the UAS-dSir2 transgenes. This suggests to us that the presence of endogenous regulatory elements may

be beneficial. Given that all of the *dSir2* expression levels for flies studied in (Burnett et al., 2011) were outside the predicted optimal range,that we report here, we hypothesized that we should be able to see life span extension in flies carrying these constructs if dSir2 expression within our predicted optimal window could be achieved.

To test this, we used the Gene Switch system to over-express *dSir2* from the myc-tagged UAS-dSir2 containing fly lines used in (Burnett et al., 2011). We used the Tubulin-Gene Switch driver in order to express *dSir2* in the same tissues as in the original study, and varied the concentration of RU-486 in the food, thereby titrating *dSir2* expression levels in these flies. We used three concentrations of RU-486 (100 uM, 200 uM, and 500 uM) to induce *dSir2* expression, and measured the *dSir2* transcript levels for each of these conditions. We then plotted the median life span increase for both our own life spans and those previously published with these constructs as a function of the measured *dSir2* levels (Fig. 2-2C). Life span was extended with these fly lines when they expressed *dSir2* within an optimal range. These results demonstrate a dose-dependent response for *dSir2* over-expression on fly life span, even for transgenic constructs previously reported not to extend life span.

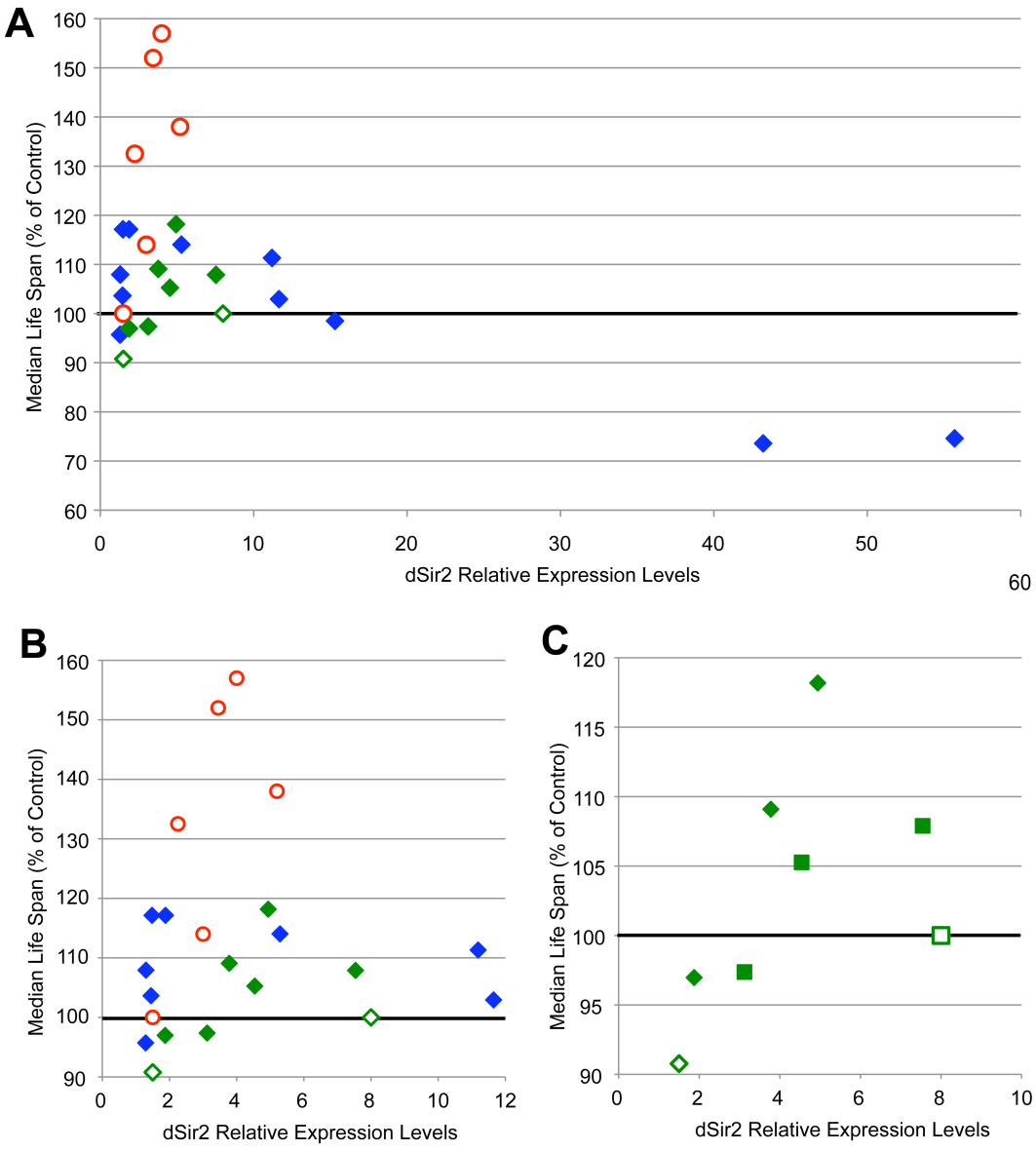

Figure 2-2.
dSir2 over-expression extends life span dose-dependently. Median life span change (shown as % of control) is plotted as a function of *dSir2* expression levels for life span experiments described in this study and in previous reports (A). Finer view of x-axis *dSir2* levels below 12-fold shown in (B). For all figures, previously published life spans are shown as open data points (Kushal Kr Banerjee, Ayyub, Ali, et al., 2012; Bauer et al., 2009; Burnett et al., 2011; Rogina & Helfand, 2004). For figures A and B, life spans using $dSir2^{EP2300}$ are shown as red circles and UAS-dSir2 transgene life spans are shown as diamonds. Life spans using the UAS-Sir2-myc lines from (Burnett et al., 2011) are shown in green, while life spans conducted using UAS-Sir2-1, Sir2-3, or Sir2-4 lines in this study are shown in blue. *dSir2* over-expression levels and median life span extension from (Burnett et al., 2011) (diamonds = Myc2, squares = Myc9, open data points indicate data from(Burnett et al., 2011)) and from our studies using the Tubulin-Gene Switch driver and three levels of RU-486 (100uM, 200uM, and 500uM) (green diamond = Tubulin-Gene Switch>Myc2, green square = Tubulin-Gene Switch>Myc9) (C). In life span experiments published in (5), additional EP lines that over-express *dSir2* from the endogenous locus also extended life span, but are not included in this graph because of the lack of data for *dSir2* expression level.

Sir2 Null Flies Respond to Dietary Restriction via Food Dilution

To test whether dSir2 is involved in the DR response, we obtained a mutant Sir2 fly line which contains a deletion of the entire dSir2 gene coding region (a Sir2 null mutant) and back-crossed it 10 times to our w^{1118} control. We then performed life span assays on the Sir2 null and control flies on four different food dilution conditions (a 10% Yeast, 10% Sugar condition was referred to as "1.0 N" food, and other foods were 0.3 fold (0.3N), 0.5 fold (0.5N), and 1.5 fold (1.5N) dilutions of that), and evaluated whether DR was still able to extend life span in the Sir2 null flies.

Both control and Sir2 null flies responded to DR in this experiment with an increase in life span, specifically on the 0.5 N food condition (Fig. 2-3). Interestingly, the Sir2 null animals were shorter lived in every food condition than the controls, confirming previous findings (Åström et al., 2003). The Sir2 null animals were more sensitive to the lowest-calorie food than the controls, with a large spike in age-dependent mortality before day 20 on the 0.3N food in both the Sir2 null males and females. This data may suggest that the relationship between diet and life span is altered in these flies, supporting some role for dSir2 in the interaction, although further testing is required. Additionally, the 0.5N condition showed the lowest difference in life span between the conditions, with a 10% reduction in the life span of the Sir2 null animals on this food. In terms of age-specific mortality, the Sir2 null females had changes in age-independent mortality with the different diets, as did the controls, consistent with the known effect of DR on mortality. The result with males was slightly less clear, due a change in the age-dependent mortality in flies on 0.5N food in comparison to the other foods, but with no

change in the slopes of 0.3N, 1.0N, or 1.5N foods relative to each other (Fig. 2-4). These data may not have the necessary number of flies to definitively interpret the mortality curves of these life spans, leading to difficulties in the interpretation of these results. In addition, these experiments were only performed once, and so would need repeating with larger numbers of flies to interpret the mortality results of DR on these *dSir2* nulls.

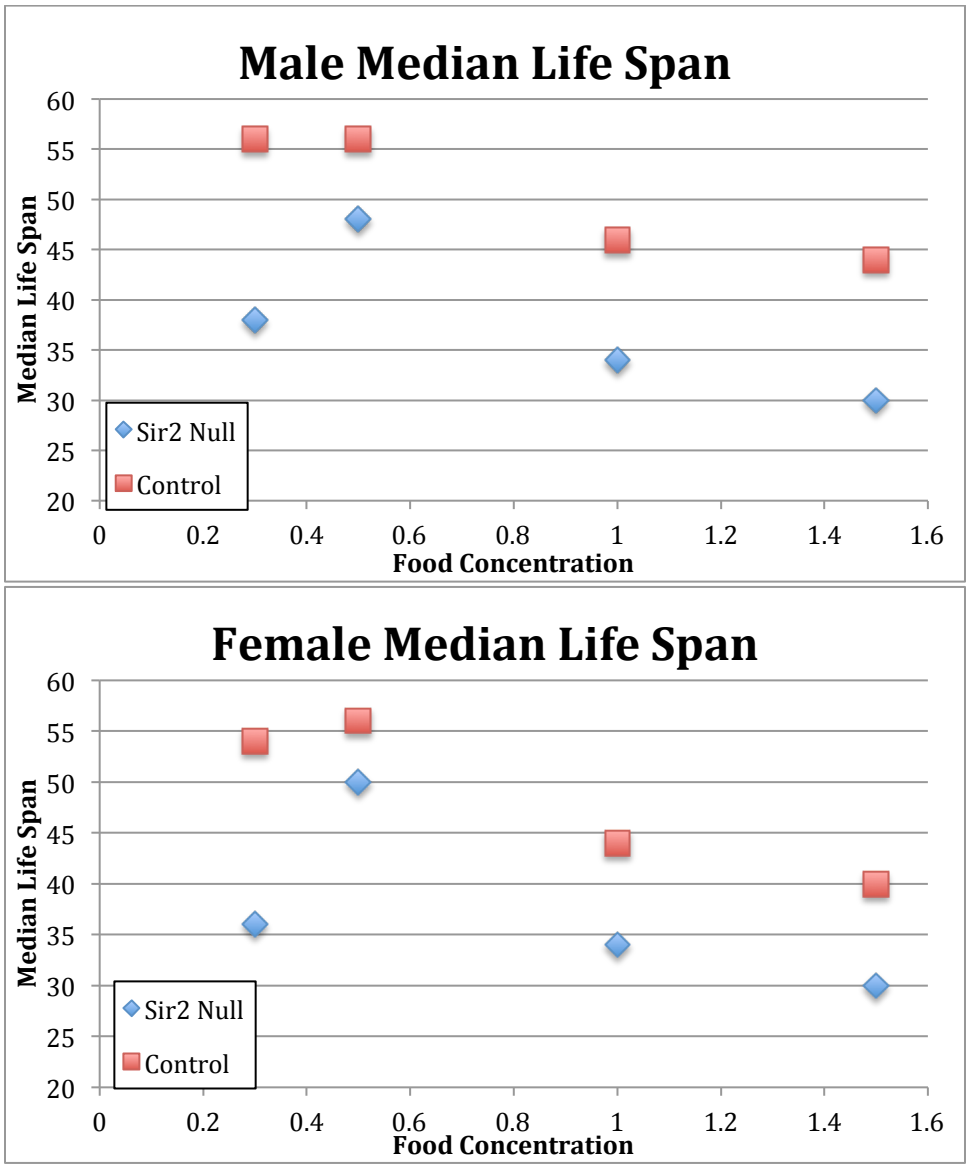

Figure 2-3.
Sir2 null mutant flies respond to DR. Median life span as a function of the dietary concentration of the food (1.5 = 15% sugar/15% yeast). Both males (A) and females (B) responded to dietary restrictions with an increase in median life span at the lower food concentration of 0.5. Sir2 null flies have lower life span at all food conditions than control flies.

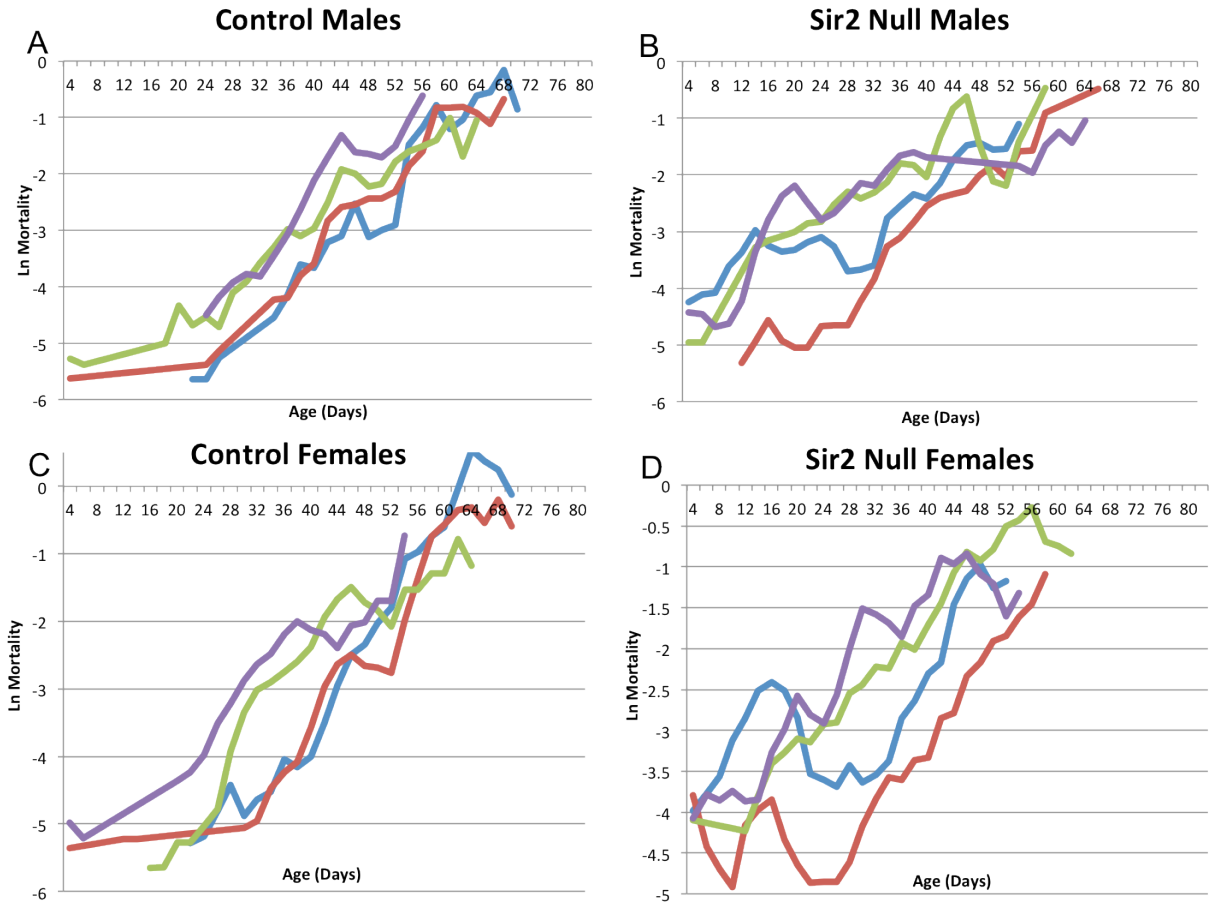

Figure 2-4.
Sir2 null females and Control males and females all responded to DR with a change in age-independent mortality (A, C, and D). However, Sir2 null males on 0.5N food responded to DR with a change in age-specific mortality (B).

High levels of dSir2 expressed from transgenes induce cellular toxicity and dnaJ-H expression.

In *Drosophila*, the endogenous dSir2 locus overlaps with the 5' UTR of a gene that is transcribed in the opposite direction, dnaJ-homologue, or dnaJ-H. The UAS insertion in the dSir2^{EP2300} over-expression line is therefore located within the 5' UTR of this gene. It was reported that over-expression of dSir2 from a UAS-dSir2 transgene was lethal when driven constitutively using either the ubiquitous actin-5C-Gal4 or neuronal ELAV-Gal4 driver (Griswold et al., 2008). When this same construct was driven in the eye using the GMR-Gal4 driver, the JNK signaling pathway and apoptosis were induced (Griswold et al., 2008). Additionally, GMR-Gal4>dSir2^{EP2300} induced 12-fold over-expression of *dSir2* and a 2-fold increase in *dnaJ-H* levels in the eye (Griswold et al., 2008). The UAS-dSir2 transgene line contains an extra dSir2 gene copy in the genome and therefore does not over-express dSir2 from the endogenous genetic location as dSir2^{EP2300} does. The observation of lethality when expressing this transgene, in combination with the increased dnaJ-H levels seen in the GMR-Gal4>dSir2^{EP2300} flies, led to the conclusion that dSir2 over expression from the transgene was lethal and that co-over expression of *dnaJ-H* in the dSir2^{EP2300} line rescued this phenotype. However in a subsequent study, when dSir2 overexpression was driven from the endogenous dSir2 locus by ELAV-Gene Switch> dSir2^{EP2300} at two different doses of RU-486, there was a dose-dependent increase in life span and *dSir2* levels, but no change in *dnaJ-H* expression levels(Bauer et al., 2009). These findings conflict with the reported induction of *dnaJ-H* in (Griswold et al., 2008) when dSir2^{EP2300} was over-expressed in the eye. This led us to question whether the expression of *dnaJ-H* in the eye was due to promiscuous expression induced by the dSir2^{EP2300} EP element, or might be due to other causes.

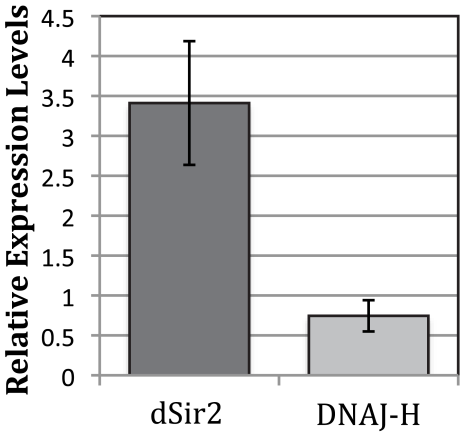

Figure 2-5.
dnaJ-H levels are not increased in ELAV-Gal4>dSir2^{EP2300} flies over-expressing *dSir2* from the endogenous *dSir2* locus.
Error bars represent SD of 3 biological replicates.

We measured *dnaJ-H* and *dSir2* transcript levels in the heads of ELAV-Gene Switch>dSir2^{EP2300} flies and confirmed that induction of dSir2^{EP2300} from the native dSir2 locus as in (Rogina & Helfand, 2004) leads to a 3.5-fold increase in *dSir2* and no increase in *dnaJ-H* levels (Fig. 2-5). We also measured *dSir2* and *dnaJ-H* transcripts in three of our own UAS-dSir2 transgene containing fly lines (Sir2-1,Sir2-3, and Sir2-4),driven by the ELAV-Gal4 driver (Fig. 2-6A). We found that *dnaJ-H* is increased in some of the UAS-dSir2 transgenic over-expression conditions, and the primary determinant of whether *dnaJ-H* increases appears to be the level of *dSir2* expression achieved (Fig. 2-6A, diamonds). In order to explore the mechanism by which *dnaJ-H* is elevated in response to transgenically expressed *dSir2,* we measured the level of expression of the JNK signaling gene, puc phosphatase (*puc*), in these conditions and found that *puc* follows the same pattern as *dnaJ-H*, exhibiting increased expression when *dSir2* reaches high levels (Fig. 2-6A, squares). This suggests that expression of *dSir2* at high levels may induce *dnaJ-H* as part of a cellular stress response and is not due to its genomic location, as suggested in (Griswold et al., 2008). Importantly, moderate levels of increased dSir2, such as those typically seen under conditions associated with life span extension, do not induce *dnaJ-H* expression.

These results led us to question whether the lethality previously observed in (Griswold et al., 2008) might have resulted from toxically high levels of *dSir2*. Since crossing the UAS-dSir2 line from that study (referred to here as Sir2(G)) (Griswold et al., 2008) to constitutive GAL4 drivers results in developmental and cellular lethality, we used the ELAV–Gene Switch driver in to induce expression from the Sir2(G) transgene in neurons for 10 days post-eclosion, and measured *dSir2* levels in fly heads. We compared *dSir2* expression from the ELAV-Gene Switch>Sir2(G) flies with ELAV-Gene Switch>Sir2-1 and ELAV-Gene Switch>Sir2-3 flies under the same conditions, and found that Sir2-3, which has moderate levels of over-expression, and Sir2-1, which shows very high *dSir2* levels, both express *dSir2* at much lower levels than the Sir2(G) line, which showed a 50-fold increase in mRNA levels under these conditions (Fig. 2-6B). We conclude that the lethality of the Sir2(G) transgene resulted from extremely high *dSir2* levels.

Since the Sir2(G) transgene expresses *dSir2* at very high levels, and *dnaJ-H* is elevated in our highly expressing dSir2 transgenes, we would predict that under conditions of high dSir2 induction, the Sir2(G) transgene should also induce *dnaJ-H*. Additionally, we expect that induction of dSir2 expression from Sir2(G) at lower levels should not induce *dnaJ-H*. To test this, we crossed the lethal Sir2(G) line to the ubiquitous Tubulin Gene-Switch driver and induced *dSir2* at different levels by using two doses of RU-486 (Fig. 2-6C). At a low concentration of RU-486 (50 uM), dSir2 is increased 3-fold and *dnaJ-H* levels are not elevated, while at a higher concentration of RU-486 (200 uM), *dSir2* is induced 25-fold over control levels and *dnaJ-H* expression is

increased almost 3-fold. Therefore, the Sir2(G) transgene induces dnaJ-H induction when dSir2 levels are high, but not when dSir2 is mildly induced.

