1-1-2010

Drosophila Nicotinamidase And Sir2 In Longevity Regulation And Oxidative Stress Response, And The Implications For Aging Theory

Gregory Miller
Wayne State University

DROSOPHILA NICOTINAMIDASE AND SIR2 IN LONGEVITY REGULATION AND OXIDATIVE STRESS RESPONSE, AND THE IMPLICATIONS FOR AGING THEORY

by

GREGORY MILLER

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2011

MAJOR: BIOLOGICAL SCIENCES

Approved by:

_____

ADVISOR **DATE**

_____

_____

_____

TABLE OF CONTENTS

Chapter 1

Background on Aging theory, Dietary Restriction, and the Protein Deacetylase Sir2

Over the last decade, the field of biological gerontology has demonstrated exceptional progress in understanding the complex phenotype of aging, paralleled by an equally prodigious increase in public interest of the science behind this universal phenomenon. The advent of molecular techniques with improved precision and rapid advances in the field of genetics has resulted in a concomitant increase in our ability to uncover the biochemical pathways that initiate and regulate aging. The growing proportion of elderly persons in the developed world correlates with a dramatic increase in the incidence of age related diseases, and is pertinent to the burgeoning public interest in aging research. While there have been exciting discoveries in diverse areas of aging research, there are still enormous gaps in our understanding of the etiology of aging, and why the rate of aging, or senescence, varies significantly both between species and within individuals of the same species. The most profound and important contribution of modern biology to our understanding of aging is the discovery of conserved molecular mechanisms that can regulate senescence across vastly different organisms. Interestingly, many of these mechanisms were discovered based on theories and observations made long before the discovery of DNA and the subsequent study of individual genes. This thesis will summarize the evolution of aging theory, discuss the contributions of molecular biology to our understanding of the aging phenotype, and utilize novel experimental data of the

genetic analysis of aging to perform a critical assessment of the current zeitgeist of the field of biogerontology.

From an evolutionary standpoint the reason for organism aging is simple. Once an organism has reached reproductive maturity and is capable of passing its genes to offspring there is no longer selective pressure to combat the inexorable momentum toward biochemical equilibrium and death. The life history of an organism can be divided into three discrete phases useful in discussing the etiology of aging; development and growth phase, health and reproductive phase, and senescence and mortality phase. The first two phases resulting in the reproductive fitness of an organism are under intense selective pressure for survival, while the third senescent phase is evolutionarily invisible. During the development and growth phase, most organisms exhibit a decreasing rate of mortality as they approach sexual maturity and enter the health phase, when the rate of mortality is at its lowest. The reproductive phase is associated with a variable period of low age associated mortality, which transitions to the senescent phase that is characterized by a marked increase in age related mortality. Analysis of survival throughout the lifespan of a group of organisms shows how changes in mortality rate during each life history phase manifest over time. It has been shown that the lifespan of most organisms is highly plastic, and can be both increased and decreased by differing environmental and genetic manipulations. Often these manipulations affect changes in the duration of the health phase; or in the observed rate of mortality during the senescent phase. Modern aging research seeks to characterize the phenotypic changes that occur during the transition from the health phase to the senescent phase, and elucidate how environmental manipulations and

gene mutations affect organism biochemistry so as to alter lifespan. Careful analysis of the aging phenotype and pathways that contribute to increased or decreased rate of senescence are integrated into theories that seek to formulate a consensus etiology of aging applicable to all organisms.