Finally, we tested whether *dnaJ-H* induction was specific to elevated *dSir2* by expressing GFP using the strong, ubiquitous, constitutive *daughterless-Gal4* driver and measuring *dnaJ-H* levels (Fig. 2-7). Interestingly, expressing GFP with this driver led to a 2.5-fold increase in *dnaJ-H*. The induction of a strong dnaJ-H expression response in the absence of increased dSir2 indicates that the induction of *dnaJ-H* observed when *dSir2* is highly over-expressed is likely part of the cellular stress response itself, and not due to a specific function of dSir2.

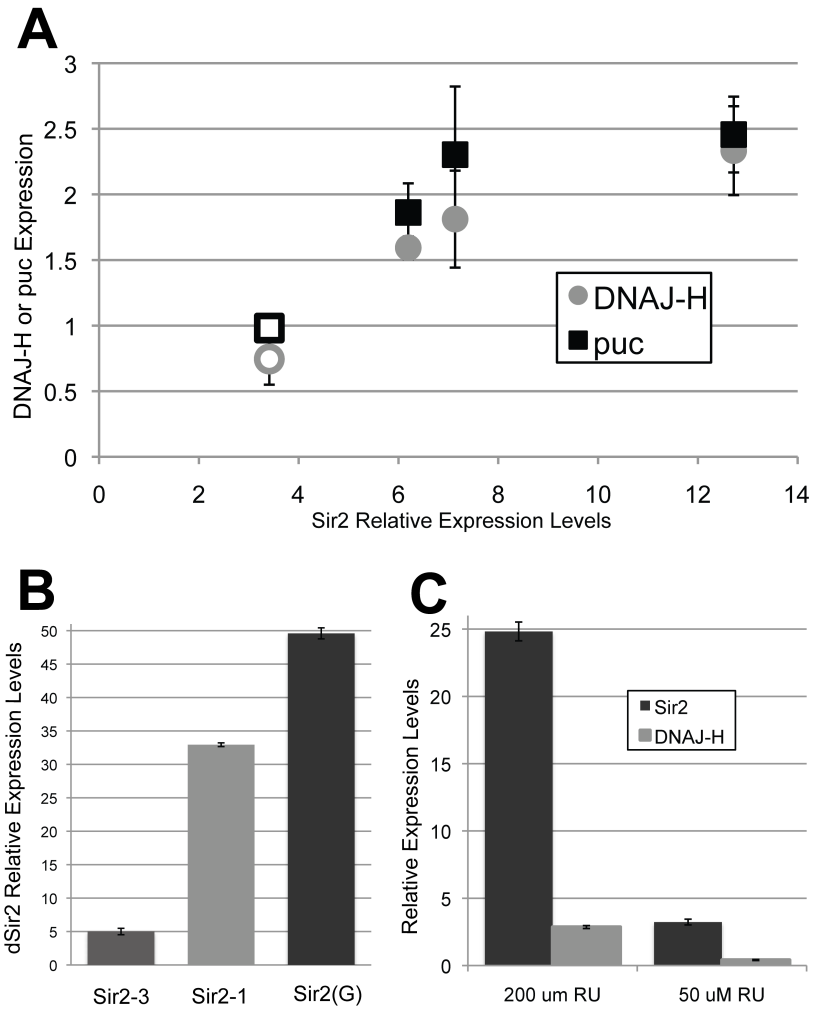

Figure 2-6.
High levels of *dSir2* induce both *puc phosphatase* and *dnaJ-H* expression, but lower levels of *dSir2* expression do not. Over-expression of *dSir2* at high levels using the constitutive neuronal driver Elav-Gal4 leads to *dnaJ-H* induction when expression is high, even when expression is not from the native *dSir2* site (A, filled diamonds = UAS-dSir2 transgenes, open diamond = $dSir2^{EP2300}$). Levels of *dSir2* expression that induce *dnaJ-H* also induce expression of *puc phosphatase*, a JNK-signaling target gene (A, filled squares = UAS-dSir2 transgenes, open square = $dSir2^{EP2300}$). With the neuronal inducible ELAV-Gene Switch driver and 500 uM RU-486, *dSir2* over-expression from the *dSir2*(G) transgene from (Griswold et al., 2008), induces *dSir2* at levels that are extremely high, even when compared to a highly over-expressing line from our own studies (Sir2-1) (B). When the Sir2(G) transgene is driven by the ubiquitous inducible Tubulin-Gene Switch driver at two different concentrations of RU-486, it can be seen that a high level of *dSir2* expression (with 200 uM RU-486) induces *dnaJ-H* expression, while a lower level of expression (with 50 uM RU-486) does not (C). Error bars represent SD of 3 biological replicates.

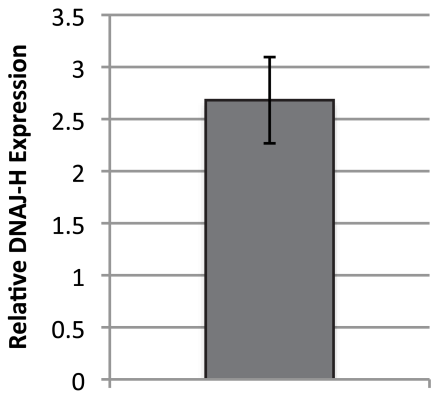

Figure 2-7.
When UAS-GFP is highly expressed using the strong, ubiquitous Daughterless-Gal4 driver, *dnaJ-H* levels are
increased. Error bars represent SD of 3 biological replicates.

dnaJ-H expression levels are not increased under many stresses

DNAJ-H has known homology to Heat Shock proteins, however very little is known about its function in vivo. Interestingly, we see an increase in DNAJ-H levels when animals are stressed with overproduction of Sir2, indicating that DNAJ-H may be involved in this stress response. In order to increase our knowledge of the response of DNAJ-H to known cellular stresses, we subjected Wild Type (Canton S, w^{1118}, or YW) flies to a variety of stresses, and measured the levels of DNAJ-H as compared to other known cellular stress response genes and normalized to GAPDH.

First, we tested whether heat shock of flies resulted in elevated DNAJ-H levels by heat shocking the flies for 15, 30, 60, or 240 minutes and measuring dnaJ-H levels via qPCR. Interestingly, none of these conditions resulted in elevated levels of dnaJ-H in either sex, showing that dnaJ-H is not induced in response to short-term heat stress. Next, we tested whether dnaJ-H might be induced under starvation in these flies by starving them and collecting the flies at 90% survival (a time at which 10% of the flies undergoing starvation were dead). Again, dnaJ-H levels were unperturbed with this intervention.

In order to test the affect of cellular toxicity on dnaJ-H induction, we also tested the response of dnaJ-H levels to Rotenone, H_2O_2, or Paraquat exposure. We exposed the flies to these stresses and collected flies at 10% mortality, in the hopes of catching the flies that were highly stressed, but still alive. The Rotenone and H2O2 tests again showed no increase in dnaJ-H transcript levels, however, Paraquat treatment resulted in an approximately 2-fold increase in DNAJ-H levels in w^{1118} males and females and in Canton S females (Fig. 2-8A). We compared this increase in dnaJ-H to the increase in Hsp27 levels to confirm activation of stress response genes (Fig. 2-8B). These results represent two biological replicates of the experiment, however a third and fourth biological replicate revealed no change in dnaJ-H levels with paraquat treatment.

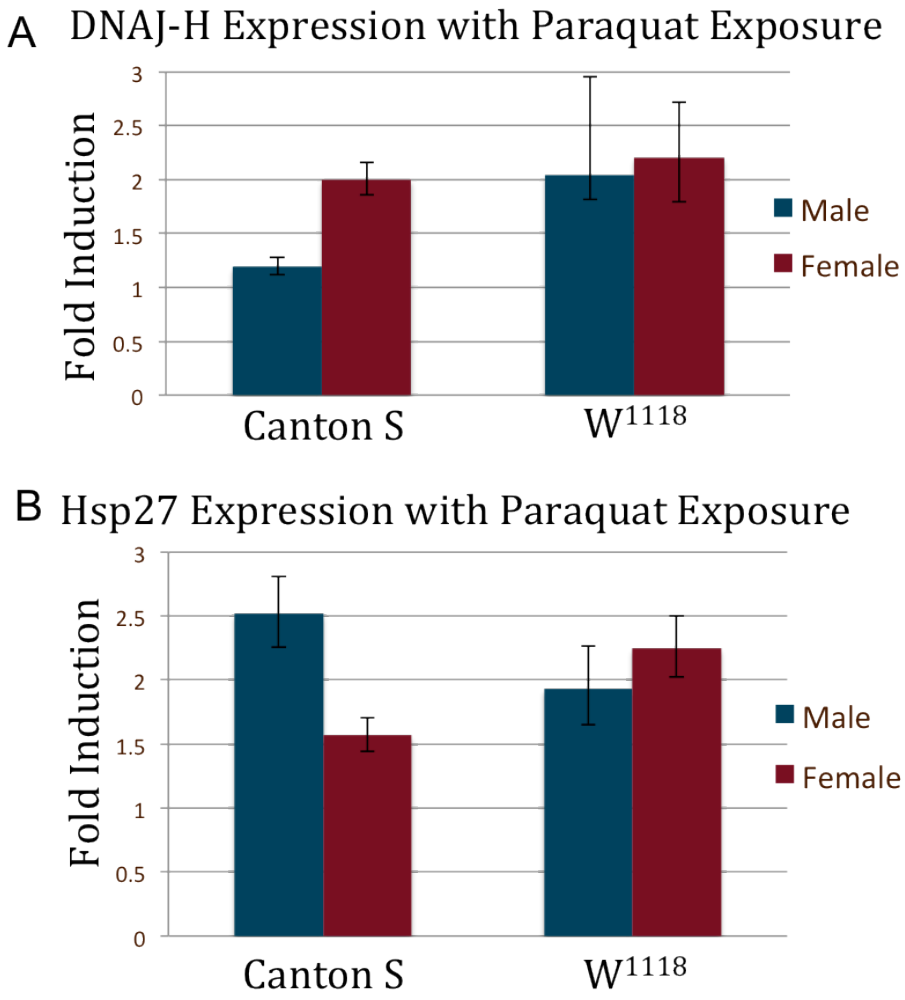

Figure 2-8.
Paraquat treatment leads to a 2-fold increase in dnaJ-H levels in Canton S and w^{1118} females, and in w^{1118} males (A). A similar increase is seen in Hsp27 as a positive control for induction of cellular stress response (B). Error bars represent SD of 2 biological replicates.

Sir2(G) transgenic line contains a V5 tag in-frame with UAS-Sir2

In addition to the lethality caused by the toxically high levels of dSir2 over-expression seen with expression of the Sir2(G) transgenic flies, I also wanted to conduct sequencing of the dSir2 insertion to determine whether there might be any toxic mutations or other irregularities within the sequence. In order to isolate the Sir2 sequence, I first attempted amplification of the construct using primers for the minimal Hsp70

promoter that is associated with the UAS sequence used in the production of the construct. However, despite trying several different primers designed against the Hsp70 promoter and the UAS sequence itself, I was unable to successfully amplify the 5' end of the Sir2(G) sequence. However, after a close reading of the manuscript, it became apparent that the cloning vector (pAc5.1) used to clone the dSir2 sequence originally contained an in-frame C-terminal V5 tag. In order to test whether the Sir2(G) flies still contained this V5 tag, I designed a reverse primer containing the V5 sequence from the pAc5.1 vector and performed PCR to amplify a fragment from 715 bp into the sir2 gene to the V5 tag. As a negative control, the same reactions were performed on the Sir2-1 line, which contains an extra Sir2 transgene of known sequence. Interestingly, the sequence was successfully amplified. To confirm that the V5 sequence was in-frame with the Sir2 gene, the resulting fragment was sent for sequencing using the same reverse V5 primer. Sequencing confirmed that the Sir2 gene contained a C-terminal V5 tag.

Discussion

Here we show that in *Drosophila,* increased expression of *dSir2* extends life span in a dose-dependent manner. By measuring life span while directly titrating the increase in *dSir2* expression through use of a series of new and available UAS-dSir2 transgenes, and by determining the level of dSir2 expression under conditions for previously published life span studies, we found that when *dSir2* expression is increased to moderate levels (approximately 2-5 fold increased over control levels), life span is consistently extended.. Expression below this range (less than 2-fold increase), or slightly above it (between 5-10 fold increase) inconsistently extends life span, while higher levels of

expression are detrimental to life span, and can induce JNK signaling and dnaJ-H expression (Fig. 2-9). Consistent with our findings that the dose of dSir2 is important in longevity determination, previous work has shown that a reduction in dSir2 levels shortens life span (Åström et al., 2003). Our demonstration that the dose of dSir2 is critical to life span extension suggests an explanation for the contrary findings reporting an inability to detect life span extension when using $dSir2^{EP2300}$ or two new UAS-dSir2 transgenes (Burnett et al., 2011). In that report (Burnett et al., 2011), dSir2 expression from the Sir2-myc2 and $dSir2^{EP2300}$ transgenes were both reported to be below the critical level we determined is necessary for life span extension (~1.5-fold increase), and in the Sir2-myc9 transgene, the level of expression is likely too high (~8-fold). To confirm the hypothesis that the optimal range for dSir2 expression was not achieved in this study (Burnett et al., 2011), we used these same two lines (Sir2-myc2 and Sir2-myc9) in conjunction with the conditional Gene Switch system to increase expression of dSir2 between 2 and 5-fold. In this report we demonstrate that when dSir2 expression is increased to within the optimal range, both of these UAS-Sir2 transgenes extend life span compared with their genetically identical sibling cohorts, in which dSir2 was not increased.

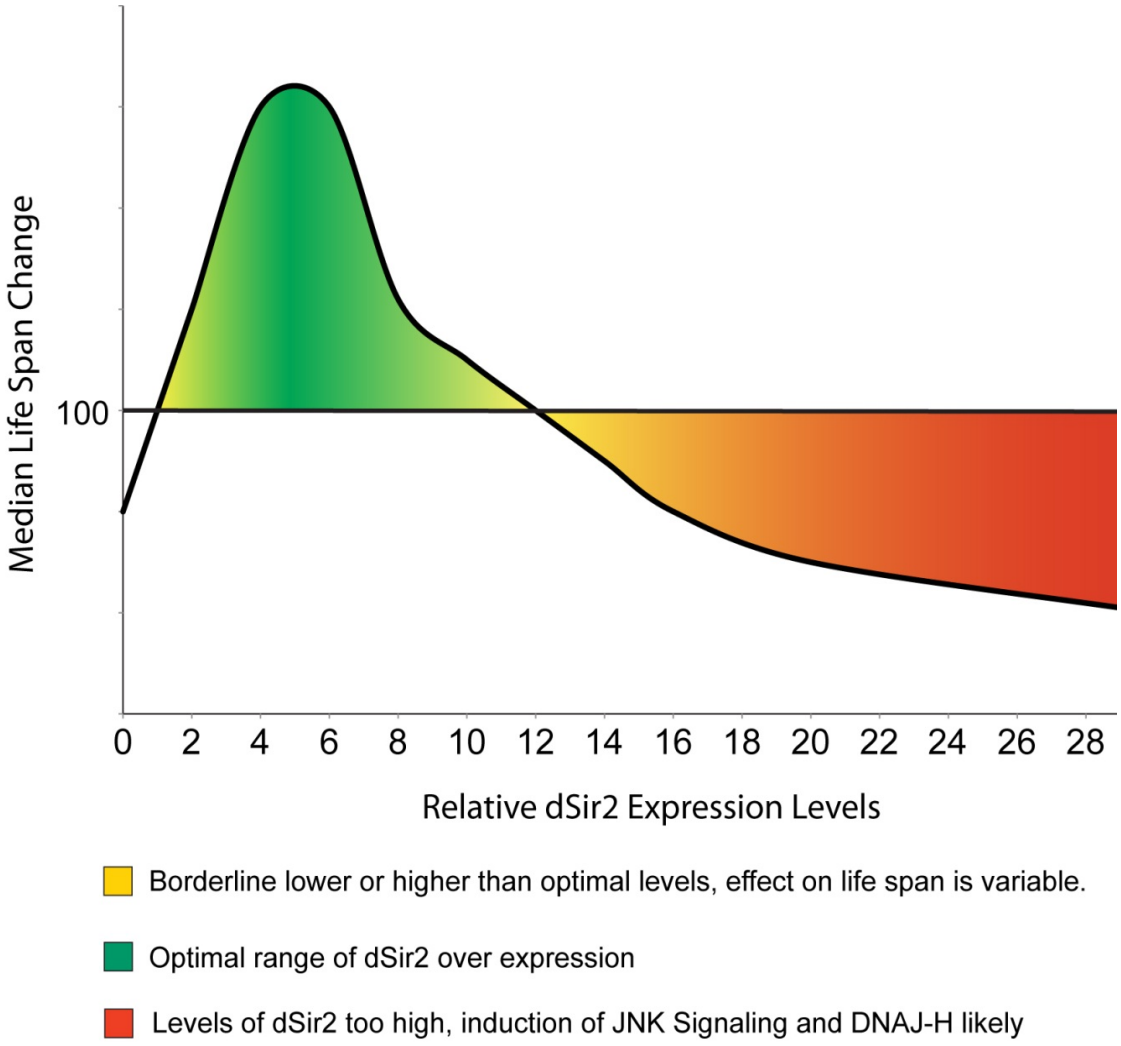

Figure 2-9.
Model for the effect of increasing dSir2 expression on life span in *Drosophila*. Between 2-5 fold increased dSir2 expression has a consistently beneficial effect on life span (green). However, lower increases below 2-fold, and higher increases above 5-fold, have more inconsistent effects on life span (yellow). A high level of expression induces JNK signaling and dnaJ-H expression is increased (red).

Sir2 null flies in our experiment appear to be shorter lived than controls, and respond to calorie restriction at the 0.5N dilution of the food. However, this data is tentative at best, given the confusion in the literature over the effect of dSir2 null mutations on life span. Important to note is that on 0.5N food, there appears to be no difference in mortality, age-specific or age-independent, between the Sir2 null and controls, although the Sir2 nulls still had a reduced life span compared with the controls

on that food type and all others. In addition to suggesting that Sir2 may not be required for the calorie restriction response, the result that Sir2 nulls were shorter lived than their genetically matched controls confirms that Sir2 nulls have reduced life span, as shown in (Åström et al., 2003). Given that Sir2 interacts with a large host of proteins, many of which are known to be involved in life span as well, it is somewhat surprising that an animal lacking Sir2 is able to survive through development, and still be able to respond to DR, but is not surprising than a Sir2 null has a reduced overall life span on any food type.

Although dSir2 mutants appear to respond to DR in these preliminary experiments, there is a large amount of evidence that suggests that dSir2 extends life span through mechanisms that overlap (either partially or entirely) with that of DR. When transcription profiles of flies under DR and those with a life span extending dSir2 increase are compared, there is a highly significant overlap in the gene expression changes that are shared between the cohorts, and this shared overlap is higher than that of another life span extending intervention, the expression of a dominant-negative Dmp53 protein (Antosh et al., 2011). Additionally, the original study showing life span extension when dSir2 was over-expressed showed that the life span of these long-lived flies was not further extended by a low calorie diet (Rogina & Helfand, 2004), indicating that dSir2 and DR use overlapping mechanisms to extend life span.

The conflicting literature over whether dSir2 is required for the DR response (and uses an overlapping mechanism to extend life span) is likely muddled by the use of many different genetic backgrounds and null/hypomorphic mutations in these studies. Additionally, all of the published mutants are hypomorphs with imprecise p-element excisions that remove a portion of the dSir2 coding sequence, but in different genetic

backgrounds, some with second-site lethal mutations, and with varying degrees of disruption of dSir2 expression (Åström et al., 2003; Burnett et al., 2011; Newman et al., 2002; Rogina & Helfand, 2004). The benefit of a precisely excised dSir2 gene, as used in this study, is that it does not disrupt neighboring loci, introduce second-site mutations, or leave behind partial coding regions. However, the caveat of all of these techniques is that these flies have disrupted or absent dSir2 during development, as well as in the germline, likely leading to other abnormalities that are unrelated to dSir2's function in DR, but are due to dSir2's role during development. An additional consideration is that since dSir2 is a histone modifying gene, it is possible that subsequent generations of flies that are missing dSir2 may present problems in the germ line that complicate these studies, especially considering some of these mutations were isolated over 10 years ago (and thus, many hundreds of generations ago).

A technical solution to the problem of hypomorphs and null mutations does exist, however. As shown in (Kushal K Banerjee et al., 2013a; Kushal Kr Banerjee, Ayyub, Sengupta, & Seetharam, 2012; Kushal Kr Banerjee, Ayyub, Ali, et al., 2012), RNAi can be used to target a reduction in dSir2 expression in a tissue-specific and adult-specific way using the gene-switch system to induce RNAi expression during adulthood. Under these more optimal conditions, dSir2 knock down led to increased Triacylglyceride (TAG) levels, increased oil red o staining of fay body, a decrease in starvation survival, and a less nuclear localization of FOXO—all phenotypes which are shared by a richer diet, and which oppose the effects of DR. Finally, using RNAi to knock down dSir2 expression in the fat body, (Kushal Kr Banerjee, Ayyub, Ali, et al., 2012) shows that DR response to changing yeast concentrations in the food is abrogated when RNAi is used to

KD dSir2. This is the kind of result that would be expected if dSir2 is necessary for the DR response, and the improved dSir2 knock down technique lends credibility to these results over those obtained with hypomorphs and null mutants.

The dnaJ-H gene in Drosophila, while its function is unknown, has homology to heat shock proteins and chaperone proteins, and contains a J-domain. In order to better understand its functions in Drosophila, we tested whether several interventions elevated DNAJ-H transcript levels. We did not find any response in DNAJ-H levels with heat shock, starvation, rotenone, or H2O2 treatment, and found a transiently occurring induction with paraquat treatment. These results can suggest several possibilities for the role of DNAJ-H.

First, the lack of an induction in transcript levels does not necessarily mean that the gene plays no role in these responses, simply that the transcript levels are not increased. It is possible that an increase in DNAJ-H may be observed at the protein level due to post-transcriptional regulation, or that the protein is activated through chemical modifications, and is rarely increased in transcription. The fact that we see dnaJ-H levels increase with toxically high Sir2 and high GFP expression seems to indicate some possible role for dnaJ-H in protein-toxicity, and based on its transcriptional similarity to puc phosphatase transcription, may indicate a possible connection with JNK signaling. Additionally, that dnaJ-H induction under paraquat treatment is difficult to reproduce may indicate that timing is of a critical importance in being able to observe the increase in DNAJ-H levels during a stress response. In order to more accurately assess the role of DNAJ-H in drosophila, several lines of study are possible. First, to define the conditions under which DNAJ-H is induced, a time course of all of the above experiments should be

performed, with samples taken at early and late-stage time points during treatment. Second, other treatments mimicking protein stress, such as DTT, which causes induction of the unfolded protein response, should be tested for DNAJ-H induction.

We note that induction of *dSir2* from its native locus using the dSir2^{EP2300} insertion allele extends life span more reliably and to a greater extent than over-expression from the dSir2 transgenes at other genomic locations. This may be due to the more modest level of *dSir2* induction seen with dSir2^{EP2300}, but it may also suggest the presence of favorable endogenous regulatory elements that are maintained when *dSir2* is expressed from its native genomic location.

In (Griswold et al., 2008), an increase in *dnaJ-H* was observed when dSir2^{EP2300} was used to over express *dSir2* in the eye using a GMR-Gal4 driver, and it was concluded that dSir2^{EP2300} over-expressed both *dnaJ-H* and *dSir2* due to their overlapping genetic locus, and that this accounted for the life span extension. However, Bauer et al. (Bauer et al., 2009) have shown that *dnaJ-H* is not induced when dSir2^{EP2300} is used to over express *dSir2* in life span-extending conditions in neurons(Bauer et al., 2009), and Banerjee et al. (Kushal Kr Banerjee, Ayyub, Ali, et al., 2012) confirmed this result when over-expressing dSir2 in fat body without any increase in dnaJ-H. Furthermore, we found that *dnaJ-H* induction was observed when *dSir2* or GFP was highly expressed from transgenic lines that are not located near the native dSir2 / dnaJ-H genomic location. The dSir2 expression conditions that exhibited an increase in *dnaJ-H* levels also showed elevated transcripts of *puc phosphatase*, a target of JNK signaling. Taken together, these results show that *dSir2* over-expression can induce JNK signaling/puc phosphatase as previously reported, but only when *dSir2* is expressed at

toxically high levels. The V5 tag on the Sir2(G) line may indicate that there is enhanced lethality with the level of over-expression of the construct, since the V5 tag is relatively large (compared to a smaller HA or Flag tag), and it is not known whether the incorporation of this sequence may affect dSir2 activity, folding, or turnover.

Our observation that very high over expression of dSir2 is toxic is consistent with previously published results showing that expression of *dSir2* from a transgene induced lethality and activated JNK signaling(Griswold et al., 2008). In this previous publication(Griswold et al., 2008) it was reported that any increased expression of *dSir2* from a transgene distant from the native dSir2 site is lethal (Griswold et al., 2008). However, we found, using the Gene Switch inducible system, that dSir2 expression from this transgenic line is at very high levels, confirming that cytotoxicity is only seen at high levels of dSir2 expression and that lethality occurs because the transgene used in (Griswold et al., 2008) expresses *dSir2* at very high levels.

The finding that high levels of dSir2 expression can lead to cytotoxicity is perhaps not surprising given the many known interacting partners of dSir2, including proteins central to metabolism (FOXO), mitochondrial biogenesis (PGC1-a), and genomic defense (p53, KU-70)(Haigis & Guarente, 2006). It is important to note that lethality in the previous study (Griswold et al., 2008) was shown and measured using the eye-specific GMR-Gal4 driver, which has previously been shown to induce mild developmental defects and apoptosis in the *Drosophila* eye, even without over-expression of another gene. This suggests that the GMR-Gal4 system that was used to demonstrate lethality may have already been partially sensitized to apoptotic phenotypes (Kramer & Staveley, 2003).

We conclude that increasing *dSir2* expression in *Drosophila* can extend life span, but caution that experiments increasing expression of *dSir2* should ensure that *dSir2* levels are both sufficient to induce a phenotype, and are not at toxically high levels. The conflicting reports in the literature over whether *dSir2* over-expression extends life span are resolved when the dosage of *dSir2* is taken into consideration.

Materials and Methods

***Drosophila* Stocks and Maintenance**

All flies were maintained at 25˚C in a temperature-controlled incubator at 50% humidity with a 12-hour light/dark cycle. The Tubulin-Gal4 (5138), ELAV-Gal4 (458), and Daughterless-Gal4 (5460) lines were from Bloomington Stock Center. ELAV Gene Switch was a gift from H. Keshishian (Yale University, New Haven, CT). dSir2^{EP2300} from the Bloomington stock center was backcrossed 9 times into the w^{1118} control line before use in qPCR studies. The lethal UAS-dSir2(G) line was a kind gift from KT Min. The Tubulin Gene Switch and Myc-tagged UAS-dSir2 lines (Myc2 and Myc9) were kind gifts from S. Pletcher.

Construction of UAS-dSir2 lines

To generate the UAS-dSir2 lines (Sir2-1, Sir2-4 and Sir2-4), full length dSir2 was cloned from an adult *Drosophila* cDNA library into pDONR221 (Invitrogen) and then the pTW vector (Drosophila Gateway Vector Collection, Carnegie Institute) using the Gateway system. Primers used were dSir2-GW-F:

GGGGACAAGTTTGTACAAAAAAGCAGGC

TGCACCATGATGGAAAATTACGAGGAA, and dSir2-GW-R:

GGGGACCACTTTGTACAAGAA AGCTGGGTCCACTGCTGCTAACTGTCCTGG, amplifying a 2.4 kb fragment. Embryo injection of pTW-dSir2 into the w^{1118} background was performed by Bestgene, Inc. (Chino Hills, CA). Flies were selected for germline transformation, and kept as homozygous stocks. Different lines were established from five separate integration events.

Life span Assays

All life span experiments were performed on food containing 150 g/L sucrose, 150 g/L autolysed yeast, and 20 g/L agar, all w/v. Life span experiments using UAS-Sir2 Myc2 or UAS-Sir2 Myc9 lines were performed on food containing 150 g/L dextrose, 150 g/L autolysed yeast, and 20 g/L agar, all w/v. Life span experiments using Gene Switch drivers were conducted on food containing these ingredients plus either RU-486 (Cayman Chemical, Ann Arbor, MI) in ethanol at the stated concentration, or the diluent alone (20 ml/L ethanol) for the control. Flies were collected under light CO_2 anesthesia, randomly divided into treatment groups, and housed at a density of 25 males and 25 females per vial. Flies were passed onto fresh food every other day (Experiments labeled "EOD" in Table 2-1) or every day (labeled "ED" in Table 2-1), and the number and sex of dead flies recorded.

RNA Isolation and qPCR

Flies for qPCR analysis were collected in a 24-hour window and grown under the same conditions as flies in life span experiments. They were passed onto new food every other day (or every day for life spans passed every day), and on day 10, were snap-frozen in liquid nitrogen and stored at -80˚C. For mRNA isolation, 30 whole male flies (for

ubiquitious driver experiments) or male fly heads (for ELAV-Gal4 and ELAV-Gene Switch experiments) were used to isolate mRNA using the Dynabeads mRNA DIRECT kit (Life Technologies). mRNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad). 50 ng of cDNA was used as qPCR template. qPCR was performed on a ABI 7500 FAST Real-Time PCR machine, using SYBR Green PCR Master Mix (Life Technologies). Each qPCR run was performed using three biological replicates in triplicate. Primer sequences used for amplification can be found in Table 2-4. GAPDH was used as a normalizing gene.

Sir2 Null Life Spans

Sir2 nulls from Bloomington (8838) were backcrossed 10 times into w^{1118} using PCR to validate genetic identity. Life span flies were sorted on light CO2, randomly divided into treatment groups, housed at a density of 25 males and 25 females per vial, and the flies were passed onto new food every other day and dead flies sexed and counted. “1.5N” food contained 150 g/L sucrose, 150 g/L autolysed yeast, and 20 g/L agar, all w/v. “1.0N” food contained 100 g/L sucrose, 100 g/L autolysed yeast, and 20 g/L agar, all w/v. “0.5N” food contained 50 g/L sucrose, 50 g/L autolysed yeast, and 20 g/L agar, all w/v. “0.3N” food contained 30 g/L sucrose, 30 g/L autolysed yeast, and 20 g/L agar, all w/v.

Paraquat, Rotenone, H_2O_2, and Starvation Stress of flies for dnaJ-H analysis

Flies were treated with starvation by syncing them (4 hours on 2% agar, then 2 hours on 1.5N food), then were placed onto 2% agar (or 1.5N food for controls) for 16 hours and collected by snap freezing in LN_2. Flies were heat shocked at 37 degrees C by incubation in a water bath for 15 min, 30 min, 1 hour, or 4 hours (control flies were incubated for the same length of time at 25 degrees in a water bath) and collected by snap freezing in LN_2.

Flies for Rotenone experiments were synced (2% agar for 4 hours, then 2% 1.5N food for 2 hours) before being placed into treatment vials. Treatment vials contained a 1" whatman disc soaked with either rotenone solution or control solution. For rotenone solution, rotenone was dissolved in DMSO to 1 mM and added to 5% sucrose solution with 15 mM tegocept. Control solution contained the equivalent volume of DMSO and tegocept in the 5% sucrose solution, without the addition of Rotenone. For Paraquat treatment, flies were synced as above, then placed into vials containing 20 mM paraquat in a 5% sucrose solution, or just a 5% sucrose solution for control flies.

Flies for hydrogen peroxide experiments were synced as above, then placed into treatment vials containing a 1" whatman disc soaked with 5% sucrose (for control flies) or 5% sucrose and 5% H_2O_2. Flies for the Rotenone, Paraquat, and H_2O_2 experiments were raised in mixed-sex vials on 1.5N food until 5 days of age, then sorted into 10 single-sex vials per treatment group, each vial containing 10 flies. In order to ensure that desiccation did not occur, treatment vials were re-treated with fresh solution every 12 hours. After treatment, the number of dead flies was recorded every 4-8 hours until the percent remaining alive was less than 90%, and then flies were snap-frozen on LN_2 and mRNA was isolated and qPCR performed as above.

Statistical Analysis

All life spans were analyzed in a Log Rank test using the Survival package in R.

Student's t-test

was used to analyze the statistical significance of qPCR data.

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Table 2-1: Life Span Information and Statistics

Control	Condition	Sex	Mean Life Span Condition/Ctrl	% Increase in Mean Life Span	Median Life Span Condition/Ctrl	% Increase in Median Life Span
Tub-W1118	Tub-Sir2-1	M	38.20/54.75	-30.228	39/53	-26.415
Tub-W1118	Tub-Sir2-3	M	59.51/54.75	8.694	59/53	11.321
Tub-Sw-W1118-etoh	Tub-Sir2-3-etoh	M	69.03/61.77	11.753	68/63	7.937
Tub-Sw-W1118-etoh	Tub-Sw-W1118-200RU	M	59.99/61.77	-2.882	63/63	0.000
Tub-Sw-Sir2-1-etoh	Tub-Sw-Sir2-1-200RU	M	47.82/54.74	-12.642	47/63	-25.397
Tub-Sw-Sir2-3-etoh	Tub-Sw-Sir2-3-200RU	M	69.04/69.03	0.014	70/68	2.941
Tub-Sw-Sir2-4-etoh	Tub-Sw-Sir2-4-200RU	M	66.19/67.37	-1.752	66/67	-1.493
W1118	Het Sir2-1	M	65.38/68.72	-4.860	67/70	-4.286
W1118	Het Sir2-3	M	77.28/68.72	12.456	82/70	17.143
W1118	Het Sir2-4	M	77.99/68.72	13.490	82/70	17.143
Elav-Sw-W1118	Elav-Sw-Sir2-3	M	57.36/54.11	6.006	57/55	3.636
Elav-Sw-Sir2-3 etoh	Elav-Sw-Sir2-3-500RU	M	64.01/57.36	11.593	65/57	14.035
Tub-Sw-Myc2-etoh	Tub-Sw-Myc2-100	F	62.3/65.4	-4.740	64/66	-3.030
Tub-Sw-Myc2-etoh	Tub-Sw-Myc2-200	F	70.3/65.4	7.492	72/66	9.091
Tub-Sw-Myc2-etoh	Tub-Sw-Myc2-500	F	73.3/65.4	12.080	78/66	18.182
Tub-Sw-Myc9-etoh	Tub-Sw-Myc9-100	F	68.14/69.14	-1.446	74/76	-2.632
Tub-Sw-Myc9-etoh	Tub-Sw-Myc9-200	F	76.9/69.14	11.224	80/76	5.263
Tub-Sw-Myc9-etoh	Tub-Sw-Myc9-500	F	78.1/69.14	12.959	82/76	7.895

Life Span Information and Statistics
(Continued-2)

Control	Condition	Sex	Control # Flies	Condition # Flies	chisq	p-value	ED/EOD
Tub-W1118	Tub-Sir2-1	M	315	206	205	0	EOD
Tub-W1118	Tub-Sir2-3	M	315	296	20.7	<0.0001	EOD
Tub-Sw-W1118-etoh	Tub-Sir2-3-etoh	M	300	282	56.9	<0.0001	ED
Tub-Sw-W1118-etoh	Tub-Sw-W1118-200RU	M	300	294	1.6	0.199	ED
Tub-Sw-Sir2-1-etoh	Tub-Sw-Sir2-1-200RU	M	293	266	51	<0.0001	ED
Tub-Sw-Sir2-3-etoh	Tub-Sw-Sir2-3-200RU	M	282	276	0.2	0.63	ED
Tub-Sw-Sir2-4-etoh	Tub-Sw-Sir2-4-200RU	M	289	297	17.3	<0.0001	ED
W1118	Het Sir2-1	M	279	301	10.6	0.00112	ED

W1118	Het Sir2-3	M	279	296	87.9	0	ED
W1118	Het Sir2-4	M	279	290	75.8	0	ED
Elav-Sw-W1118	Elav-Sw-Sir2-3	M	358	281	17.5	<0.0001	EOD
Elav-Sw-Sir2-3 etoh	Elav-Sw-Sir2-3-500RU	M	281	293	52.5	<0.0001	EOD
Tub-Sw-Myc2-etoh	Tub-Sw-Myc2-100	F	252	264	4.1	0.0433	EOD
Tub-Sw-Myc2-etoh	Tub-Sw-Myc2-200	F	252	245	14.6	0.000134	EOD
Tub-Sw-Myc2-etoh	Tub-Sw-Myc2-500	F	252	246	52.8	<0.0001	EOD
Tub-Sw-Myc9-etoh	Tub-Sw-Myc9-100	F	247	238	0.7	0.415	EOD
Tub-Sw-Myc9-etoh	Tub-Sw-Myc9-200	F	247	233	31.4	<0.0001	EOD
Tub-Sw-Myc9-etoh	Tub-Sw-Myc9-500	F	247	251	51.1	<0.0001	EOD

Life Span Information and Statistics (Continued-3)

Control	Condition	Sex	Mean Life Span Condition/Ctrl	% Increase in Mean Life Span	Median Life Span Condition/Ctrl	% Increase in Median Life Span
Tub-Sw-Myc2-etoh	Tub-Sw-Myc2-100	M	60.4/57.36	5.300	64/64	0.000
Tub-Sw-Myc2-etoh	Tub-Sw-Myc2-200	M	61.04/57.36	6.416	64/64	0.000
Tub-Sw-Myc2-etoh	Tub-Sw-Myc2-500	M	63.88/57.36	11.367	66/64	3.125
Tub-Sw-Myc9-etoh	Tub-Sw-Myc9-100	M	62.60/61.78	1.327	68/64	6.250
Tub-Sw-Myc9-etoh	Tub-Sw-Myc9-200	M	67.57/61.78	9.372	70/64	9.375
Tub-Sw-Myc9-etoh	Tub-Sw-Myc9-500	M	66.06/61.78	6.928	70/64	9.375
Tub-W1118	Tub-Sir2-1	F	48.56/62.49	-22.292	49/61	-19.672
Tub-W1118	Tub-Sir2-3	F	66.55/62.49	6.497	67/61	9.836
Tub-Sw-W1118-etoh	Tub-Sir2-3-etoh	F	78.96/77.53	1.844	80/80	0.000
Tub-Sw-W1118-etoh	Tub-Sw-W1118-200RU	F	78.13/77.53	0.774	79/80	-1.250
Tub-Sw-Sir2-1-etoh	Tub-Sw-Sir2-1-200RU	F	61.25/69.55	-11.934	62/71	-12.676
Tub-Sw-Sir2-3-etoh	Tub-Sw-Sir2-3-200RU	F	82.74/78.96	4.787	85/80	6.250
Tub-Sw-Sir2-4-etoh	Tub-Sw-Sir2-4-200RU	F	71.54/70.50	1.475	72/71	1.408
W1118	Het Sir2-1	F	70.50/61.53	14.578	75/64	17.188
W1118	Het Sir2-3	F	73.06/61.53	18.739	79/64	23.438
W1118	Het Sir2-4	F	68.23/61.53	10.889	72/64	12.500
Elav-Sw-W1118	Elav-Sw-Sir2-3	F	68.53/66.37	3.254	69/69	0.000
Elav-Sw-Sir2-3 etoh	Elav-Sw-Sir2-3-500RU	F	70.74/69.18	2.255	71/69	2.899

Life Span Information and Statistics (Continued-4)

Control	Condition	Sex	Control # Flies	Condition # Flies	chisq	p-value	ED/EOD
Tub-Sw-Myc2-etoh	Tub-Sw-Myc2-100	M	228	226	0.3	0.606	EOD
Tub-Sw-Myc2-etoh	Tub-Sw-Myc2-200	M	228	252	0.1	0.7	EOD
Tub-Sw-Myc2-etoh	Tub-Sw-Myc2-500	M	228	239	7.5	0.00626	EOD
Tub-Sw-Myc9-etoh	Tub-Sw-Myc9-100	M	245	247	7.7	0.00547	EOD
Tub-Sw-Myc9-etoh	Tub-Sw-Myc9-200	M	245	230	20.3	<0.0001	EOD
Tub-Sw-Myc9-etoh	Tub-Sw-Myc9-500	M	245	240	8.7	0.00321	EOD
Tub-W1118	Tub-Sir2-1	F	349	200	94.3	0	EOD
Tub-W1118	Tub-Sir2-3	F	349	289	6.9	0.00881	EOD
Tub-Sw-W1118-etoh	Tub-Sir2-3-etoh	F	297	282	0.1	0.805	ED

Tub-Sw-W1118-etoh	Tub-Sw-W1118-200RU	F	297	304	4.6	0.0317	ED
Tub-Sw-Sir2-1-etoh	Tub-Sw-Sir2-1-200RU	F	291	271	102	0	ED
Tub-Sw-Sir2-3-etoh	Tub-Sw-Sir2-3-200RU	F	282	285	48.2	<0.0001	ED
Tub-Sw-Sir2-4-etoh	Tub-Sw-Sir2-4-200RU	F	301	301	0.8	0.381	ED
W1118	Het Sir2-1	F	310	297	28	<0.0001	ED
W1118	Het Sir2-3	F	310	298	73.1	0	ED
W1118	Het Sir2-4	F	310	310	11	0.000929	ED
Elav-Sw-W1118	Elav-Sw-Sir2-3	F	366	293	4.7	0.0309	EOD
Elav-Sw-Sir2-3 etoh	Elav-Sw-Sir2-3-500RU	F	293	297	0.3	0.564	EOD

Table 2-2.
dSir2 expression levels in reported conditions.

Control	Over expression condition	dSir2 mRNA Level	p-value
Tub-W1118	Tub-Sir2-1	43.21	<0.0001
Tub-W1118	Tub-Sir2-3	11.19	<0.0001
Tub-Sw-W1118-etoh	Tub-Sir2-3-etoh	1.30	0.0078
Tub-Sw-Sir2-1-etoh	Tub-Sw-Sir2-1-200RU	55.69	<0.0001
Tub-Sw-Sir2-3-etoh	Tub-Sw-Sir2-3-200RU	11.65	<0.0001
Tub-Sw-Sir2-4-etoh	Tub-Sw-Sir2-4-200RU	15.30	0.00021
W1118	Het Sir2-1	1.29	0.00278
W1118	Het Sir2-3	1.48	0.00224
W1118	Het Sir2-4	1.88	<0.0001
Elav-Sw-W1118	Elav-Sw-Sir2-3	1.45	0.00234
Elav-Sw-Sir2-3 etoh	Elav-Sw-Sir2-3-500RU	4.98	<0.0001
Tub-Sw-Myc2-etoh	Tub-Sw-Myc2-100	1.87	0.00332
Tub-Sw-Myc2-etoh	Tub-Sw-Myc2-200	3.78	<0.0001
Tub-Sw-Myc2-etoh	Tub-Sw-Myc2-500	4.94	0.00029
Tub-Sw-Myc9-etoh	Tub-Sw-Myc9-100	3.12	0.01865
Tub-Sw-Myc9-etoh	Tub-Sw-Myc9-200	4.54	0.00068
Tub-Sw-Myc9-etoh	Tub-Sw-Myc9-500	7.54	<0.0001

Table 2-3.

Over-expression Condition	Control	dSir2 Level	dSir2 p-value	DNAJ-H Level	DNAJ-H p-value	puc level	puc p-value
Elav-Gal4-EP2300	Elav-Gal4-W1118	3.41	<0.0001	0.745	0.34245	0.981	0.18223
Elav-Gal4-Sir2-1	Elav-Gal4-W1118	12.72	0.00033	2.333	0.00025	2.456	0.00249
Elav-Gal4-Sir2-3	Elav-Gal4-W1118	6.196	0.00348	1.593	0.00377	1.862	0.01736
Elav-Gal4-Sir2-4	Elav-Gal4-W1118	7.136	0.00189	1.812	0.00512	2.303	0.0088
Elav-Switch-Sir2-1, 500 uM RU	Elav-Switch-Sir2-1, etoh	32.946	<0.0001				
Elav-Switch-Sir2-3, 500 uM RU	Elav-Switch-Sir2-3, etoh	4.998	0.00036				
Elav-Switch-Min UAS-Sir2, 500 uM RU	Elav-Switch-Min UAS-Sir2, etoh	49.607	<0.0001				
Tub-Switch-Min UAS-Sir2, 200 uM RU	Tub-Switch-Min UAS-Sir2, etoh	24.825	<0.0001	2.863	<0.0001		
Tub-Switch-Min UAS-Sir2, 50 uM RU	Tub-Switch-Min UAS-Sir2, etoh	3.233	0.00358	0.414	<0.0001		
Da-Gal4-UAS-GFP	Da-Gal4-YW			2.681	0.0021		

dnaJ-H and *puc* expression levels in reported conditions.