Dietary Restriction, Sir2, and Longevity

The implementation of a specialized diet is the most potent experimental paradigm of increasing lifespan that is conserved a wide variety of eukaryotic organisms. The phenomenon of "calorie restriction", as it was initially termed; mediates lifespan extension and was first reported by McCay and colleagues in 1935. Their observation was that rats fed a diet that was approximately 40% less than *ad libitum* levels resulted in an increase in lifespan of nearly 50%. Contemporary research has confirmed this result in a variety of rodents and other mammals, including dogs and primates (Weindruch and Walford 1988, Masoro 2005, Kealy et. Al 2002, Coleman et al 1998). Importantly, the increase in lifespan associated with *dietary restriction* (as it is now termed based on contemporary findings) is concomitant with an observed reduction in the incidence of many age related diseases in both rodents and primates; including cancer, diabetes, and cardiovascular disease (Weindruch and Walford 1988, Coleman 1998, Edwards et al 2001, Fontana et al 2004). The effect of food restriction on lifespan is conserved

in lower eukaryotic organisms, in which a reduction in different dietary components may be more relevant to lifespan than a reduction in total available calories per se, depending on the organism. In the yeast *S. cerevisiea*, calorie restriction can be induced by a reduction in available glucose, or by mutation of genes involved in the glucose metabolic pathway, while in the nematode worm *C. elegans* partial removal of dietary bacteria or complete removal of all food source can elicit extended longevity (Lin et al 2000, Kaeberlein et al 2006, Lee et al 2006). In the fruit fly *Drosophila*, dietary restriction mediated lifespan can be elicited by the reduction of the amino acid methionine (partridge nature pap), or augmentation of available yeast or carbohydrates (Pletcher et al 2005, Helfand et al 2008). Regardless of the variations in paradigm, the diversity of organisms that respond to dietary restriction suggests that these phenotypes may arise from a common evolutionary origin and involve conserved genes and pathways.

Mechanisms by which dietary restriction (DR) endows extended longevity are a matter of considerable debate, with plausible explanations involving a reduction in the onset of age related disease, a more resilient and efficient metabolism, and an enhanced resistance to various forms of cellular stress and damage. The one clear conclusion elucidated through the continued study of dietary restriction is that lifespan in most organisms is highly plastic and can be manipulated to promote both longevity and increased health. Because of the stringency of dietary conditions that result in extended longevity and improved health in mammals, the widespread adoption of dietary restriction by modern human populations is not likely to occur. There is therefore impetus to discover the genes and pathways that mediate the response to

dietary restriction in order to generate mimetics that could be useful in extending the human health span and preventing the onset of terminal diseases. Model organisms with proven genetic tools for determining the ability of specific genes and pathways to modulate observable phenotypes have been indispensable in characterizing the molecular basis of dietary restriction (DR) mediated lifespan extension.

Genes and pathways that are integral to the organism response to dietary restriction that are conserved in model organisms include the target of rapamycin (TOR) nutrient sensing and growth pathway, the insulin/IGF signaling pathway (IIS), and the gene encoding the protein deacetylase sirtuin family member *Sir2*. The TOR pathway is involved in mediating cell size and growth in response to a variety of internal and external environmental signals, including nutrient availability, concentration of growth factors, and conditions of stress and/or cellular damage. TOR, a serine/threonine protein kinase, has been linked to various cellular processes by responding to these cues through activation or inhibition and regulating transcription, translation, nutrient partitioning and autophagy accordingly (Reiling and Sabatini 2006). There is only circumstantial data in mammals suggesting that TOR is involved in mediating the DR phenotype, including a correlation between reduced insulin signaling and down-regulation of TOR, which results in a decrease of in the production of mitochondrial ROS in cell culture models similar to that observed in CR models (Sarbassov et al 2005, Kim et al 2005). Long-lived Ames dwarf mice display a reduction in TOR activity, which has been postulated to contribute to their lifespan extension (Sharp and Burtke 2005). In a recent study, the administration of rapamycin (an inhibitor of TOR) to mice late in life resulted in an increase in median and