Table 4.
Primers used for qPCR amplification.

Gene	**Forward:**	**Reverse:**
GAPDH	GACGAAATCAAGGCTAAGGTCG	AATGGGTGTCGCTGAAGAAGTC
dSir2	GTCGAGAACGGCATCATGAAG	GGAGCCGATCACGATCAGTAG
DNAJ-H	GGTCTCAGCCCCTTCGATAC	AGGGACTGCACTTCTTGTCG
puc	GCCACATCAGAACATCAAGC	CCGTTTTCCGTGCATCTT

CHAPTER THREE: DIETARY SWITCH REVEALS FAST COORDINATED GENE EXPRESSION CHANGES IN *DROSOPHILA MELANOGASTER*

Dietary switch reveals fast coordinated gene expression changes in Drosophila melanogaster.

Abstract:

Dietary restriction (DR) is one of the most studied interventions known to extend life span. The robustness of its effect across species suggests the existence of conserved mechanisms to reduce mortality rates and increase longevity. However, because DR elicits a large number of physiological changes, many of which are unrelated to the longevity response, it has been difficult to identify these specific mechanisms. Whole-genome gene expression studies have typically reported several hundreds to thousands of differentially expressed genes in response to DR. The fruit fly *Drosophila melanogaster* shows a remarkable response to a change in diet: within 2-4 days after a switch to DR, mortality rates drop to the same level as cohorts continuously on DR (Partridge, Piper, & Mair, 2005). Based on this observation, we utilized a novel experimental design to enrich for genes directly associated with the longevity response. By profiling gene expression in a cohort of fruit flies that were switched from normal food to DR we were able to partition genes in several classes with distinct patterns of expression. One of these classes includes 146 genes rapidly switching within 1-2 days to the same level observed in the DR cohort, i.e. within the same time frame during which mortality rates drop. Pathway analysis revealed this class was enriched in genes involved in carbohydrate and fatty acid metabolism, but not oxidative phosphorylation – one of the pathways previously identified – indicating that transcriptional changes in the latter might be a secondary response to DR. Our work shows that the combination of a dietary switch with whole-

gnome time-course profiling is a powerful method that can reveal the transcriptional landscape more closely associated with the longevity response.

Introduction:

Dietary restriction (DR), i.e. reduction of nutrient intake to roughly 65% of ad libitum levels, has been studied extensively in a range of model organisms including yeast, *Saccaromyces cerevisiae* (Lin, Ford, Haigis, Liszt, & Guarente, 2004), the nematode, *Caenorhabditis elegans* (Houthoofd, 2003), and the fruit fly, *Drosophila melanogaster* (Bross, Rogina, & Helfand, 2005; Gershman et al., 2007; Zheng, Mutcherson, & Helfand, 2005). These studies have shown that dietary restriction can extend longevity and delay the occurrence and progression of age associated diseases. The ubiquity of this effect suggests that at least some of the molecular pathways involved are conserved across species.

In particular, flies respond very quickly to nutritional changes and age-specific mortality is reduced by up to 95% within 48 hours of exposure to nutrient-restricted conditions (Partridge, Piper, et al., 2005). Moreover, mortality remains lower than that seen in fully-fed flies as long as DR is maintained, and subsequent age-dependent changes at the organism and molecular levels are significantly altered thereafter (Pletcher et al., 2002).

By studying the age-dependent changes in gene expression in normal and experimentally manipulated flies it has been possible to identify some key molecular pathways that may regulate the aging process in Drosophila. While single gene analyses have concentrated on loci related to oxidative or general stress response, microarray data have revealed there are many molecular pathways responsive to DR, including

mitochondrial function and innate immunity (Antosh et al., 2011). A primary challenge for current research is to determine how DR modulates these systems, and in turn how they affect aging.

An emerging experimental approach to address these questions looks at the acute response of transcript levels to a shift in nutrient uptake. This has primarily been done in vertebrate and mammalian systems. Zebrafish, when overfed with artemia to create diet-induced obesity (DIO), showed changes in several physiological pathways when gene expression in adipose tissue was compared with DIO profiles from mice, rats, and humans (Oka et al., 2010). These changes included blood coagulation, platelet activation, fatty acid metabolism, cholesterol efflux, and triglyceride metabolism. Additionally, dogs fed an ad-libitum diet showed gene expression changes in metabolism, oxidation-reduction pathways, extracellular matrix, and immune response (Grant, Vester Boler, Ridge, Graves, & Swanson, 2011) as compared to those on a diet designed to maintain their weight. Finally, rats fed a reduced diet for one week or one month showed an enhancement in the expression of genes involved in ATP synthesis, AMPK, and stress response (Takahashi et al., 2011). These studies are able to highlight pathways that are involved in changes in diet.

In Drosophila, this approach is motivated by a remarkable observation: when adult flies are shifted from a restricted to an ad lib diet (or vice versa), age-specific mortality rapidly adjusts to the level of characteristic of the cohort that is continuously maintained upon this diet. These demographic transitions are rapid, being complete within 48 hours, suggesting that the response to changes in nutrients occurs over a short time scale.

The first analysis of this kind in Drosophila studied the nutrient response of 2nd instar larvae when starved or when fed only sugar (Zinke, Schütz, Katzenberger, Bauer, & Pankratz, 2002). Zinke et al used a 4-fold change criterion to identify several genes with interesting properties. Higher resolution was not practical because of limitations on replicates and, we believe, because the larvae were not developmentally and physiologically synchronized prior to the nutrient manipulation; these factors produce substantial error variance relative to the differences among treatments. Thus, while Zinke et al identified exciting individual candidates for the nutrient response, there was little power to detect many if not most of the responsive messages. A key goal of our current proposal is to measure nearly all of the transcriptional changes in response to acute nutrient manipulation – only from such data can we conduct systems level analysis to understand how DR modulates aging.

In contrast to Zinke et al, (Pletcher et al., 2002) examined the chronic effect of DR across the age-span of cohorts. The first time point for transcript changes were assessed three days after transfer to low calorie diet, making it improbable that nutrient-early-response genes, those most likely to have regulatory function, would have been detected. None-the-less, this design was very powerful and identified 2079 genes whose transcript abundance associated with adult diet. The majority of the changes were relatively small, less than 1.5 fold, suggesting that a sensitive approach will indeed be needed to acquire a 'saturation' description of nutrient responsive genes (Pletcher, Libert, & Skorupa, 2005).

By studying the age-dependent changes in gene expression in normal and experimentally manipulated animals it has been possible to identify some key molecular pathways that may regulate the aging process. While single gene analyses have

concentrated on loci related to oxidative or general stress response, microarray data have revealed there are many molecular pathways responsive to DR, including mitochondrial function and innate immunity. A primary challenge for current research is to determine how DR modulates these systems, and in turn how they affect aging. In Drosophila as many as 2721 genes have been shown to change in response to DR (Antosh et al., 2011). A recent meta-analysis of 40 DR gene expression studies has identified 12214 genes differentially expressed in mouse (Swindell, 2009). It is clear that, although a large number of genes respond to DR, only a fraction is likely causal to the longevity response.

In this study we present a novel experimental design to study the transcriptional response to DR that can enrich for genes that are more closely associated with changes in mortality. The overall design was based on the following observation. The fruit fly *Drosophila melanogaster* shows a remarkable response to nutritional changes: when adult flies are shifted from a control diet to a restricted diet (or vice versa), age-specific mortality rapidly adjusts to the level characteristic of the cohort that is continuously maintained upon this diet. These demographic transitions are rapid, being complete within 2-4 days (Good & Tatar, 2001; Mair, Goymer, Pletcher, & Partridge, 2003; Partridge, Pletcher, & Mair, 2005), suggesting that the response to changes in nutrients occurs over a short time scale. By sampling mRNA from flies immediately following the diet switch through the entire duration of the mortality switch, we were able to uniquely investigate the behavior patterns of different classes of genes, with the goal of identifying genes whose expression pattern displayed a complete transition from the original to the switched food level. We then describe enrichment of functional categories in the different temporal pattern detected as well as similarities and differences with other similar studies.

Results

Dietary switch induces fast reduction of mortality rate

Many studies have identified genes and pathways that are differentially activated during dietary restriction. However, many of these differences are probably not causal to the longevity effect but are linked to other physiological changes (e.g. fertility, behavior, etc.). It is likely that some of these changes might be due to a long term adaptation to the difference in diets. It is clear that, although many genes respond to DR, only a fraction of them is likely to be implicated in the longevity response. Flies show a remarkable response to changes in diet: flies reared at on high-calorie food show, after the DR switch, a fast drop in mortality to the same level as flies on low-calorie food (Partridge, Pletcher, et al., 2005). In essence, flies that were aging rapidly reverse the accumulated consequences of past senescence and, after the DR switch, age in a way that is indistinguishable from 'younger' flies that were aging at a slow rate all along; the reversal is rapid and usually completes within 2-3 days of the change in diet.

We first confirmed that switching fully fed flies to DR induces a rapid reduction in age-specific mortality (Figure 3-1A). Canton-S flies were collected in a 24-hour window, sorted into vials of 25 males and 25 females per vial, and randomly divided into treatment groups for the study. A total of 240 vials of flies were placed on control food (CF cohort), and 120 vials were placed on restricted food (RF cohort). Flies were passed onto new food and demographies were performed for all flies every day. During the course of the experiment, the mortality rate was analyzed for both cohorts and the separation of mortality rate between the RF and CF cohorts was verified before the

switch. On day 40, 120 vials of flies were switched from control food to restricted food (Switch Food, SF cohort) and demographies were continued. We observed a sharp drop in the mortality in the SF cohort following the diet switch: within 3 days following the switch, the age-specific mortality trajectory was the same as that observed in the RF cohort (Figure 3-1A). Hence, our results recapitulate the rapid switch in mortality observed in (Partridge, Pletcher, et al., 2005).

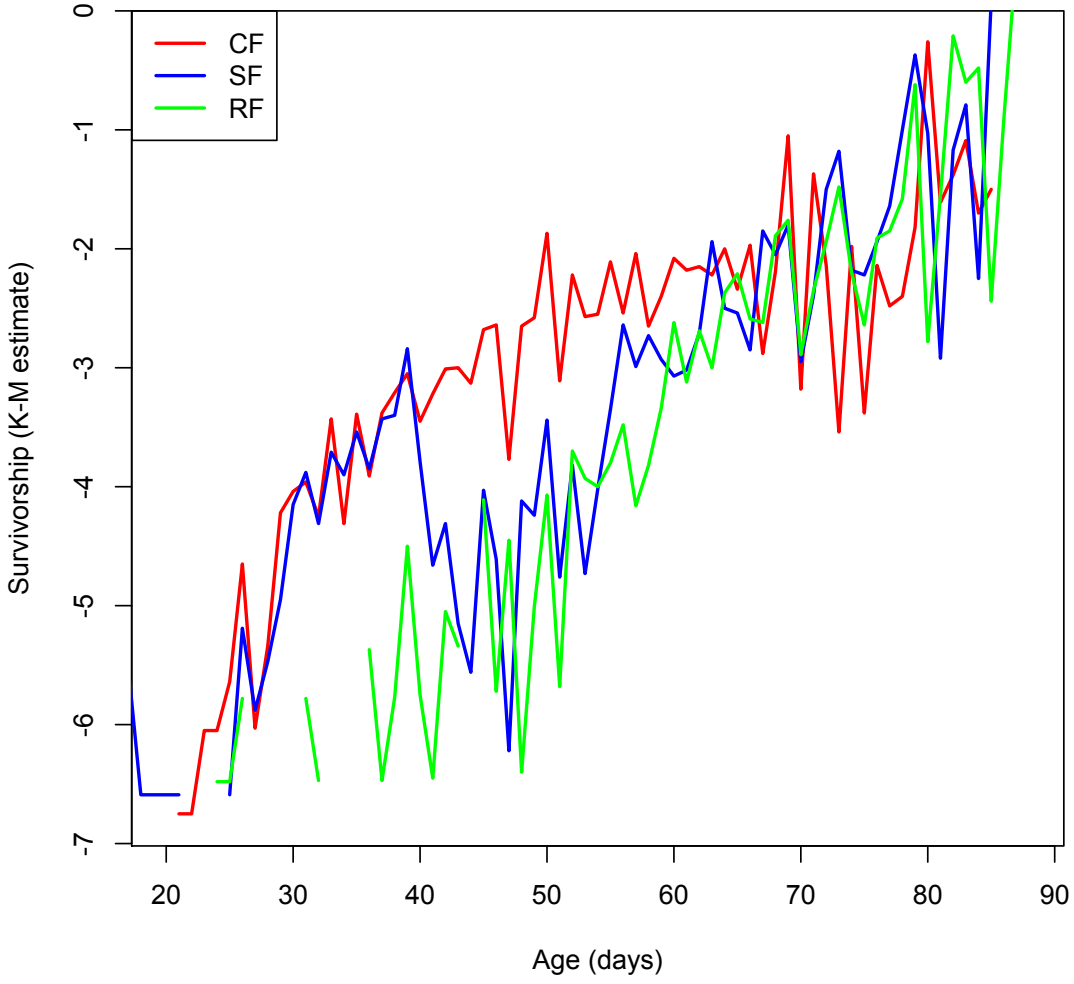

Figure 3-1. Rationale, experimental design and switch in mortality rate. (A). Age-specific mortality rates for female *Drosophila* vary according to calorie levels. Female *Drosophila* cultured in mixed-sex populations given higher-calorie diets (1.5N) show an acceleration of age-related increases in mortality rate as compared to females on lower-

calorie food (0.5N). The flies switched from high calorie to low calorie show a deceleration in mortality rate behaving as the low calorie cohort. Instantaneous Hazard of flies on normal food (Control Food, CF, red), Restricted Food (RF, green), or switched from normal food to restricted food at day 40 (SF, blue).

Gene responses to diet switch cluster into four different qualitative categories

We reasoned that most gene changes that are causal to this sudden drop in mortality must have occurred within the 3 days time window (Figure 3-1B). Hence we profiled gene expression in the three cohorts– a CF cohort continuously maintained on control food; a RF cohort continuously maintained on restricted food; and a SF cohort moved from control food to restricted food at day 40 –within the first 72 hours following the switch in food at variable intervals such that most time points were collected within the first day after the switch (0, 2, 4, 8, 12, 18, 24, 32, 40, 48, 56, 72 hours after switching) (Figure 3-1 C).

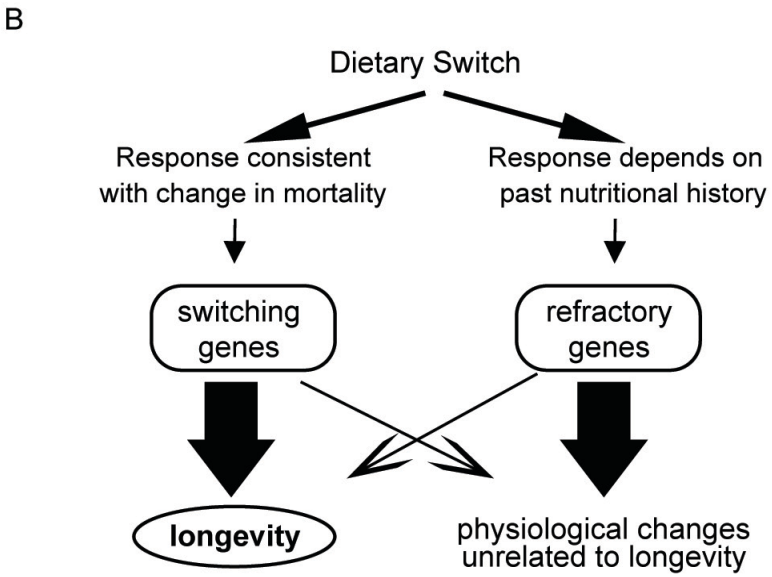

Figure 3-1B. After a dietary switch, genes will cluster into at least 2 categories: those that change their expression in a direction consistent with the change in mortality will be called switching genes, and are likely to play a role in the longevity response to diet. Genes whose expression does not change likely depend on the past nutritional history of the animals (Refractory Genes), and may

be genes that are either unrelated to longevity, or may be related to longevity but are not responsive within the time frame of the experiment. (Figure prepared by P.G.)

C

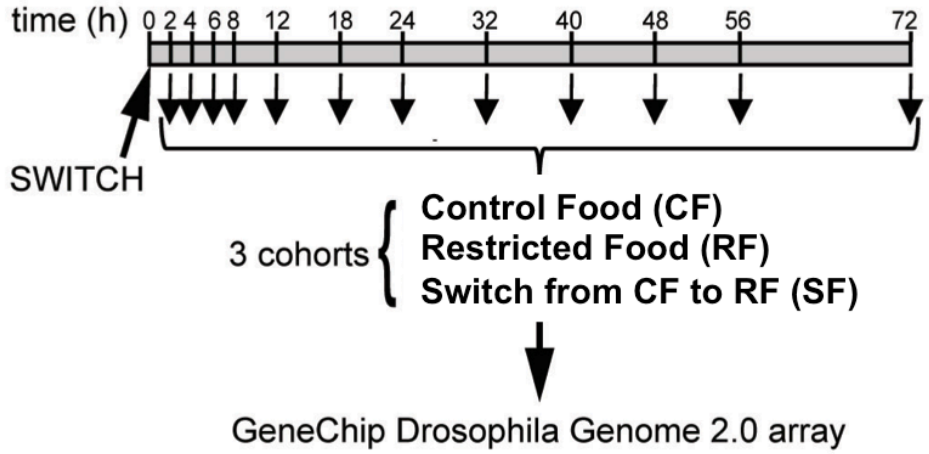

Figure 3-1C. Experimental Design. Flies were raised on Control Food or Restricted Food until day 40. At day 40, half of the flies on Control food were switched to the Restricted food. Flies were collected for microarray analysis at the time of the switch, and at 12 other time points until 72 hours after the switch. (Figure prepared by P.G.)

Female flies head/thorax was used in this study; abdomen was removed to avoid eggs, the number of which is known to be affected by DR. We first compared RF to CF using t-tests for each gene followed by p-value correction for multiple testing and detected 879 differentially expressed genes at a False Discovery Rate (FDR) of 5%. The overlap between this list and previously published data [1] in the same tissue was highly significant (35% overlap, hypergeometric test $p < 10^{-15}$), with differences likely a consequence of the different ages of flies in the two studies (40 days as opposed to 10 days), as previously reported (Antosh et al., 2011). Of the differentially expressed genes, 868 genes have been annotated as belonging to a gene ontology group (319 under-expressed and 549 over-expressed genes). Pathway analysis revealed that the differentially expressed genes were significantly enriched in 17 KEGG pathways (Table 3-1). Interestingly, we found that the metabolism of fatty acids and carbohydrates and oxidative phosphorylation were predominantly represented by enriched pathways.

We performed a similar analysis for SF versus CF, and SF versus RF. Computing the statistical significance of all three pairwise differences between cohorts, RF vs. CF, CF vs. SF, and RF vs. SF to find genes that were diet dependent (differentially expressed between CF and RF), diet responsive (differentially expressed between CF and SF), and switching (not differentially expressed between RF and SF) (Table 3-2). We found that of the 879 genes differentially expressed between RF and CF, only 146 showed a significant difference between SF and CF but no significant difference between SF and RF (Table 3-2); We refer to these genes, grouped on Category I, as "switching genes" as their response is consistent with the mortality change. Category II contains genes that are diet dependent, diet unresponsive and non-switching. We defined these genes "refractory genes", as their level depends on the past nutritional history. Category III contains genes that are diet dependent, diet responsive and non-switching. We defined these genes "responsive genes" as they respond to the diet switch but their response is not consistent with the mortality change. Category IV contains genes that are diet independent, diet responsive, and non-switching. We defined these genes "deviating genes" because they respond to the diet switch but not to the diet level. The typical expression pattern in each of the four categories are exemplified in Figure 3-2.

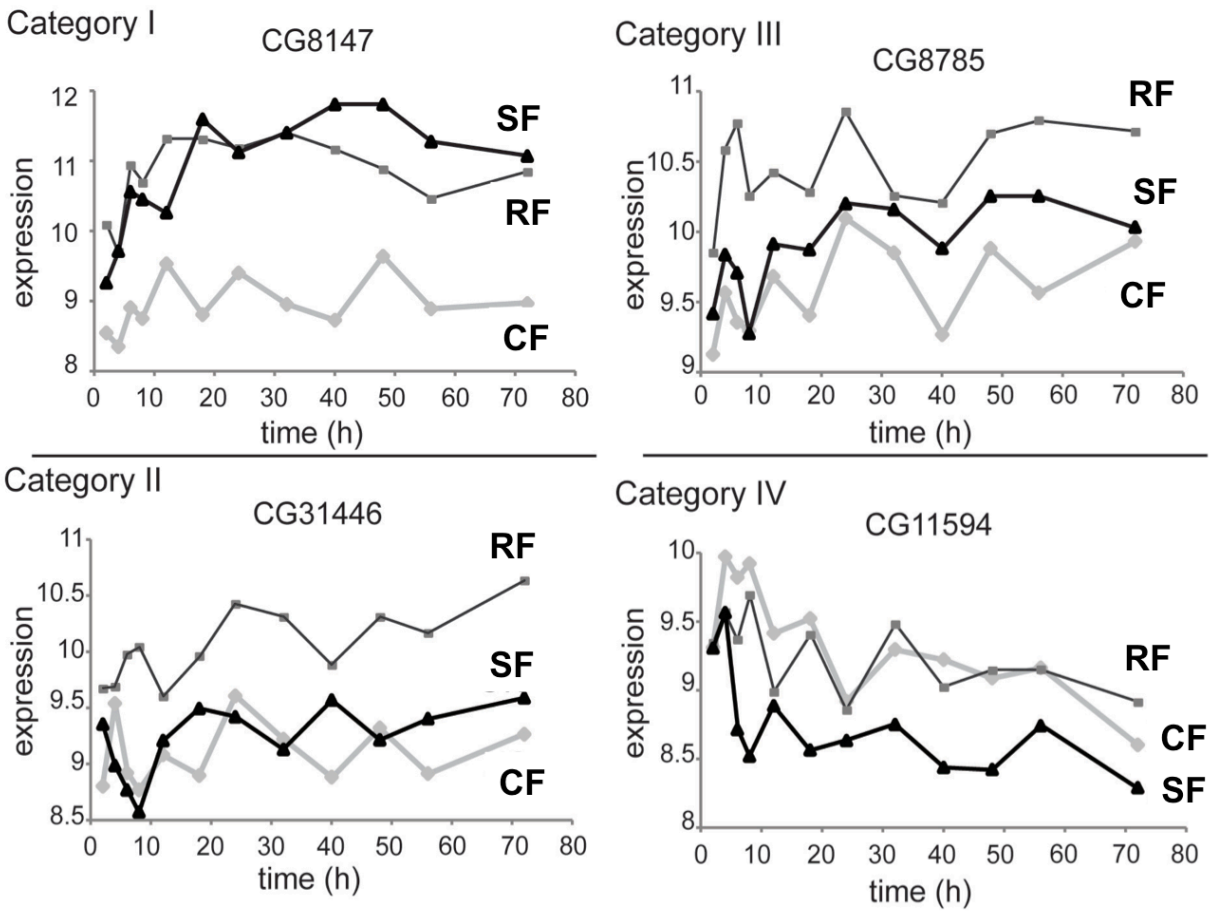

Figure 3-2. Expression plots of selected genes in each category. Shown are examples from each consistent category. Represented in abscises are time points after switch, in ordinates are shown the expression scores in the log2 scale. (Figure prepared by P.G.)

Genes and Pathways Identified

Categories I and II contain most of the genes (146 and 202 respectively) and were the main focus of our follow-up analysis. In category I, of the 146 genes that switched, 46 were up-regulated in RF (Figure 3-3, top section), while 100 were down-regulated (Figure 3-3, bottom section). In category II, the number of up-regulated genes was 148 while the number of down-regulated genes was 54.

Most of the switching genes belong to metabolic pathways with a significant enrichment in genes in carbohydrate and fatty acid metabolism. Interestingly, the genes enriched in these pathways have their expression down-regulated after DR (Table 3-3). The over-expressed genes clustered in one enriched pathway, folate biosynthesis. The down-regulated genes grouped under category II did not belong to any enriched pathway, while the up-regulated fraction had a significant role in the oxidative phosphorylation pathway (Table 3-3). Analysis of gene ontology enrichment based on biological process provided equivalent results.

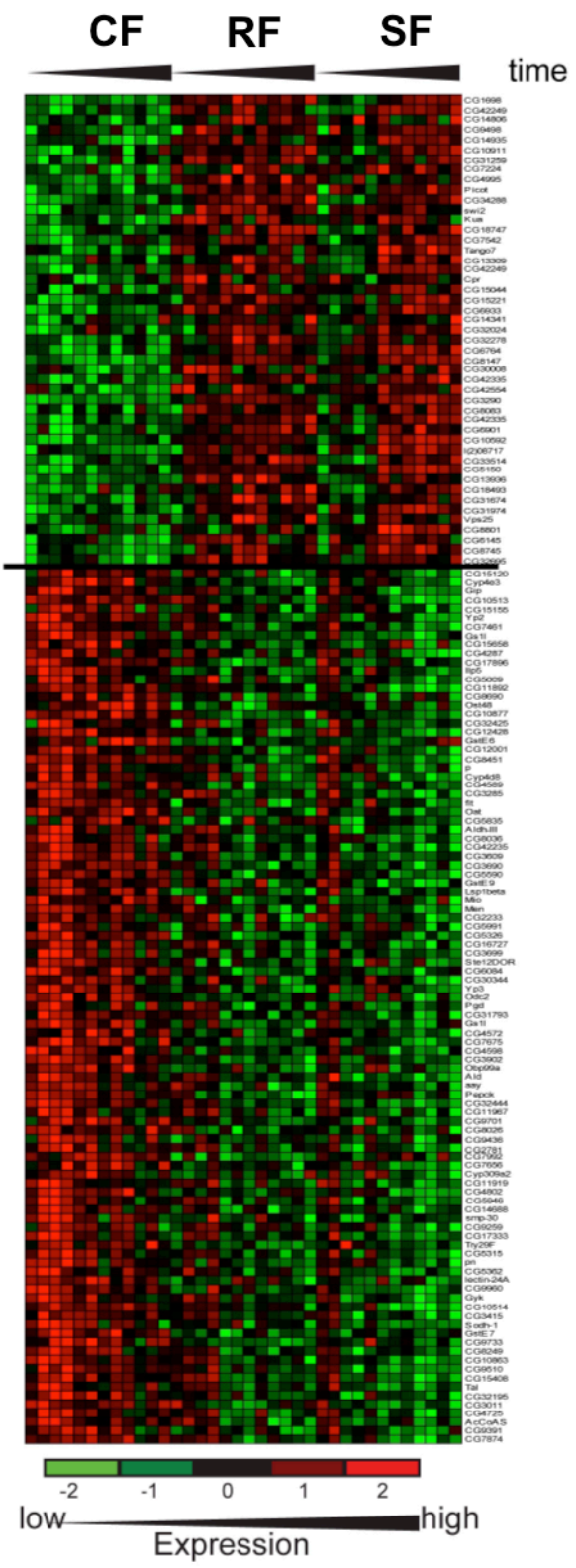

Figure 3-3. **Gene expression of switching genes.** Expression of 146 Category 1 ("switching") genes with time in each cohort. The top section represents genes that were up-regulated with RF, the bottom section contains genes with expression that was downregulated on RF food. (Figure prepared by P.G.)

Oxidative phosphorylation and in particular the increase in production of reactive oxygen species from specific sites of the electron transport chain has been extensively studied in the context of aging. Aging impairs oxidative phosphorylation while calorie restriction seems to reverse this effect, increasing mitochondrial biogenesis (Guarente, 2008). In the present study, the expression of genes implicated in oxidative phosphorylation was enhanced with DR and these genes significantly clustered on category II. Thus their response did not correlate with the mortality change, suggesting that these genes are not implicated in the longevity response to DR.

The most highly induced genes according to fold change under DR conditions, belong to the group of the alkaline phosphatases (Figure 3-4). KEEG pathway enrichment analysis grouped these genes in the folate biosynthesis pathway, particularly in the conversion of 2-amino-4-hydroxy-6-(erythro-1,2,3-trihydroxypropyl)-dihydropteridine triphosphate to dihydroneopterin. As the level of conservation of downstream steps of the folate biosynthesis pathway in Drosophila is not known, it is unclear how the observed increase in these enzymes will affect folate or other metabolite biosynthesis pathways downstream of this event.

A possible link between alkaline phosphatases and folate can be found in the conversion of piridoxal 5'-phosphate (PLP) to pyridoxal. The mammalian tissue-nonspecific alkaline phosphatase, an ortholog of the alkaline phosphatases upregulated in this study, catalyzes the cleavage of PLP, facilitating the transport of pyridoxal into the brain cells where it is rephosphorylated back into its active form (Footitt et al., 2011). The B-vitamins folate and PLP, are precursors involved in the transfer of one-carbon

groups, a necessary step for the synthesis and methylation of DNA (Depeint, Bruce, Shangari, Mehta, & O'Brien, 2006). In the present study we found that CG3011 (Glycine hydromethyltransferase), one of the enzymes that using PLP as cofactor catalyzes the conversion of 5,10-methylenetetrahydrofolate to tetrahydrofolate resulting in methyl group transfer, is a switching gene downregulated during DR (Figure 3-4B). Thus, taking together the induction of alkaline phosphatases and the under expression of the hydroxymethyl transferase we can picture a scenario where the synthesis and methylation of DNA is impaired during DR.

According to fold change, the highest ranked downregulated switching gene was Larval-serum protein 2 (Figure 3-4C). In a previous paper, Lsp2 was described as the unique significatively downregulated gene intersecting in three longevity inducing interventions: DR, dSir2 overexpression and DN-Dmp53 expressing long-lived flies (Bauer et al., 2010). In adult Drosophila Lsp-2 transcript is expressed in the adipose tissue of the head (Beneš et al., 1990) and has oxygen transporter and nutrient reservoir activities. Further investigation is required to elucidate the role of the larval serum protein complex in DR related longevity.

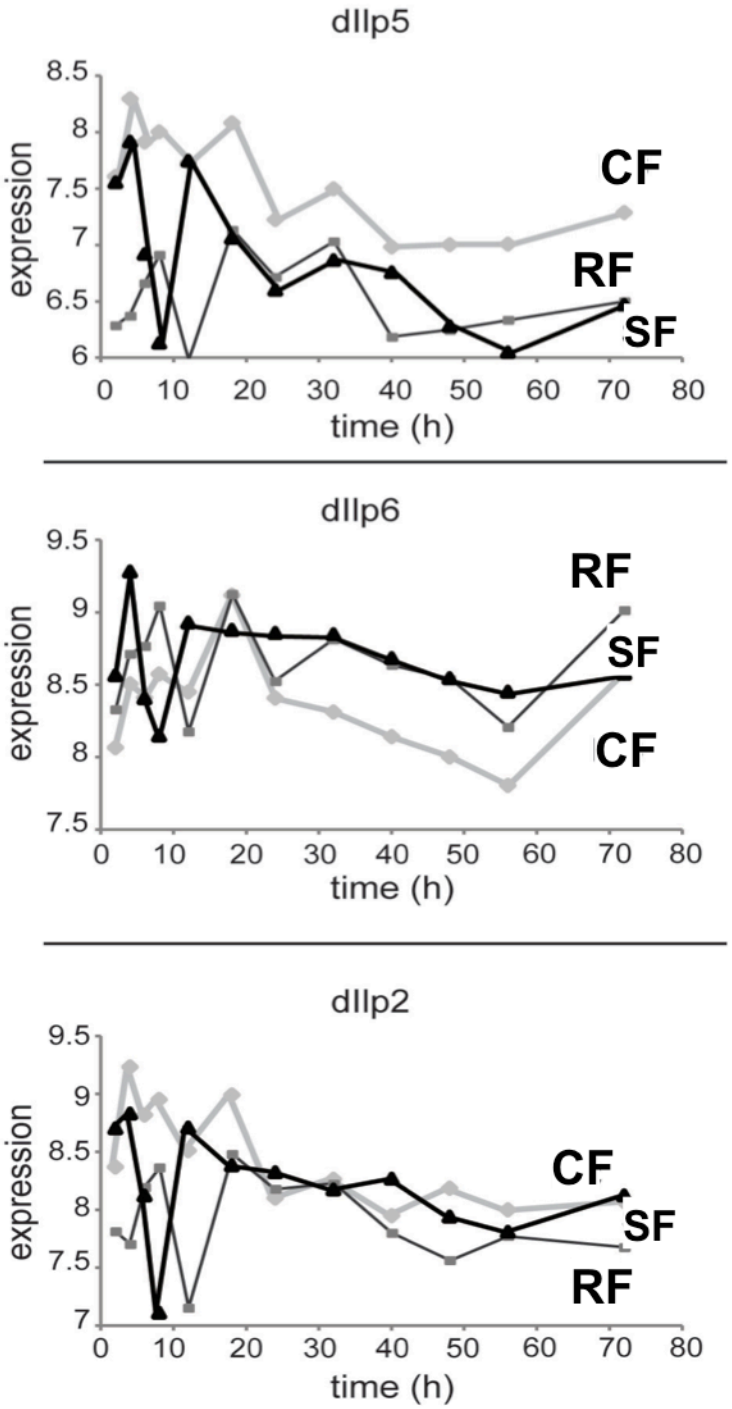

Figure 3-4. Insulin response to diet switch is consistent with previous findings. *dilp5* is reduced with DR and is a category 1 switching gene. *Dilp6* expression is increased with DR, and *dilp2* is unchanged with DR. (Figure prepared by P.G.)

Dietary Switch and Insulin Response

Reduced insulin signaling extends life span in many species, including *Drosophila* (Tatar, Bartke, & Antebi, 2003). Recently it has been shown that overnight fasting reduced the levels of the *Drosophila* insulin-like peptide 6 (*dilp6*) mRNA in *Drosophila* fat body, and *dilp5* mRNA expression was decreased in the brain while *dilp2* remained unchanged (Bai, Kang, & Tatar, 2012). These findings are consistent with expression patterns we observe; *dilp5* is a Category I gene, with an expression profile that is higher expressed in the

control food than the restricted food, and with the Switch cohort changing expression patterns of *dilp5* to resemble the new restricted diet (Figure 3-4). Additionally, we observe that *dilp6* shows increased expression in the restricted food cohort. Finally, *dilp2* levels did not change between the three groups, consistent with the previous finding that *dilp2* levels in the brain remained unchanged with fasting.

Dietary Restriction vs. Starvation

We compared the genes that changed with dietary restriction in our study with those that changed in a study examining the gene expression changes in starved flies (Fujikawa et al., 2009). Fujikawa et al starved flies for 24 hours, then examined the gene expression changes in these flies and observed 65 genes whose expression increased under starvation, and 48 genes whose expression decreased. Of the 588 genes whose expression was higher in the Restricted Food group in our study, only 3 of these correlated with genes shown to increase during starvation (Figure 3-5A and 5E). Similarly, 8 of the genes seen to increase in the RF cohort were seen to decrease in the starvation study (Figure 3-5C). Most of these genes were not readily classified as switching or refractory genes in our study.

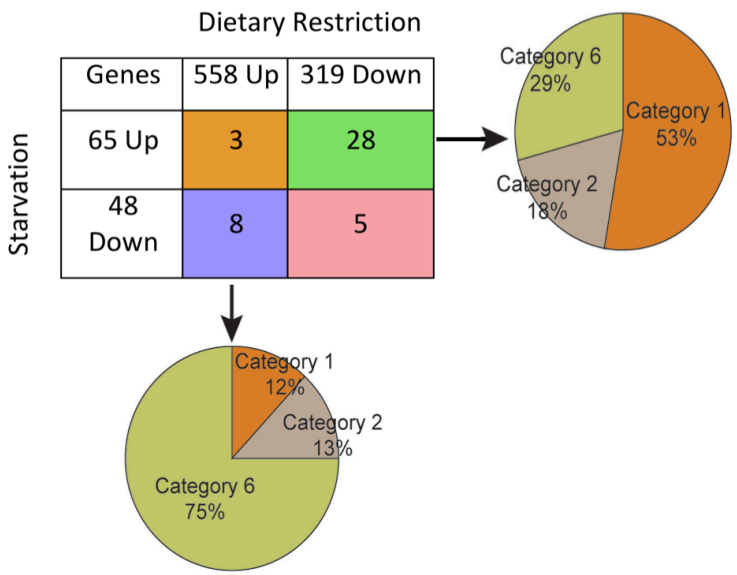

Figure 3-5. Different gene regulation between Dietary restriction and starvation. A. Summary of overlap between genes changed with starvation in Fujikawa et al, and genes changed with dietary restriction in this study. For the genes that changed in opposite directions, the breakdown for which categories they represented is shown (Category 1 = switching genes, category 2 = refractory genes, category 6 = genes that are unable to be computationally assigned to categories 1-5. (Figure prepared by P.G. and R.W.)

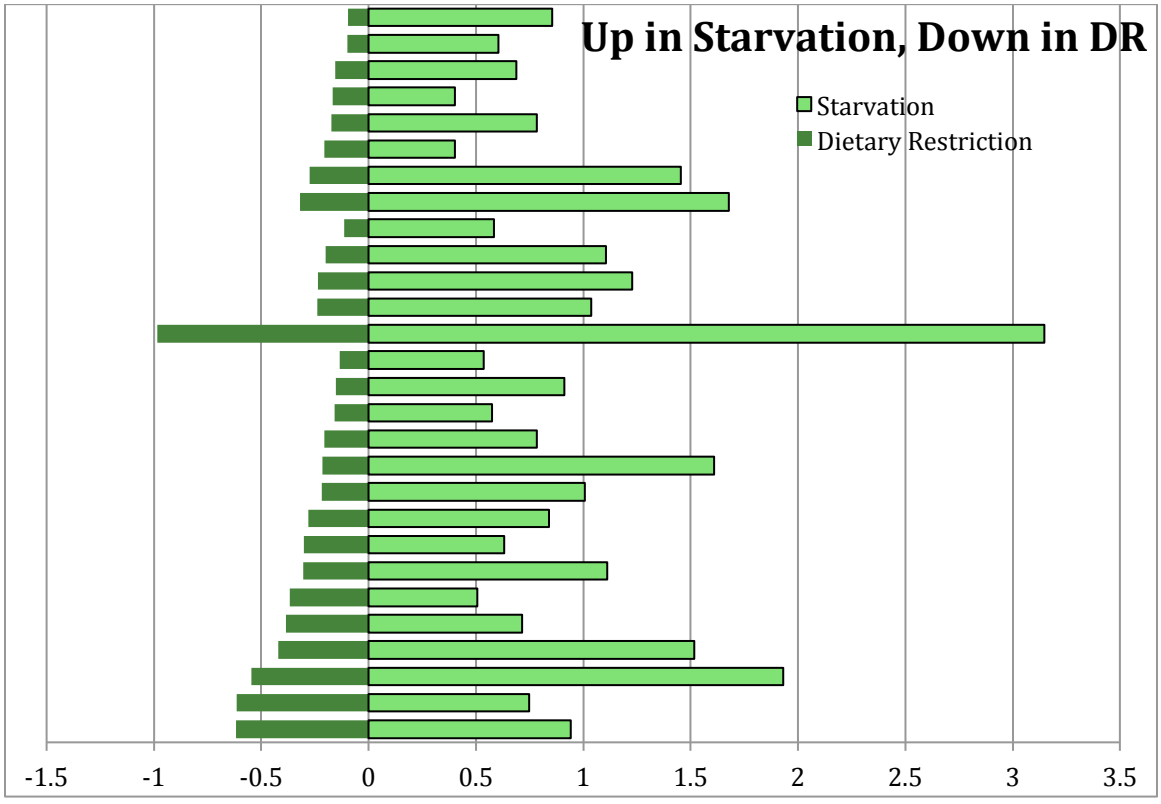

Figure 3-5B. Fold change of genes that were upregulated with starvation in Fujikawa et al, but downregulated with DR.

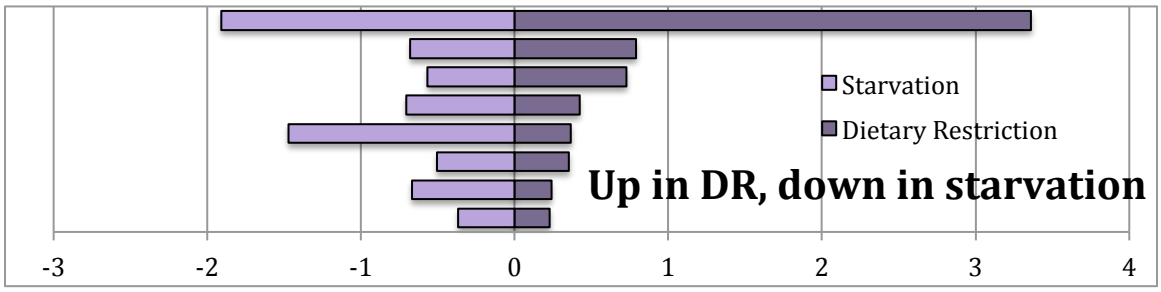

Figure 3-5C. Fold change of genes that were downregulated with starvation, but upregulated on restricted food in our study.

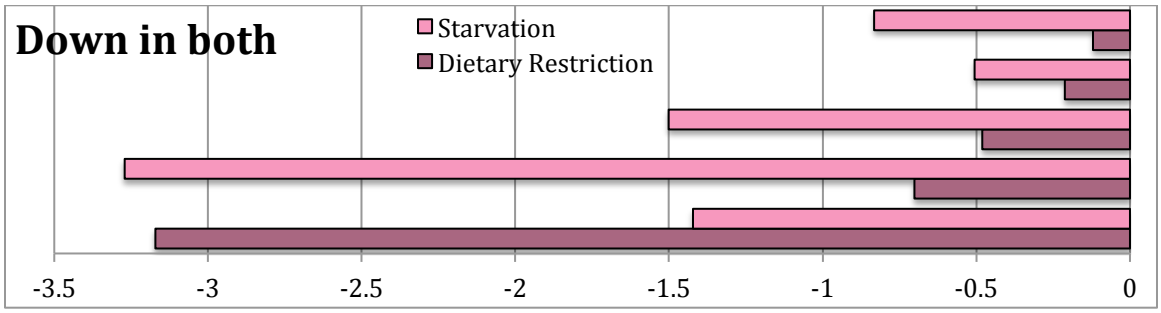

Figure 3-5D. Fold change of genes that were downregulated in both starvation and restricted food conditions.

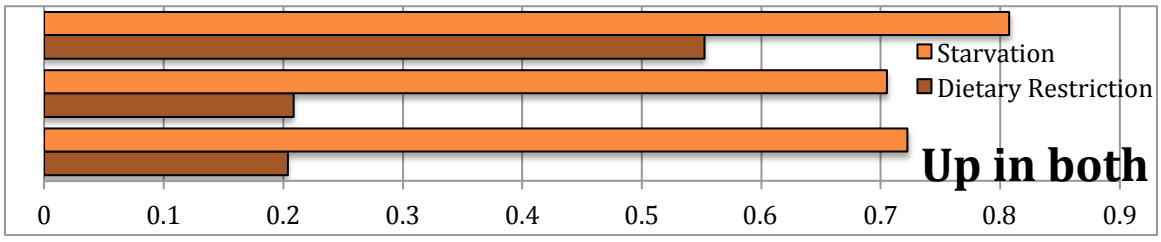

Figure 3-5E. Fold change of genes that were upregulated in both starvation and restricted food conditions.

319 genes showed reduced expression on Restricted Food in our study, and only 5 of them were also observed to decrease in the starvation data set (Figure 3-5D). Interestingly, 28 of the genes with reduced expression on restricted food were actually seen to increase in the starvation study (Figure 3-5B). Of these, 53% of the genes that had reduced expression in restricted food conditions and increased expression in the starvation study were in the switching category of genes, indicating that these genes may

be involved in the mortality switch. Interestingly, the direction of the expression change for these genes correlates with mortality in each data set.