maximal lifespan in both males and females (Harrison et al 2009). The result of increased lifespan was observed in three disparate mouse strains at three different laboratories, indicating that TOR is involved in lifespan regulation in at least three diverse genetic backgrounds in mice. The down regulation of TOR signaling is capable of increasing the lifespan of many lower eukaryotes, including yeast, *C. elegans*, and *Drosophila* (Kaeberlein et al 2005, Jia et al 2004, and Kapahi et al 2004). In nematodes, loss of function mutation or RNAi knockdown of TOR signaling components is capable of extending lifespan, however whether or not this pathway correlates with dietary restriction is unclear (Vellai et al 2003, Jia et al 2004). There is a correlation between dietary restriction and TOR signaling in *Drosophila*, as flies with genetically down-regulated TOR activity through inhibitors have increased lifespan, but do not exhibit a further increase lifespan when placed on a dietary restriction diet (Kapahi et al 2004). This, together with the observation that TOR inhibition in the fat body (the major metabolic organ in flies) is sufficient to extend lifespan, suggests that TOR is at least one element of the fly's response to dietary restriction.

Insulin and insulin-like growth factor (IGF-1) are major components of the IIS pathway in mammals, and have orthologs in *C. elegans* and *Drosophila*. The insulin/IGF-1 axis is critical to growth and cell division during development, and is a regulator of cellular metabolism by controlling the flux of metabolism between glycolysis and oxidative phosphorylation (see below). The inability of cells to respond to insulin, termed insulin resistance, is a major cause of age associated diabetes, and likely involves impaired mitochondrial function and both cellular metabolism (Wang and Wei 2010). Insulin resistance can be caused by chronically increased

insulin secretion, which occurs under conditions of excess calories. It has been hypothesized that dietary restriction would ameliorate this effect by causing a decrease in insulin secretion and therefore increasing insulin sensitivity over the lifetime of an animal. Indeed, dietary restricted rodents show a reduction in serum insulin levels of up to 50%, and a reduction in IGF-1 of up to 22% (Masoro et al 1992, Wetter et al 1999, Breese et al 1991, Sonntag et al 1999). When the IGF-1 receptor is mutated in mice, heterozygous IGF-1R/+ mice display an increase in average lifespan on 25%, and a concomitant increase in oxidative stress resistance (Holzenberger et al 2003). It is unclear if down-regulation of the insulin signaling axis results in effects on downstream targets that are consistent with dietary restricted animals; and thus whether or not insulin and IGF regulation is a major component of mammalian dietary restriction remains to be resolved. In nematodes, down regulation of insulin signaling is obtained by mutation of the *daf-2* gene, which is an insulin-like receptor, or through mutation of the *phosphoinositol-3 kinase* or *age 1* gene. These mutations result in a dramatic increase in lifespan which requires the forkhead transcription factor DAF-16 (Kenyon 2005). When insulin signaling is down-regulated, DAF-16 is phosphorylated and migrates to the nucleus where it affects transcription of genes involved in stress response, somatic maintenance, and DNA repair. Lifespan extension through dietary restriction, however, does not require DAF-16, and long lived mutant nematodes that arise from insulin signaling mutation still experience an increase in lifespan when placed on dietary restriction paradigms (Lakowski and Hekimi 1998, Kaeberlein et al 2006, Lee et al 2006). This data suggests that CR and insulin signaling utilize different mechanism for lifespan extension, and insulin signaling is not a mediator of dietary restriction in these animals. In *Drosophila*, mutation of the insulin receptor substrate *chico*

results in lifespan extension that appears to be mechanistically related to dietary restriction. However *chico* flies exhibit a shorter lifespan than controls at low food concentrations which cause a maximal lifespan through dietary restriction, (Clancey et al 2002). This seems to indicate that mutations in *chico* have effects that reduce the ability of flies to respond to dietary restriction, and more data is needed to dissect whether or not dietary restricted flies experience changes in insulin signaling outputs.