Discussion

As a well-conserved mechanism of life span extension, it is of paramount importance to understand the molecular mechanisms of dietary restriction. A difficulty of studying the differences between dietary restriction and control diets alone is that once molecular pathways are identified as being differentially regulated in the two conditions, it is difficult to assess which of these pathways are important for the mortality decrease and life span extension in these animals. In order to differentiate between pathways that play an active role in the mortality decrease on a restricted diet and those that are by-products of the dietary switch, we have until now been limited to directly testing specific genetic manipulations of each pathway to identify those whose role is important for mortality. While this approach is required to confirm the effects of genetic interventions on life span, using it as a way to narrow down the possible candidate genes and pathways that are involved in mortality changes is laborious. By examining the changes in gene expression in animals that are switched to a restricted diet during the time course of the fast mortality reduction, we are able to differentiate between pathways that are likely to play a role in the fast mortality switch and those whose regulation changes, but that are not involved in the immediate response of the organism to dietary restriction.

We raised flies on either control food (CF) or restricted food (RF) and at 40 days old, switched one cohort of flies from control food to restricted food (SF). We performed microarray analysis on female fly heads/thoraces in a time course that covered from 8 hours before to 72 hours after the switch in order to monitor gene expression changes.

We used KEGG pathway analysis to compare gene expression profiles of flies fed control food to those on the restricted diet (Table 3-1). A previous study (Antosh et al., 2011) evaluated gene changes in 10 day old dietary restricted female head/thorax. An analysis of the significance of the overlap between differentially regulated genes in our study versus those in the 2011 study showed a significant overlap between the two studies. However, it is important to note that the difference in the age of the organisms in the two experiments may highlight differences in the response to dietary restriction in young (10 day) versus older (40 day) flies.

We sorted the genes that were differentially regulated between the different conditions into four categories. We sorted the genes based on three criteria: whether they were diet-dependent (i.e., where expression was different between RF and CF cohorts), diet-responsive (i.e., where expression changed in the Switched Food cohort during the course of the experiment), and Switching, where the direction of the change of the genes in the switch cohort switched from levels resembling the control diet to those resembling the levels seen in restricted fed animals. We then categorized the gene sets based on which qualifications they met. For example, a gene that is diet-dependent, diet-responsive, and switching, was called a Category I, or "Switching," gene. Those genes which were diet-dependent, but whose expression levels did not change in the switch cohort (aka were non-diet responsive and non-switching) were called Category II, or refractory genes, since their expression levels during the course of our study depended on the past nutritional history of the fly. Genes which were diet-dependent and diet-responsive, but whose direction of change did not correlate with the mortality change were called

Category III Responsive Genes. Category IV "Deviating Genes" contains genes which are not diet-dependent, but do change their expression levels in the switch cohort.

The benefit of this study design is that we can look at biochemical pathway changes that change with DR that switch in response to a diet change as well as those that are different between DR and Control food but do not play a role in the mortality switch. Of the pathways that were differentially regulated in DR, many of them were also shown as significant in the switch cohort, indicating that they play a role in the mortality decrease after the switch. The only significantly over-expressed switching pathway was folate biosynthesis. Especially important to note is that while Oxidative Phosphorylation was seen as a differentially regulated pathway with DR in our study and in previous work (Antosh et al., 2011), it was not a category I Switching pathway.

An example of the benefit of examining gene changes in the first 72 hours of the switch in diet is the ability to differentiate between genes that show differentiated expression between control food and restricted food from those that show differentiated expression and are also likely to be involved in the change in mortality. A key example of this is our finding that genes in the oxidative phosphorylation pathway increased in restricted food conditions as previously reported. However, these genes were not in the switching category of genes, indicating that while the oxidative phosphorylation process may be differentially regulated in dietary restricted flies, it is not involved in the immediate change in mortality that is observed when flies are switched to a restricted diet.

Another interesting finding is that the expression of genes in the folate biosynthesis pathway, particularly genes involved in the conversion of Dihydroneopterin to 7,8-Dihydro-pteroate, increase with dietary restriction and are switching genes. While

many of the enzymes mediating the folate biosynthesis pathway in Drosophila are not yet identified, it is worth noting that the more downstream steps of the folate biosynthesis pathway that have been identified have also been implicated in the aging process in several organisms. In particular, folate is a precursor to methionine, which is then converted into s-adenosyl methionine (SAM), a cell-wide donor of methyl groups. It is through this pathway that folate levels have been associated with DNA and histone methylation levels and aging. Additionally, folate is a source of carbon for the addition of methyl groups on DNA and histones, and folate deficiency can result in hypomethylation of DNA. Finally, mice that were fed a folate-deficient diet had disruptions in their circadian rhythms that resembled those of aged mice. If the production of 7,8-Dihydro-pteroate is linked to the induction of folate biosynthesis in the more downstream part of the pathway as predicted, this induction could be a potential mechanism for upregulation of methylation in the cell, and a potential mechanism for a fast response to dietary alterations.

Comparison of our results with a data set which investigated gene expression changes during starvation in Drosophila show that of the genes that change in both starvation and a diet switch to restricted food, very few of these genes changed in the same direction in both conditions. Of the 44 genes that were observed to change in both our study and the starvation data set, only 8 of these genes changed in the same direction with starvation and with the switch to restricted food, 3 with increased expression and 5 with decreased expression in both conditions (Figure 3-4A). However, 36 of the genes changing in both studies changed in opposite directions in response to starvation vs dietary switch. Of these, 28 genes were upregulated under starvation conditions and down

regulated in DR, and 8 were upregulated in DR and downregulated in Starvation. Especially interesting to note is that a large number of these genes were category I switching genes. Therefore, these genes may play a role in the mortality response in both the restriction and starvation conditions, where the level of expression of these genes correlates with the anticipated change in mortality.

Materials and Methods

Flies line and conditions.

Canton-S flies were obtained from the Bloomington *Drosophila* Stockcenter at Indiana University (Bloomington, IN) and kept at 25˚C in a temperature-controlled incubator at 50% humidity with a 12 hour on/off light cycle. The parental generation of flies was raised on food containing 30 g/L autolysed yeast, 120 g/L sucrose, 50 g/L cornmeal, 10 g/L agar with added dry live yeast. Experimental flies were collected in a 24-hour window, anesthetized under light CO_2, sorted into vials at a density of 25 males and 25 females per vial, and randomly divided into treatment groups. The flies were then passed every day on either Control Food (CF, 150 g/L sucrose, 150 g/L autolysed yeast, and 20 g/L agar, all w/v) or Restricted Food (RF, 150 g/L sucrose, 150 g/L autolysed yeast, and 20 g/L agar, all w/v) and the number of dead flies recorded. On day 40, half of the flies on Control Food were switched to the Restricted Food. During the course of the experiment, age-specific instantaneous mortality rate was analyzed and the separation of mortality rate between the food conditions was verified before the switch. Flies were sorted under light CO_2 and collected at fixed time intervals before (8, 4, and 2 hours before), during (0 = time of switch), and after (2, 4, 8, 12, 18, 24, 32, 40, 48, 56, 72 hours

after) the switch time point via snap freezing in liquid nitrogen and were stored at −80°C. Heads and thorax of female flies were collected for microarray experiments.

Life span determinations. Demographies were performed on all experimental flies (total number of flies in demography: CF = 2892, RF = 2987, SF = 2278) and flies collected for microarray analysis were censored from the study on the day of their collection (total number of flies censored: CF = 1650, RF = 1825, SF = 1126).

Log rank tests were performed using the survival package in R to obtain Chi-square and p-values. Maximum life span was calculated as the mean life span of the longest surviving 10% of the population. Age-specific instantaneous mortality rate was estimated as $\ln(-\ln[1-D_x/N_x])$ where D_x is the number of flies that died in each scoring interval and N_x is the total number of flies still alive.

Microarrays. Total head and thorax RNA was isolated from at least 75 females using Trizol (Invitrogen) and further purified using RNeasy columns (QIAGEN). 5 μg total RNA was used with Affymetrix One Cycle DNA conversion Kit (Cat # 900431) and all steps were carried out according to the Affymetrix manual. Briefly, first RNA was converted to double stranded cDNA followed by a clean-up step using spin columns. The double stranded cDNA was amplified in an in-vitro transcription reaction overnight at 37 °C using Affymetrix IVT labeling kit (cat # 900449), resulting in biotin labeled cRNA. After clean-up of the labeled cRNA with spin columns, 15 μg of cRNA were fragmented using metal induced hydrolysis. 10 μg of the fragmented RNA were hybridized to Drosophila 2.0 arrays overnight at 45 °C, 60 rpm. The array was stained using Affymetrix Hybridization-Wash-Stain kit and Fluidics Script FS450_0002 on the

Affymetrix 450 fluidics station and finally, the arrays were scanned using an Affymetrix 3000 G7 scanner.

Probes were mapped to CG numbers using the drosophila2.db annotation package from Bioconductor. The data was PVAC filtered, quantile normalized and summarized using Robust Multichip Analysis (RMA) to obtain expression scores in the $\log_2$ scale.

Statistical analysis. Paired t-test, two sided, was used to test for differential expression between LC and HC, SW and HC and SW and LC, using all time points. A q value correction was applied to the pooled p values from all tests and comparisons. The FDR cutoff was set to 0.05 and genes were selected into different categories depending on *qvalues* between cohorts.

Data analysis. Heat map visualizations were conducted with Expander6Win analysis software [Ulitsky I, Maron-Katz A, Shavit S, Sagir D, Linhart C, et al. (2010)]. Functional enrichment and pathway analysis were performed using Flymine [Lyne R, Smith R, Rutherford K, Wakeling M, Varley A, et al. (2007)] and KEGG database (http://www.genome.jp/kegg/)

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Tables

Table 3-1. Functional profiling of dietary restriction. Statistically significant GO terms derived from upregulated and downregulated genes in RF versus CF cohorts using Flymine database. The Benjamini and Hochberg method was used for Multiple Hypothesis Test correction allowing a maximum value cutoff of 0.05. The ontology used was based on biological process.

Pathway	q value	#genes
underexpressed genes		
Galactose metabolism	1.4137E-7	12
Valine, leucine and isoleucine degradation	0.0002862	9
Fatty acid metabolism	0.0014377	8
Fatty acid elongation in mitochondria	0.0031803	4
Starch and sucrose metabolism	0.0036617	10
Metabolism of xenobiotics by cytochrome P450	0.0036617	10
Pentose and glucuronate interconversions	0.0044247	8
Pentose phosphate pathway	0.0049176	6
Pyruvate metabolism	0.0107608	8
Drug metabolism - cytochrome P450	0.0109795	9
Glutathione metabolism	0.0129466	9
Peroxisome	0.0170669	9

Fructose and mannose metabolism	0.0198534	6
overexpressed genes		
Oxidative phosphorylation	6.2119E-10	28
Ubiquinone and other terpenoid-quinone biosynthesis	0.0252233	3
Folate biosynthesis	0.0002213	7
Riboflavin metabolism	0.0063198	5

Table 3-2. Pairwise comparison between cohorts. Paired t-test, two sided, was used to test for differential expression between RF and CF, SF and CF and SF and RF, using all time points. A qvalue correction was applied to the pooled p values from all tests and comparisons. The FDR cutoff was set to 0.05 and genes were selected into different categories depending on q values between cohorts. The numbers of genes found in GO categories and KEGG pathways correspond to unique non-redundant genes as obtained from Flymine and KEGG database.

Categories	**Behavior**	**Interpretation**	**genes**	**# GO***	**# KEGG**
I	diet dependent & diet responsive & switching	SWITCHING GENE: Response consistent with mortality change	149 48↑ 101↓	73	58
II	diet dependent & diet unresponsive & non-switching	REFRACTORY GENE: Level depends on past nutritional history	198 145↑ 53↓	123	55
III	diet dependent & diet responsive & non-switching	RESPONSIVE GENE: Response not consistent with mortality change	7 4↑ 3↓	6	1
IV	diet independent & diet responsive & non-switching	DEVIATING GENE: Responds to change but not to diet level	12 4↑ 8↓	10	1

*Gene Ontology Enrichment: Biological process / Multiple test correction: none

Table 3-3. Functional profiling of genes expressed in the consistent categories. Statistically significant GO terms derived from upregulated and downregulated genes grouped in the different consistent categories. Enrichment analyzed using Flymine database with Benjamini and Hochberg test for Multiple Hypothesis Test correction, maximum value cutoff: 0.05, ontology: Biological process.

Pathway	q value	#genes
Category I		
underexpressed genes		
Pyruvate metabolism	0.001033	7
Pentose phosphate pathway	0.001674	5
Fructose and mannose metabolism	0.007134	5
Glycolysis / Gluconeogenesis	0.041416	5
Fatty acid metabolism	0.043466	4
Galactose metabolism	0.04722	4
overexpressed genes		
Folate biosynthesis	2.02E-04	4
Category II		
underexpressed genes		
No Enrichment found		
overexpressed genes		
Oxidative phosphorylation	9.74E-06	12

CHAPTER FOUR: DISCUSSION

Discussion

Further insights into the molecular mechanisms of aging in "lower" organisms such as flies and worms is crucial to the advancement of understanding the process itself, and identifying potential therapeutic targets for slowing or preventing the process in humans. The short life span and large array of genetic tools available in these organisms enables researchers to make direct mutations and genetic manipulations that are time consuming and difficult in long-lived vertebrate systems. Additionally, the identification of pathways such as Sir2 that are largely conserved across the animal kingdom may be a key to narrowing down the potential mechanisms responsible for the aging process.

Drosophila in particular has a remarkable record in terms of its contribution to the field of aging research. Genetic tools such as homologous recombination and the UAS-Gal4 system, as well as the large number of publically available resources and manipulated fly lines, makes genetic manipulation in Drosophila very accessible. Additionally, the life span of flies enables these studies to be completed in a short amount of time, and multiple replicate experiments can be performed. Finally, the ease of culturing flies allows researchers to use hundreds or even thousands of animals in a single life span. Given the importance of being able to use Drosophila as a model system for understanding the aging process, it was imperative to investigate and resolve the confusion over whether dSir2 played a role in mediating life span in Drosophila.

Through careful experimental design and analysis of previously published research, the controversy over whether dSir2 mediates life span in Drosophila has been resolved. Previous studies that reported life span extension when dSir2 expression is

increased over-expressed dSir2 from the endogenous dSir2 locus, and induced dSir2 expression at optimal levels (Banerjee et al., 2012; Bauer et al., 2009; Rogina & Helfand, 2004) (between ~2-5-fold increased over wild type). Studies that reported toxicity (Griswold, Chang, Runko, Knight, & Min, 2008) or an absence of life span extension (Burnett et al., 2011) when increasing dSir2 expressed it from an ectopic transgenic locus, and either slightly increased dSir2 expression (Burnett et al., 2011) or induced dSir2 too highly (Burnett et al., 2011; Griswold et al., 2008). By examining these results and combining them with our own studies on transgenic UAS-dSir2 lines, we observe that moderate over expression of dSir2 extends life span, although inducing this expression from the endogenous locus via the dSir2EP2300 line is more successful than induction from UAS-dSir2 transgenes. Additionally, dSir2 expression that is too highly induced can have no effect on life span, or can be detrimental to life span, inducing JNK signaling and the heat shock homology protein dnaJ-H.

Future studies into the mechanism of dSir2-mediated life span extension should include genetic controls both through the use of gene-switch drivers, and by crossing flies of the same background (without any balancer chromosomes) as the transgenic flies to the driver condition. Expression levels of dSir2 should be monitored, and at least 3 doses of dSir2 expression should be tested if the absence of an effect is being reported. Just as more than two dietary conditions should be used to test whether an intervention is related to dietary restriction (Tatar, 2007), more than two levels of increased expression should be tested in studies that are increasing expression of a protein of interest in order to ensure that the dose is high enough to induce a phenotype, but not so high as to induce un-related toxicity effects.

There are several possible explanations for the lethality of very high doses of dSir2. First off, given the very large interaction network of Sir2 (Haigis & Guarente, 2006), there are many pathways that can be disrupted by large increases in Sir2. Additionally, Sir2 may have cellular effects through its function as a histone deacetylase, affecting gene transcription and regulation. Finally, as Sir2 is an NAD-dependent deacetylase, having large quantities of dSir2 may act as a sink for NAD, converting it to NADH and changing the availability of NAD for its many crucial metabolic functions. Too much dSir2 may also induce a protein-related damage response, inducing ER stress and the unfolded protein response. In support of this is the data from (Griswold et al., 2008) that shows induction of the JNK signaling pathway in response to what we later discovered were very high levels of dSir2, and JNK signaling is a downstream effect of ER stress (Ozcan et al., 2004).

Some future studies regarding the role of dSir2 in life span extension in Drosophila may include further research into the relationship between dSir2 and DR. Since dSir2 null animals are still able to extend life span under DR, DR and dSir2 may have overlapping mechanisms of life span extension, but dSir2 is likely not required for the lifespan component of the DR response. However, there is an abundance of hypomorph and null mutants used in these studies and those in this work (Åström, Cline, & Rine, 2003; Burnett et al., 2011; Newman, Lundblad, Chen, & Smolik, 2002; Rogina & Helfand, 2004), which may be contributing to the confusion over whether dSir2 is required for the DR response. These studies span a variety of mutations and genetic backgrounds, and the use of hypomorphs or null mutants is not ideal, since appropriate levels of dSir2 are not preserved during development or in the germ line. The effect of

genetic background in these studies is likely to be high, given that even in yeast, the genetic background and differing genetic alleles therein can completely change whether (and how) a yeast strain responds to DR without the presence of Sir2 (Kaeberlein, Kirkland, Fields, & Kennedy, 2004).

Given that dSir2 is a nexus point for many different molecular pathways involved in longevity, characterizing which of the other main life span-extending interventions result in increased activation or expression of dSir2 may reveal insights into dSir2's involvement in and overlap with those pathways. Additionally, given that dSir2 is a chromatin modifier, chromatin immunoprecipitation of a tagged dSir2 (or using a dSir2 antibody) followed by DNA-sequencing to determine how dSir2's localization changes with age will likely be informative. A new finding that human SIRT1 is activated by resveratrol only in the presence of Lamin A (Ghosh, Liu, & Zhou, 2013; Liu et al., 2012) also hints at new, intriguing possibilities as to how increased dSir2 activity may be localized to heterochromatic regions, since Lamin A is known to tether heterochromatin to the nuclear envelope (Solovei et al., 2013). It is currently unknown whether the interaction of Sir2 and Lamin A is present in Drosophila or other systems. If this interaction is conserved, it would be intriguing to know whether the interaction between Sir2 and Lamin A changes with age using a simple IP and western blot, and whether this interaction can be further characterized to identify which region of dSir2 interacts physically with Lamin A. Even if this interaction is not conserved in Drosophila, it would be interesting to identify the domain of dSir2 that interacts with Lamin A. If a mutation of this domain does not disrupt the activity of dSir2, but disrupted its ability to interact with Lamin A, it would be very interesting to see whether disrupting this interaction

would abrogate the ability of Sir2 to extend life span, change its interaction with chromatin, or affect the longevity of the organism. If dSir2 cannot be modified in this way without disrupting its activity, it might be possible to instead disrupt the Lamin A domain that interacts with Sir2. In either case, the finding that Lamin A interacts with SIRT1, coupled with the role Lamin A plays in anchoring heterochromatic regions to the nuclear envelope, opens up an exciting new field of study in Sir2 biology and mechanism.

The function of the dnaJ-H gene is largely uncharacterized in Drosophila, and though it has homology to the heat shock protein 40 family of proteins, it appears not to be induced in response to heat shock, rotenone treatment, starvation, or hydrogen peroxide treatment. However, it is induced when dSir2 levels are increased to toxic amounts, and when GFP is highly expressed, indicating that it may play a role in protein-induced stress. In support of this, dnaJ-H levels may increase with paraquat treatment, although this result is tenuous, as it was not repeatable. Given its potential role in stress response, it will be interesting from a basic science perspective to understand the function of dnaJ-H in vivo. First, a more thorough investigation into the conditions that result in increased dnaJ-H expression is needed. In order to investigate what the binding partners of dnaJ-H are and where it is cellularly located, homologous recombination could be a valuable tool to introduce an HA or FLAG tag onto the 5' end of the sequence (although care will have to be taken not to disrupt the dSir2 locus in the process).

Dietary restriction is obviously one of the most highly studied and fascinating processes in the aging field, and a better understanding of the molecular pathways involved in that process is absolutely essential to identifying new targets for longevity-inducing therapeutics. However, not all of the cellular and organismal changes that occur

with DR are involved in the longevity response. Therefore, studies such as ours, which uses a time-course to look at changes in gene expression when animals are switched to a restricted diet, are important. This study allows us to differentiate between gene pathways that are changed due to DR, and those that are changed with DR *and* involved in the almost immediate decrease in mortality seen with this diet switch.

Importantly, we performed these experiments in older flies, at 40 days of age. Using older flies may account for some of the differences seen between our study and previous studies into the transcriptomic changes in diet restricted flies seen in a previous study, which used flies that were only 10 days of age (Antosh et al., 2011). An example of the difference this may make can be seen in the response of the oxidative phosphorylation pathway in the two experiments. In (Antosh et al., 2011), the oxidative phosphorylation pathway was decreased in female head/thorax under DR, however in the 40 day old flies in our mortality switch study, the oxidative phosphorylation pathway increases in response to the switch to DR. This indicates that the responses to DR are age-dependent, and poses that the response to DR should be investigated at several different ages, since comparing responses in older animals may not correlate with the response in young animals. In this way, we will not only be able to get a better understanding of the gene changes in response to DR, but we will be able to determine whether the direction or magnitude of those changes is different in young vs old flies, and how this might relate to the aging process and the importance of these pathways to mortality with age.

There are several reasons why the response to DR may be different in young vs old individuals. First off, the general changes in lots of pathways that are observed with

DR may be very different in an older animal that has already been susceptible to accumulated damage and increased mortality. Since a younger animal has not had to endure these conditions before being switched to a restricted diet, the transcriptomic history of the younger fly is likely to be fundamentally different than that of an older fly. In this way, a study that uses a time course to observe the changes in transcription with a switch to a restricted diet at many different ages can be very informative. Specifically, it may allow us to define the transcription changes with DR that are crucial to the prevention of accumulated damage (as would be expected to be observed in an animal switched at young ages) to the reversal of this damage or a compensatory response aimed at controlling it, as we may expect to see in older animals switched to a restricted diet.