Perhaps the most well studied mediator of dietary restriction in lower eukaryotes, and possibly mammals as well, is the sirtuin member protein deacetylase Sir2, and its mammalian ortholog SIRT1. Sirtuins are evolutionarily conserved nicotinamide adenine dinucleotide (NAD) dependent protein deacetylases that can regulate a variety of genes and pathways via interactions with transcription factors and histones (Imai and Guarente 2010, Haigis and Sinclair 2010). Sir2 has been linked to several physiological processes relevant to senescence in eukaryotes; including metabolism, stress resistance, and apoptosis. Sir2 protein can affect these processes through its ability to deacetlyate and modify the activity of transcriptional regulators that are key modulators of these phenotypes; including PGC-1α, FOXO, and p53 (Bauer and Helfand 2009, Donmez and Guarente 2010). Sir2 expression has been linked to lifespan regulation and stress response in model organisms ranging from yeast to humans, and in some organisms appears to be required for the response to dietary restriction. While there is no link to date between lifespan extension and increased SIRT1 expression in mice, it appears that in some strains SIRT1 is required for dietary restriction mediated lifespan extension (Bioley et al 2008). Importantly, when SIRT1 is deleted from the mouse brain hallmarks of dietary

restriction including down-regulation of the IGF-1 and GH axis and increased physical activity do not occur (Chen et al 2005, Cohen et. al 2009). This shows that at least some outputs of that help mediate lifespan extension require a functional SIRT1. Over-expression of SIRT1 improves metabolic health in obese mice, which exhibit improved insulin sensitivity, glucose metabolism, protection from age associated diabetes, and a reduction in lipid-induced inflammation (Banks et al 2008, Pfluger et al 2008). Screening for small molecule activators of SIRT1 (STACs) resulted in the discovery of resveratrol, which was shown to activate SIRT1 *in vitro* (Howitz et al 2003). Mice fed resveratrol were subsequently shown to engender protection from the metabolic consequences of a high fat diet, and show a transcriptome highly similar to CR animals in heart, skeletal muscle, and brain tissues (Baur et al 2006, Langouge et al 2006, Barger et al 2008). Recently the fidelity of resveratrol to activate SIRT1 has come into question, and there is contention as to whether resveratrol may have targets other than SIRT1 (Dasgupta and Milbrandt 2007), thus the data requires further analysis before a connection between STACs, SIRT1, and dietary restriction can be made.

In the yeast *S. cerevisiae,* longevity is typically measured in terms of replicative lifespan; which is the number of mother cell divisions to produce daughter cells before senescence results in the termination of this division process (Mortimer and Johnston 1959). Recently, it was demonstrated that the etiology of aging in yeast is the buildup of extrachromosomal rDNA circles, which are toxic to the yeast cell due to their impairment of replication and transcription factor activity (Sinclair and Guarente 1997). When Sir2 expression is increased through the introduction of extra gene copies, ERC formation decreases and yeast lifespan increases; while

when *Sir2* is mutated ERC formation increases and lifespan decreases (Kaeberlein et. al 1999). Thus Sir2 is required in yeast for a regular lifespan due to its function in down-regulating ERC formation. Because ERC formation is not a cause of aging in higher eukaryotes, the ability of Sir2 to reduce ERCs is unremarkable when considering senescence outside of yeast; however this is not the only link between Sir2 and yeast aging. Sir2 has a prominent role in the response of yeast to dietary restriction. Dietary restriction in yeast can be imposed by either diluting the concentration of glucose in the growth media, or by mutating genes responsible for glucose metabolism (Lin et al 2000). In either paradigm, replicative lifespan is increased, and the effect of on lifespan is dependent on the enzymatic activity of Sir2 and concomitant reduction of ERCs (Lin et al 2000). Despite the fact that ERCs are not implicated in the lifespan of other organisms, this result raised the possibility that Sir2 may be an evolutionarily conserved regulator of the response to CR.