It is a common goal of research into the aging process to try to identify the "master regulator" of the aging process or of the DR response. Such a protein would have the capability of extending life span when manipulated, and would be required for the DR response to extend life span, resulting in an ideal single drug target for targeting the aging process in humans. However, just as dSir2 may not be required for the DR response in Drosophila, many other proteins have been shown to be involved in pathways that affect longevity and metabolism, yet may not be required for life span extension via dietary restriction. For example, over-expression of FOXO and dSir2, as well as reduction in the TOR and ILS pathways, extends life span, yet none of these pathways seem to be required for the life span response to DR (see introduction chapter for review). These studies are usually based on two types of experiments—one where the life span-extending genetic intervention is applied, and then combined with DR to evaluate whether the effects are additive, or a second approach, which uses a genetic null or

hypomorphic mutant to test whether DR still increases life span in the absence of that protein. While it would indeed be of paramount importance to identify a "master regulator" protein that would be capable of mimicking a DR response to life span, the importance of establishing that a pathway is not necessary for the DR response may be over-estimated. The very conserved response to DR throughout the animal kingdom, combined with the very wide interaction network of pathways implicated in the DR response, indicates the likelihood that there may not be a "master regulator" gene that can be wholly responsible for the DR pathway. Instead, it seems much more likely that many different molecular pathways all work in tandem to translate the reduction in available food into the many physiological responses, including life span extension, that are associated with DR. Given this, while it is informative to identify which genes might be required for the DR response, the ability of DR to extend life span even without the presence of one or more proteins should in no way be interpreted as that protein having no relationship to the DR response.

In understanding the importance of dosage in dSir2's mediation of life span, and in the use of a time course to understand the changes in transcription during DR that correlate with mortality, it is clear that careful and deliberate experimental design is crucial to researching the aging process. One of the best qualities surrounding the use of Drosophila as a model organism is the ability to create and use ideal genetic controls, as can be seen with our use of the gene-switch genetic system of over-expression to increase dSir2 levels. In addition, Drosophila life span is extended when dSir2 is over-expressed, confirming its importance in future research into the role that dSir2 plays in life span. The ability to create large cohorts of genetically isogenic flies allows for complicated time-

course experiments, and allowed us to perform demographic studies at the same exact time as the collection of flies for biochemical study, controlling for inter-experimental variability. These studies confirm the power, importance, and relevance of this invertebrate system to the study of the biology of aging.

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CHAPTER FIVE: OTHER CONTRIBUTING WORKS AND PROJECTS

Position Effect Variegation of a GFP reporter

Introduction:

Heterochromatic gene silencing markers such as Heterochromatin Protein 1 (HP1) and H3K9Me3 are known to have reduced enrichment in heterochromatic genomic regions with age in Drosophila melanogaster (Wood, Hillenmeyer, & Lawrence, 2010). However, it is still unclear whether there are functional consequences to these changes at the level of gene expression. Preliminary data in the lab has used a reporter which contains a LacZ gene under the control of a heat shock promoter to show that when this reporter is located near the boundary of heterochromatic/euchromatic regions, expression of the LacZ gene increases with age (Personal communication with N. Jiang, Helfand Lab). This data indicates that the reduction in the enrichment of HP1 and H2K9Me3 markers at heterochromatic boundary regions may correlate with a loss of heterochromatic gene silencing with age.

One of the caveats of using the LacZ position effect variegation lines to view the changes in gene expression with age is that the flies need to be sacrificed for staining and visualization of the reporter. A more recent method of visualizing gene expression in vivo is the use of GFP reporters. Ahmad et al (Ahmad & Henikoff, 2001) created several UAS-GFP lines that displayed Position Effect Variegation (PEV) effects due to genomic relocations. In addition to showing PEV of the mini-white gene that is present in the construct, these lines were also shown to display some GFP PEV when driven with an eye-specific GMR driver (that did not contain a mini-white marker). We hypothesized that GFP PEV expression from these UAS-GFP reporters could be used to visualize gene expression changes in the eye or in other external tissues with age. To examine this, we

crossed several of the UAS-GFP lines from (Ahmad & Henikoff, 2001) to ubiquitous, oenocyte-specific, or GMR-Gal4 drivers, and monitored the amount of visible GFP in the flies with age.

Results:

We crossed UAS-GFP lines 18.3.1, 18, 21, and 18.4.1, along with a positive euchromatic GFP control, to many different driver lines. Some of the drivers were modified versions of normal Gal4 drivers with the mini-white gene excised. This is useful, since it prevents the pigmentation of the ommatidia from obscuring GFP fluorescence. These lines, referred to as mw-, were A5CE1, GMR, and Daughterless-Gal4 drivers, and were obtained from Ahmad, et al. We also crossed the UAS-GFP lines to several RU-486-inducible Gene-switch Gal4 drivers, Tubulin-Gene Switch, Daughterless-Gene Switch, and ELAV-Gene Switch. Adult progeny from these crosses was raised on 1.5N food, and flies were anesthetized on CO_2 and visualized under a florescent dissecting microscope for fluorescence in the wings, legs, eyes, abdomen, and thorax at several ages between 2-40 days of age.

In each case, most fluorescence that was observed in the euchromatic control line was absent in the PEV lines, regardless of their age. While the PEV lines had the appropriately colored eyes for the crosses (confirming that the crosses were performed correctly), there was very little fluorescence visible in any of the crosses when compared to their negative and positive controls. Additionally, the eyes almost never showed PEV of the GFP shown in the publication, indicating that such visible GFP in the eye may be

incredibly rare. Occasionally, one particular PEV GFP line (18 x Daughterles-Gal4 mw-) showed wing vein fluorescence, but the percentage of flies with at least one + wing vein was approximately the same as the percentage of negative (female) control flies showing the same, indicating that the wing vein fluorescence observed was likely due to spontaneous autofluorescence.

Discussion:

In general, the absence of fluorescence that was visible under CO_2 from the outside of the fly under a dissection microscope in the PEV GFP lines indicates that the PEV may not be strong enough to be useful for the type of assay we are interesting in developing. An assay looking at the change in fluorescence in a single fly with age relies first on the presence of such PEV, and second on it happening with enough frequency that a sufficient population of fluorescent flies could be created and observed. Since neither of these requirements were met with any of the conditions tested here, it is unlikely that these UAS-GFP lines can be used to monitor gene expression in whole flies during their adulthood.

Changes in Reactive Oxygen Species, but not Mitochondrial Electron Transport Activities, with DR in Drosophila head/thorax.

Introduction:

Caloric restriction has been shown to effect mitochondrial physiology and biogenesis. In several rat and mouse tissues, a reduction in mitochondrial ROS production was observed with CR. Additionally, electron transport chain activities have been evaluated under CR, and (Layman, Merdian-Bender, Hegarty, & Swan, 1981) reported that ETC complex II had reduced activity in muscles from rats on a severely restricted diet for 9 days. (Baker, Betik, Krause, & Hepple, 2006) reported a reduction in citrate synthase activity with CR. However, (Hempenstall, Page, Wallen, & Selman, 2012) observed that a 40% CR diet in mice increased cytochrome oxidase (Complex IV) activity but did not increase citrate synthase (Complex I) activity. While it is clear that mitochondrial physiology changes with CR, there is confusion over the specific phenotypes. In drosophila, it has been shown that mitochondrial biogenesis changes with DR (Magwere et al., 2006). We hypothesized that DR may affect mitochondrial ROS production and Electron Transport Chain activities. We grew flies on a high calorie (15% sugar/15% yeast) or low calorie (5% sugar/5%yeast) diet and measured Reactive Oxygen Species (ROS) and Electron Transport Chain Complex activities after 10, 20, 30, or 40 days.

Results:

We first measured the production of Reactive Oxygen Species via the production of H2O2 in isolated mitochondria from Canton S head/thorax samples from males and females on high calorie (1.5N) or low calorie (0.5N) food at ages 10, 20, 30, and 40 days. We found that there was an ~ 20% reduction in the rate of H2O2 production in the low

calorie flies at all ages as compared to the high calorie fed flies in both males and females (Figure 1A and 1B), showing that ROS production is lower in calorie restricted flies. We next tested whether there were differences in mitochondrial electron transport chain complex activities in complexes I, II, III, and IV by measuring the enzyme activity levels in the same conditions as in the ROS study. We found that there were no differences in the complex activities of any of these conditions when normalized to citrate synthase activity to control for active mitochondria in the samples (Males, Figure 2A, 2B, 2C, and 2D. Females, Figure 3A, 3B, 3C, and 3D).

Discussion:

The reduction in ROS production in CR flies indicates an improved mitochondrial function, however our observation of no change in complex activities is in contradiction with previous findings. The standard deviation of our complex activity assays was very high, even after extensive assay optimization, which may have led to a compromised ability to detect changes in the activity levels of these enzymes.

Figures:

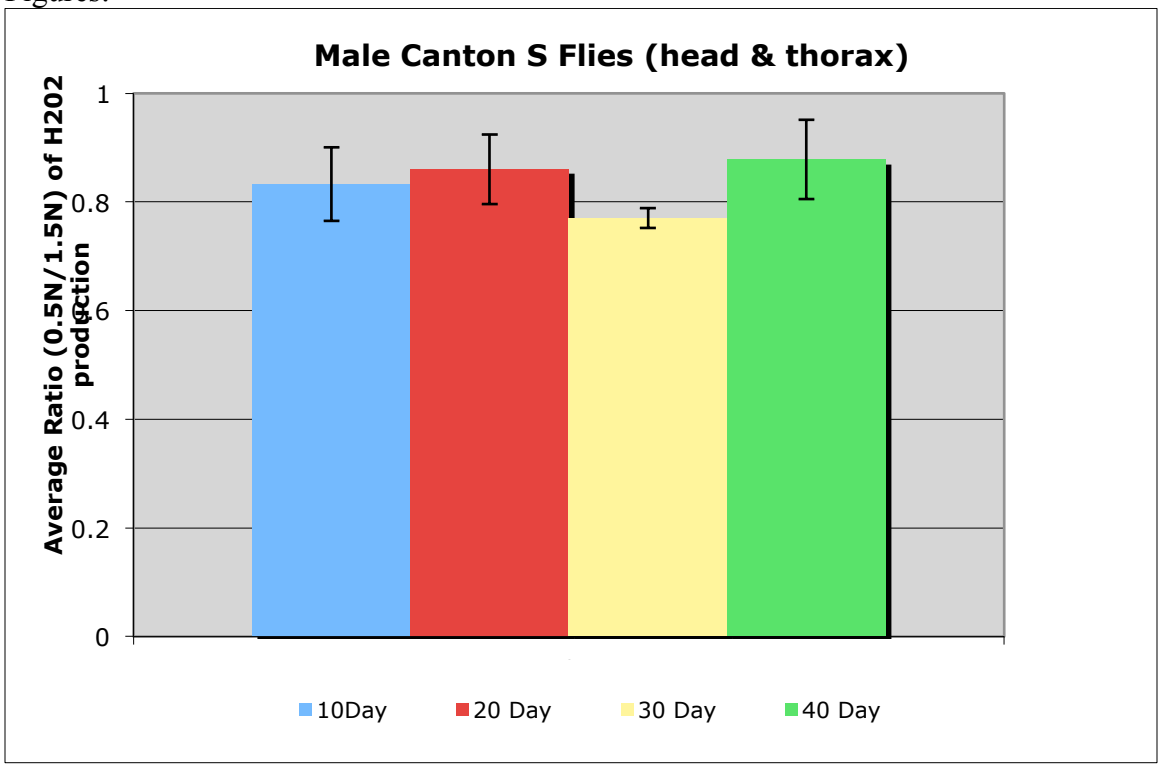

Figure 5-1A. ROS production in male flies is reduced on a low-calorie diet. Error bars represent SEM of 3 biological replicates.

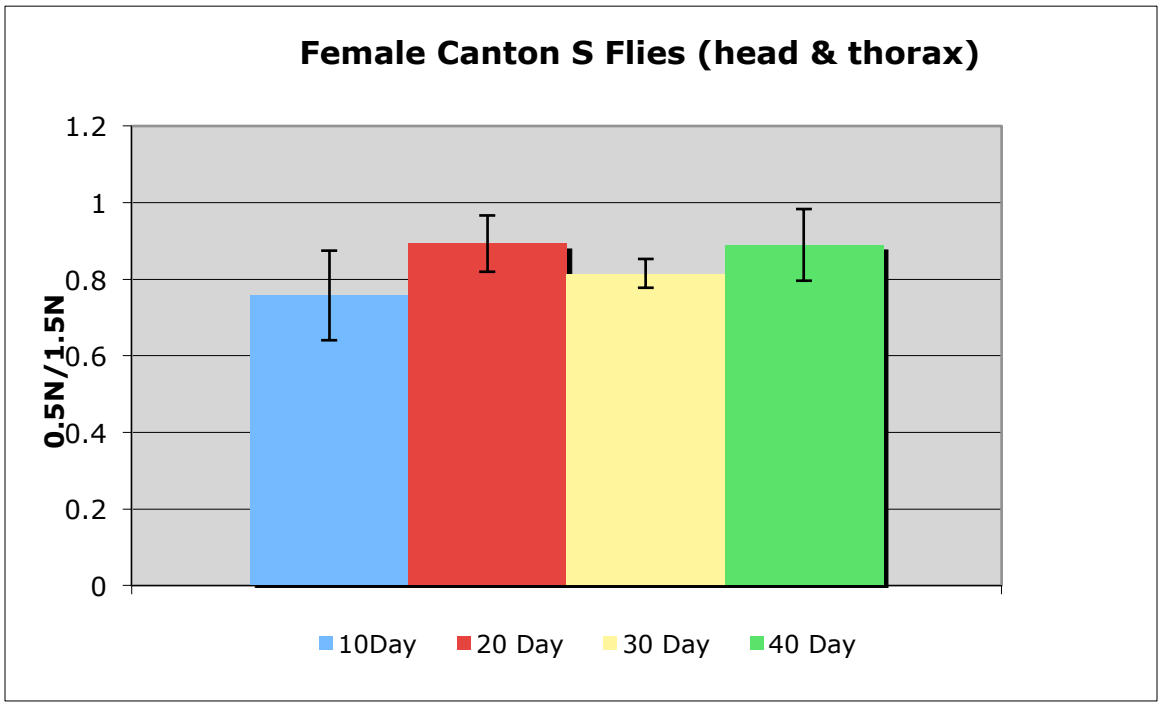

Figure 5-1B. ROS production in female flies is reduced on a low-calorie diet. Error bars represent SEM of 3 biological replicates.

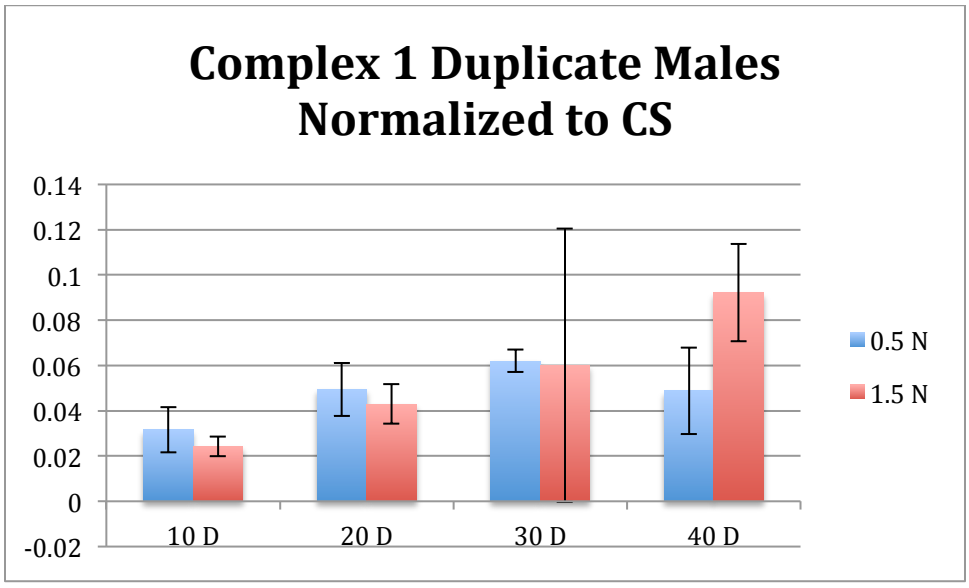

Figure 5-2A. Electron Transport Chain Complex 1 activity (normalized to citrate synthase activity) with age in high calorie vs low calorie fed male flies. Error bars represent SEM of three biological replicates.

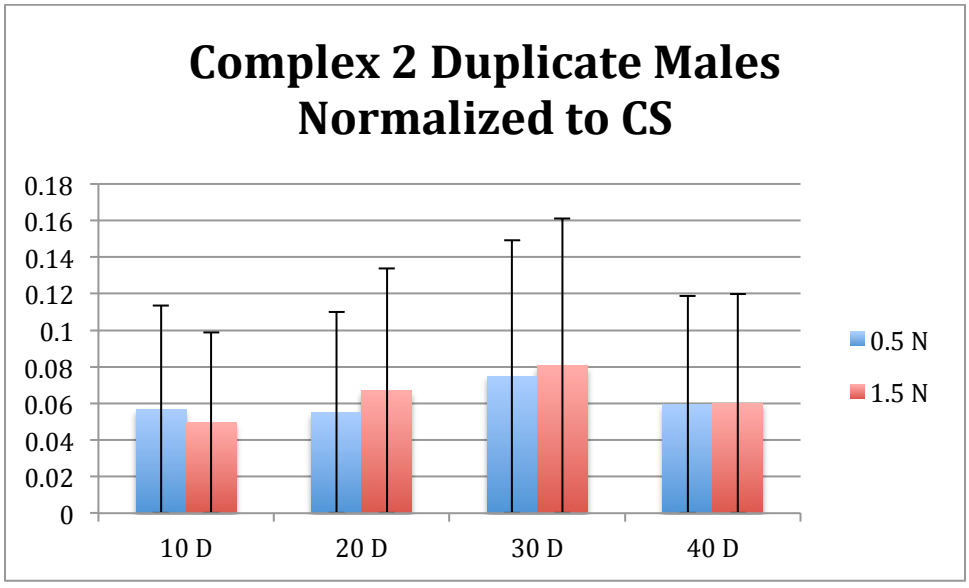

Figure 5-2B. Electron Transport Chain Complex 2 activity (normalized to citrate synthase activity) with age in high calorie vs low calorie fed male flies. Error bars represent SEM of three biological replicates.

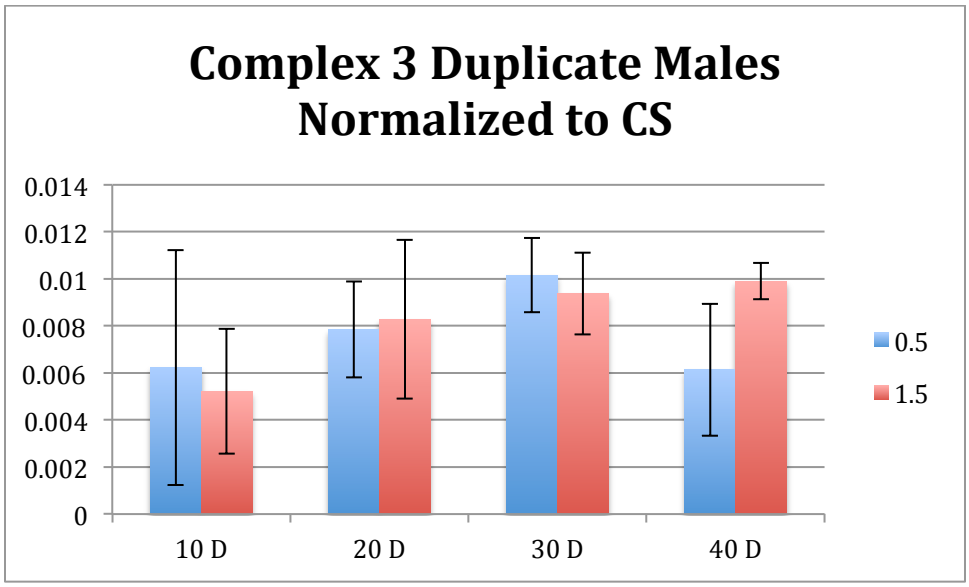

Figure 5-2C. Electron Transport Chain Complex 3 activity (normalized to citrate synthase activity) with age in high calorie vs low calorie fed male flies. Error bars represent SEM of three biological replicates.

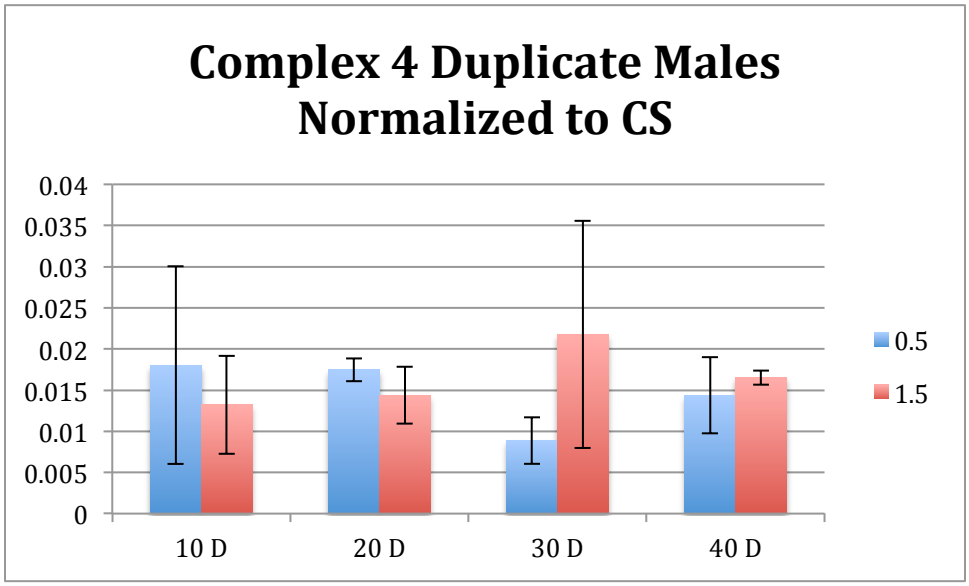

Figure 5-2D. Electron Transport Chain Complex 4 activity (normalized to citrate synthase activity) with age in high calorie vs low calorie fed male flies. Error bars represent SEM of three biological replicates.