In the nematode *C. elegans*, there are multiple orthologs of yeast Sir2, and the ortholog with the highest homology, *sir2.1*, has a prominent role in lifespan regulation (Tissenbaum and Guarente 2001). In nematodes the dilution or removal of the bacterial food source, as well as mutation in genes that control pharyngeal pumping and food intake cause dietary restriction induced lifespan extension (Klass 1977, Kaeberlein et al 2006, Lakowski and Hekimi 1998). In the case of defective feeding induced by the *eat-2* mutation, simultaneous mutation of *sir2.1* suppresses CR-mediated longevity (Wang and Tissenbaum 2006); however *sir2.1* mutation does not suppress lifespan extension in food dilution experiments (Bishop and Guarente 2007). This apparent discrepancy may result from the primary involvement of *sir2.1* in stress response

rather than dietary restriction. Heat stress promotes the interaction of Sir2.1 protein with the forkhead transcription factor DAF-16, which then initiates DAF-16 dependent stress response genes (Berdichevsky et al 2006, Wang and Tissenbaum 2006). Interestingly, the DAF-16 gene product is also required for insulin signaling mutation mediated lifespan extension, which as previously discussed is *not* required for dietary restriction mediated longevity. Transgenic *C. elegans* with over-expression of Sir2.1 show increases in lifespan of up to 50% which is dependent on DAF-16 function (Wang and Tissenbaum 2006). Mutation of *sir2.1* reduces lifespan consistently only when nematodes are simultaneously under conditions of elevated stress (Viswanathan et al 2005, Wang and Tissenbaum 2006). Taken together, it remains ambiguous as to whether or not sirtuins are actively involved in nematode dietary restriction, or are part of a broad stress response network that controls the response to nutrient deprivation along with other various environmental and cellular stresses.

As discussed previously, dietary restriction in *Drosophila* can be achieved through dilution of yeast and/or glucose in the food source, or by reducing the amino acid methionine (Clancy et al 2002, Partridge nat pap). Lifespan is enhanced in dietary restricted flies, and this has been shown to coincide with the reduction of the class I histone deacetylase Rpd3 *(*Rogina et al 2002). Heterozygous mutation of *rpd3* is capable of extending *Drosophila* lifespan and results in the increased mRNA expression of *Drosophila* Sir2 (dSir2). The model for DR mediated lifespan extension in flies is as follows: DR mediated repression of Rpd3, resulting in increased expression of dSir2, and lifespan extension affected through increased dSir2 activity. Two experimental observations pertinent to this model are the fact that over-expression of

dSir2 in both the nervous system and ubiquitously in all tissues results in lifespan extension similar to that observed under conditions of , and the inability of to extend lifespan in a *dSir2* mutant fly (Rogina and Helfand, 2004). The ability of *dSir2* to modulate normal lifespan in *Drosophila* is augmented by genetic background, as homozygous mutation of *dSir2* has been shown to either decrease lifespan or have no significant effect on lifespan, depending on the strain and allele of mutation tested (Newman to Smolik 2002, Astrom Rine 2003, Pallos et al 2008). Similarly, the sirtuin activator resveratrol enhances lifespan in at least one genetic background, but has been shown to have no effect in other backgrounds under similar food and dosage conditions (Wood et al 2004, Bass to Partridge 2007). However, the fact that different Sir2 over-expression systems tested in independent laboratories result in increased lifespan, and the observation that both lifespan and increased mobility induced by dietary restriction do not occur in *dSir2* mutant animals strongly supports the idea that Sir2 is an important regulator of the *Drosophila* response to DR (Rogina and Helfand 2004, Parashar and Rogina 2009, reviewed in Frankel et al 2010).

A possible mechanism for dSir2 mediated lifespan extension in *Drosophila* is through its deacetlyation and down-regulation of the tumor suppressor p53. Mammalian p53 has been shown to regulate DNA repair, cell cycle progression, and apoptosis; and contributes to lifespan and tumor formation in mice (Maier et al 2004, Tyner et al 2002). The ability of p53 to modulate lifespan in *Drosophila* is well documented, with effects seen under conditions of over-expression, mutation, and chemically induced inhibition. Flies mutant for *p53* exhibit reduced lifespan, however when dominant negative constructs that obliterate DNA binding are