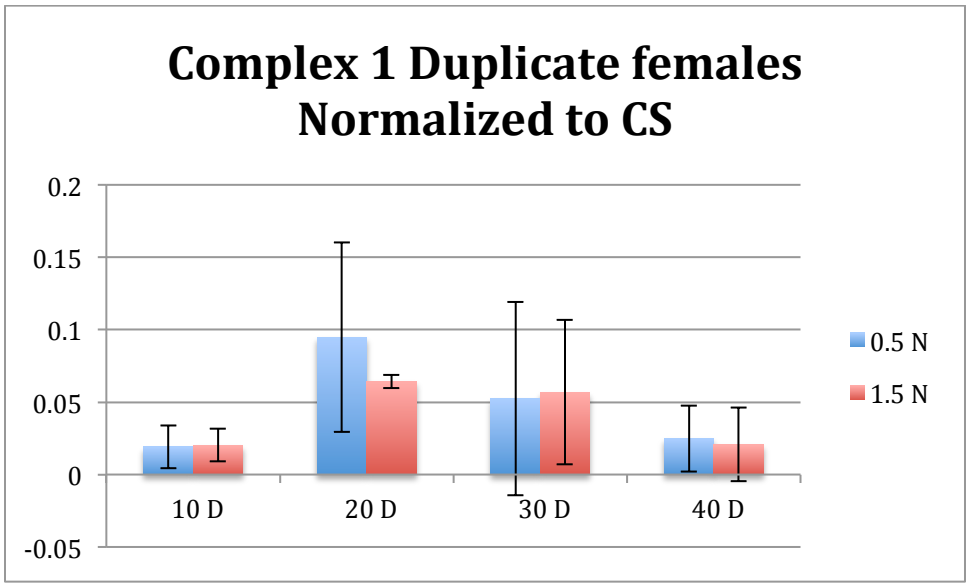

Figure 5-3A. Electron Transport Chain Complex 1 activity (normalized to citrate synthase activity) with age in high calorie vs low calorie fed female flies. Error bars represent SEM of three biological replicates.

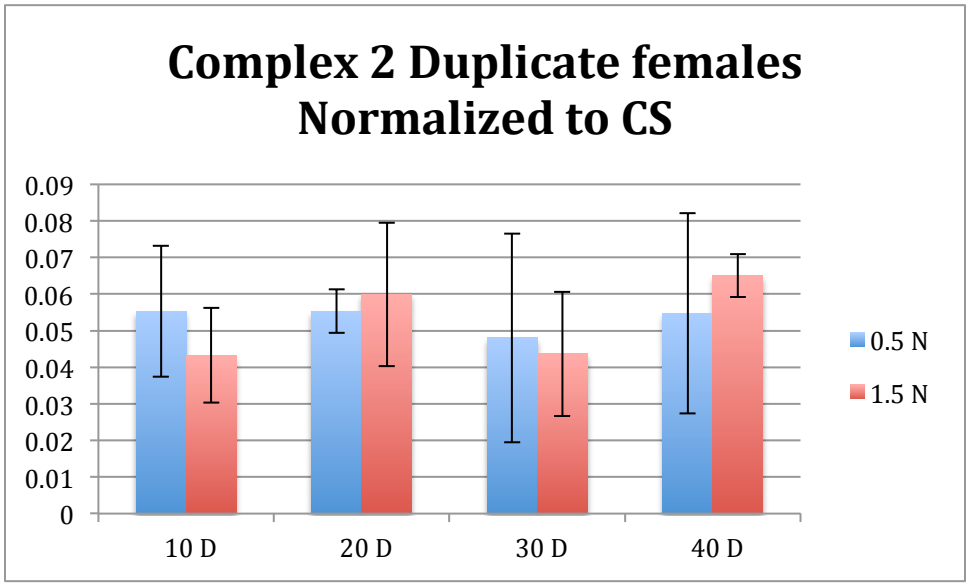

Figure 5-3B. Electron Transport Chain Complex 2 activity (normalized to citrate synthase activity) with age in high calorie vs low calorie fed female flies. Error bars represent SEM of three biological replicates.

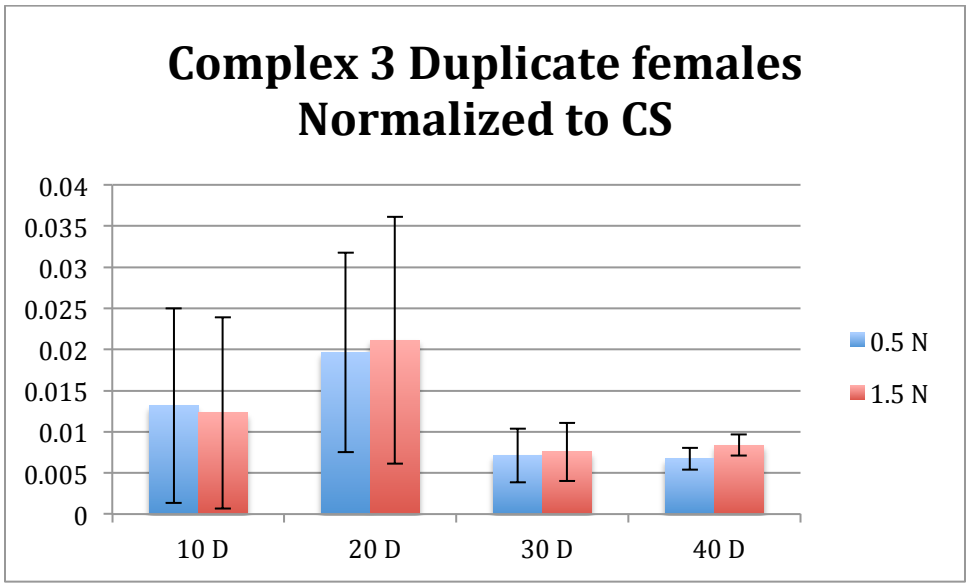

Figure 5-3C. Electron Transport Chain Complex 3 activity (normalized to citrate synthase activity) with age in high calorie vs low calorie fed female flies. Error bars represent SEM of three biological replicates.

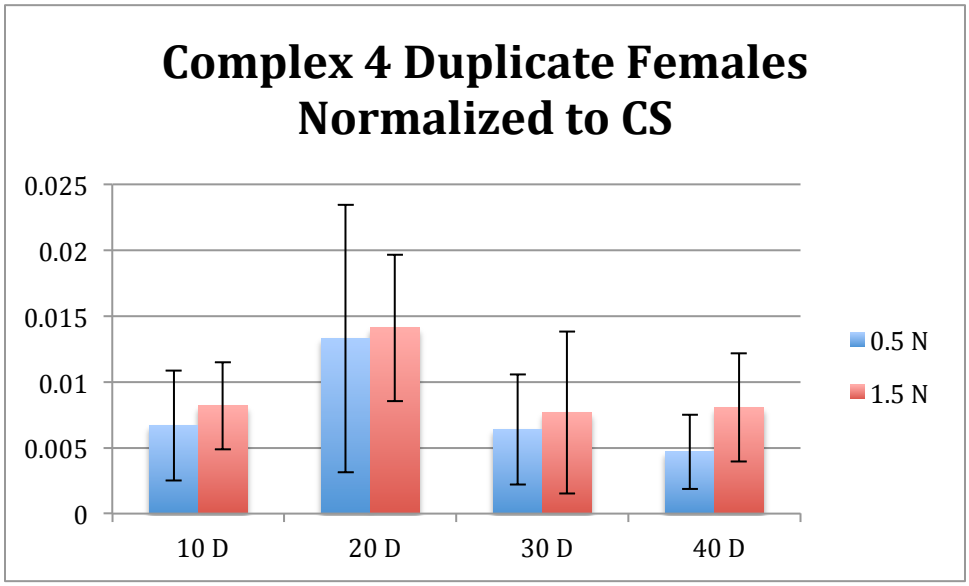

Figure 5-3D. Electron Transport Chain Complex 4 activity (normalized to citrate synthase activity) with age in high calorie vs low calorie fed female flies. Error bars represent SEM of three biological replicates.

Developing an assay to screen for inhibitors of Indy protein.

Abstract:

The scope of this project was to develop an assay that would allow for a drug screen for inhibitors of mouse (mINDY) or human (SLC13a5) homologues of the Indy protein. This was attempted in *S. cerevisiae* by cloning the mouse and human Indy homologues into the w303 strain of yeast, and screening for any growth phenotypes that would enable the development of an inhibitor assay. However, there did not appear to be any differences in the growth of yeast containing Indy homologues on many different concentrations of media containing dextrose, citrate, and/or succinate as a carbon source at 25, 30, 37, or 42 degrees Celsius. GFP or HA tagging of human and mouse Indy homologue clones did not reveal any location-specific staining, indicating that any Indy protein being made in yeast is not being inserted into the plasma membrane. Additionally, Indy homologues were cloned into *E. coli* and expressed using an IPTG inducible *E. coli* expression vector. These clones were not able to grow using citrate as a carbon source, as determined using the Simmons Citrate Agar test. In summary, it is likely that the Indy protein, when expressed in Yeast or *E. coli*, is not inserted into the plasma membrane, either due to improper folding, degradation, or the absence of important post-translational modifications or cofactors.

Methods:

Verification of whether S. cerevisiae can grow using citrate or succinate as a sole carbon source:

W303 strain of S. cerevisiae was quadrant streaked onto SC + Ura yeast media (see media methods section) containing 2% dextrose (positive growth control), 1% citrate, 2% citrate, 4% citrate, 2% succinate, or no carbon source, and plates were cultured at 30 degrees C for 72 hours.

Using the Gateway cloning system to clone Indy homologues from human (SLC13a5) and mouse (mINDY) into the w303 strain of S. cerevisiae.

RNA from human (gift from XXX) or mouse (gift from J. Sedivy, Brown University) liver samples was reverse transcribed into cDNA using the Invitrogen Superscript 3 first strand synthesis kit with random hexamer and poly-A primers. cDNA was then amplified for further cloning using primers containing kozak sequences (for forward primers) and AttB flanking sequences (see table for all primers). Indy was amplified using Phusion Taq polymerase. Fragments were ligated (Human SLC13a5 large isoform, SLC13a5 small isoform, and mouse mINDY) into pDONR221 entry vector using the Life technologies Gateway Cloning protocol for the BP reaction. NEB 5alphaF'Iq E. coli cells were transformed with the pDONR221-indy plasmids using the NEB protocol and selected on plates containing LB + 50 ug/ml kanamycin. After growth at 37 degrees C for 12-18 hours, transformed colonies were picked and grown in LB + Kan at 37 degrees C. Plasmid DNA was isolated using Promega PureYield Plasmid Miniprep kit. Clones were initially verified using digest with diagnostic restriction enzymes, and eligible clones sent to eurofins mwg operon for sequencing of the Indy gene using primers found in table 1. Plasmid DNA from sequence-verified clones was ligated into the pAG306 yeast expression vector using the Gateway Cloning LR reaction protocol from Life

Technologies. pAG306-Indy (containing either human SLC13a5 large isoform or mouse mINDY) plasmid DNA was isolated and diagnostic restriction digests performed to confirm identities.

Transformation of W303 S. cerevisiae with pAG306-Indy.

W303 yeast were transformed with the pAG306 plasmid, which recombines with the endogenous URA-3 locus and allows for selection of yeast containing the Indy construct via the ability to grow on media that lacks Ura. (Yeast transformation protocol used can be found at home.cc.umanitoba.ca/~gietz/method/html). Basically, yeast were grown in 2x YPAD media to $2x10^7$ cells/ml, washed in sterile water, incubated with Transformation Mix containing 33% PEG 3500, 0.1M LiAc, 139 ul/ml Boiled Salmon Sperm carrier DNA, and plasmid DNA at 42 degrees C for 40 minutes. Cells were washed with sterile water, and plated onto SC media without Ura and incubated at 30 degrees C for 2-4 days. Empty pAG306 vector was used as a negative control.

Looking for growth phenotypes in Indy-expressing yeast

"Frogging" Technique: Cells were growth to log phase in 2x YPAD media, washed in sterile PBS, and concentration of cells was adjusted using OD measurements and washes until OD measurements for individual cultures were within the error of the spectrophotometer from each other. Then, samples were serially diluted in a plate until 8 dilutions. A 20-ul multichannel pipettor was used to deposit 3ul (for plates containing citrate or succinate) or 5 ul (for all other plates) of the cultures onto the plates. All

samples of the same dilution were pipetted at the same time to ensure the accurate comparison between different conditions.

Testing for growth phenotypes on variable concentrations of dextrose, citrate, or succinate.

Frogging assays were performed for transformants on the ratios of dextrose, citrate, or succinate seen in table 2.

Determining whether HA-tagged or GFP-tagged versions of the human or mouse Indy sequence in Yeast localize to the cell membrane

Cloned Human SLC13a5 (both large and small isoforms) and mouse mINDY into pAG306 vectors with either HA tags or GFP tags in order to tag Indy being made and visually determine whether the Indy protein is being incorporated into the cell membrane. LR reactions to clone Indy from pDONR221 entry vectors into expression vectors were performed as above and validated with restriction enzyme digests, and w303 S. cerevisiae were transformed as above to incorporate the tagged versions of Indy into the genome at the URA3 locus. Clones were selected by plating on SC media minus Ura for further microscopic analysis.

For visualization of GFP-tagged Indy: Cultures were grown to log phase in rich media (2xYPD) with added Adenine, washed 2x with sterile PBS, and stained with Hoescht dye for visualization of the nucleus. Cells were visualized using a Zeiss axioimager light microscope with axiovision software.

For HA-tagged Indy: Cultures were grown to log phase in rich media (2xYPD) with

added Adenine, washed 2x with sterile PBS, and fixed with 5% paraformaldehyde for 5 min at RT. Cells were then washed once in sterile PBS and split into 4 conditions: Permeablized and blocked, non-permeabilized and blocked, permeablized and no blocking buffer, non-permeabilized with blocking buffer. Permeablized cell solutions all contained 0.1% Triton after the fixing step. Non-permeabilized cell solutions did not contain Triton. Permeabilized cells were incubated with a 0.2% Triton solution in PBS with shaking for 30 min at RT, then incubated with Rab-anti-HA antibody (1:500 in blocking buffer OR 1:500 in PBS) for 1 hour at RT. Non-permeabilized cells went from the post-fixation wash step to incubation with the Rab-anti-HA antibody as above. After primary antibody incubation, cells were washed 2x with sterile PBS, and incubated with Alexa-488-conjugated goat anti-rabbit antibody in either PBS or blocking buffer for 1 hour at RT. Finally, cells were washed once in PBS, 5ul of Hoescht dye was added for staining of nuclei and incubated for 15 minutes, and cells were rinsed once more in sterile PBS, then 5 ul of cells was deposited onto microscope slides, covered with a coverslip, and visualized under a light microscope. Cells were visualized using a Zeiss axioimager light microscope with axiovision software.

Using the Gateway cloning system to clone Indy homologues from human (SLC13a5) and mouse (mINDY) into E. coli.

Plasmid DNA from sequence-verified pDONR221 clones containing either the large human SLC13a5 gene or the mouse mIndy gene was ligated into the pDEST14 E. coli expression vector using the Gateway Cloning LR reaction protocol from Life Technologies. pDEST14-Indy (containing either human SLC13a5 large isoform or mouse

mINDY) plasmid DNA was isolated and diagnostic restriction digests performed to confirm identities. One Shot BL21 Star (DE3) Chemically Competent E. coli from Life Technologies were transformed with pDEST14 plasmid DNA to create IPTG-inducible indy-expressing E. coli and selected for using Ampicillin.

Inducing Expression of of Indy homologues in E. coli and monitoring ability to grow on Citrate as a carbon source

E. coli clones were grown in liquid culture (LB + Ampicillin), and induced in one of two ways.

First, E. coli cultures were induced to express Indy by incubation in LB + 0.5 mM IPTG for 2-3 hours at 37 degrees, and streaked or stabbed into test tubes containing 5 mL of Simmons Citrate Agar with or without IPTG, poured and cooled in a slant so that deep butts were formed. Cultures were placed in 37 degree C incubator for up to 9 days post-innoculation, and monitored for growth of E. coli via monitoring the color of the media. As a positive control, enterobacter (which do grow on SCA) were cultured in the same conditions and monitored for growth at 37 degrees C.

Second, E. coli cultures were streaked onto plates containing LB + Agar + Amp + 0.5mM IPTG and grown at 37 degrees overnight. Then, colonies of E. coli were streaked directly onto Simmons Citrate Agar tubes with IPTG and monitored for growth as above for 10 days.

Results:

Project A: Develop an assay in S. cerevisiae that will be used for inhibitor screen.

The objective of this project was to incorporate Indy homologues into *S. cerevisiae* and screen for the ability to grow on media containing only citrate as a carbon source. Failing any obvious phenotypes/increased ability to grow, a screen for temperature-dependent phenotypes or differences in the sensitivity to media containing toxically high levels of citrate will be performed in an attempt to identify conditions in which the Indy-expressing yeast are phenotypically different than their control cohort.

First, it was necessary to determine whether *S. cerevisiae* were able to grow on media containing citrate or succinate as the sole carbon source. The w303 *S. cerevisiae* strain was streaked onto plates containing no carbon, dextrose (as a positive growth control), citrate, or succinate as a sole carbon source, and monitored for growth at 30 degrees C. Growth was seen on the dextrose control plates as early as 24 hours, and growth in all 4 quadrants was seen at 72 hours. Citrate media growth was not seen at 30 degrees until a total of 5 days of growth, and the growth seen was minimal (and probably indicates using agar as a carbon source, not citrate). Succinate only media growth was also slow and mirrored the growth on the citrate plates. Agar-only (no carbon) plates also showed visible growth in all 4 quadrants after 5 days, indicating that growth seen on citrate-only and succinate-only plates is due to the growth using agar, not citrate or succinate.

Next, w303 *S. cerevisiae* were transformed with the normal (full-length) human SLC13a5 isoform or mINDY, and growth of the Indy-expressing yeast was monitored in a variety of temperature (25, 30, 37, and 42 degrees c) and carbon-source conditions for

any difference in growth as compared to controls that were transformed with an empty expression vector. No consistent differences were seen under different dextrose concentrations, citrate concentrations, or temperatures. Yeast were tested for growth ability by "frogging" assays (serial dilutions of samples with identical optical densities) onto 0.01%, 0.05%, 0.1%, 0.5%, 1%, 2% dextrose, with or without the addition of 2% citrate or 2% succinate, and while occasionally small differences in the growth ability of one of the clones was seen, this difference was not reproducible.

Next, we tested whether *S. cerevisiae* containing Indy homologue genes (or controls without the gene) were able to grow on media containing 5% or 10% citrate (with 2% Dextrose) to determine if they were more susceptible to citrate-induced toxicity. No growth was observed in any condition, including controls, on plates containing 5% or 10% citrate at 30 degrees C, even after several weeks.

Finally, to test whether the *S. cerevisiae* were expressing Indy and if so, whether it was being localized to the cellular membrane, both the full-length and the small human SLC13a5 isoforms and mINDY were GFP or HA-tagged. When viewed under a fluorescent microscope and nuclear stained with Hoechst 33342, GFP-tagged full-length Indy homologues displayed a diffuse GFP signal in comparison to negative controls, but that signal was not localized to the cell membrane or to any other cellular location. The smaller human SLC13a5 variant was GFP-negative, indicating that the smaller version of the Indy protein may be rapidly degraded.

HA-tagged Indy-expressing yeast were stained with anti-HA primary antibody, and an Alexa-488 conjugated secondary antibody, and the nucleus was stained with Hoechst 33342 and viewed under a fluorescent microscope. Four different staining

conditions were attempted with these yeast, however under all conditions, there was no HA-specific staining.

Project B: Develop an assay in E. coli expressing human or mouse Indy homologues that will be used in an inhibitor screen.

The objective of this project was to incorporate Indy homologues into *E. coli* and screen for the ability to grow on media containing only citrate as a carbon source using the Simmons Citrate Agar test. The Simmons Citrate Agar test consists of an agar containing the pH-sensitive dye, Bromthymol Blue, and Citrate as a sole carbon source. When bacteria are able to grow on the media using citrate, the pH of the media changes, and the color of the media changes from green to blue. Normal *E. coli* are unable to grow using citrate as a sole carbon source. We used the Gateway cloning system to clone human full-length SLC13a5 and mINDY into *E. coli*, induced expression using IPTG, and monitored *E. coli* for the ability to grow using citrate as a carbon source using the Simmons Citrate Agar test. This was tested using two different culture methods (liquid-culture induction, using growth of *E. coli* in liquid culture including the induction agent IPTG, or solid-phase induction, where the *E. coli* were grown on solid LB media containing IPTG), and were applied to Simmons Citrate Agar in two different methods (streak of culture across the surface of the media, or "stabbing" of the culture into the middle of the media). No growth of *E. coli* was seen on the Simmons Citrate Agar under any of these conditions, even after incubation at 37 degrees for 10 days. The positive control bacteria (Enterobacter) was observed to grow on Simmons Citrate Agar after one day (when "stabbed" with colonies) or three days (when colonies were streaked).

Discussion

We tested both *S. cerevisiae* and *E. coli* for conditions in which growth of an Indy-expressing cell culture was phenotypically different from negative controls, in an effort to design an assay that could be used to screen for inhibitors of Indy. In *S. cerevisiae*, the w303 strain of *S. cerevisiae* that we used did not readily grow using citrate or succinate as a sole carbon source, making it an ideal candidate for our further studies. However, there were no changes in the ability of the yeast containing Indy expression vectors to grow in any of the many conditions we tested. Additionally, when human SLC13a5 or mINDY were expressed in *E. coli*, there was no increased ability of the *E. coli* to grow on media containing citrate as a sole carbon source. There are several possible explanations for this result. First, the Indy protein may not be appropriately modified/folded, or may not be present in the membrane. The lack of an available Indy antibody prevents direct visualization of the protein, but the lack of localization to the membrane in the yeast GFP and HA-tagged Indy indicates that it is likely that the Indy protein in these cells is not being inserted into the cell membrane. This may indicate a misfolding event, or that a critical chaperone, co-factor, or post-translational modification may be missing in these cells that is required for Indy's membrane localization. It is also possible that the protein is being degraded before insertion into the membrane. Therefore, it was not possible to develop an assay for an inhibitor screen of Indy in *S. cerevisiae* or *E. coli.*

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