expressed in the nervous system (DN-p53) flies exhibit an increase in lifespan (Bauer et al 2005, Bauer et al 2009). Intriguingly, expression of DN-p53 outside of the nervous system results in decreased lifespan, and over-expression of wild type p53 has differential effects on lifespan of male and female flies depending on whether it is expressed ubiquitously or in the nervous system; indicating that p53 mediated lifespan is at least in part regulated by the fly nervous system (Shen and Tower 2009, Bauer et al 2005). The effect of ubiquitous, but not neuronal expression of p53 on *Drosophila* lifespan is obliterated in dSir mutants, indicating complex and overlapping effects of these two genes depending on the pattern of tissue expression (Shen and Tower, 2010). Importantly, p53 and dSir2 have recently been shown to physically interact, and the lifespan extension resulting from DN-p53 and dSir2 expression is not additive, suggesting that they may reside in the same pathway of lifespan regulation. There is evidence that dSir2 also interacts genetically or physically with two other transcription factors in *Drosophila*, FOXO and Hairy; and cell culture experimentation has shown that dSir2 over-expression results in changes in the expression of over 300 genes, thus there is the possibility of many different components of Sir2 signaling integrating to regulate the DR response (Griswold et al 2008, Rosenberg amd Parkhurst 2002, Bauer et al 2009, Cho et al 2005).

There are at least two potential mechanisms of regulating Sir2 enzymatic activity that are conserved in model organism and could contribute to the DR response by directly influencing Sir2 protein activation rather than expression. First, all sirtuins have a conserved NAD binding pocket and utilize the metabolite as a cofactor for the deacetylase reaction, and second, the byproduct of this reaction, nicotinamide, is a potent inhibitor of sirtuin activity

(Smith et al 2000, Bitterman et al 2002). Accordingly, Sir2 activity is expected to be up-regulated under conditions of elevated NAD; and down-regulated in conditions of increased nicotinamide. There is evidence supporting regulation of Sir2 from both mechanisms, however there is some contention as to which mechanism is more physiologically relevant. In reality, the assessment of a differential contribution between these two mechanisms to regulation of Sir2 depends strongly upon the experimental paradigm and organism tested. The ratio of NAD/NADH has been proposed to act as an indicator of energy metabolism and nutrient partitioning, proposed to shift toward higher NAD concentration and lower NADH under conditions of dietary restriction where glycolysis is limited and there is increased flux through the electron transport chain (reviewed in Wallace 2008). Because Sir2 requires NAD and is inhibited by NADH, it is proposed that DR affects changes in metabolism manifested through the NAD/NADH ratio that result in increased Sir2 activity, which then mediates changes in gene activity required for lifespan extension (Lin et al 2004, Picard and Guarente 2005). Indeed in *S. cerevisae* dietary restriction shifts metabolism away from glycolysis and toward respiration thereby increasing the NAD/NADH ratio by reducing intracellular NADH (Lin et al 2002). Furthermore, deletion of elements in the electron transport chain, such as cytochrome c, abolishes the effect of DR; while over-expression of NADH dehydrogenase genes increase the NAD/NADH ratio and yeast replicative lifespan (Lin et al 2002, Lin et al 2004). However, a direct correlation between these effects and increased Sir2 activity or expression has not been made. Interestingly, the yeast *npt1* gene, which initiates NAD synthesis, is required for dietary restriction mediated lifespan, however NAD production does not increase when Npt1 is over-expressed and the NAD/NADH ratio does not change during glucose restriction mediated

lifespan extension (Lin et al 2000, Anderson 2002). In *C. elegans*, rearing in media containing NAD increases lifespan in a *Sir2* dependent manner, however this phenotype also requires the *DAF-16* gene, which is not required for dietary restriction mediated lifespan (Hashimoto et al 2010). Thus while NAD and NADH levels may affect the activity of *Sir2*, it is unclear if this is the mechanism through which DR initiates Sir2 activation.

The second mechanism of *Sir2* regulation involves activation through the metabolism and thus intracellular depletion of the potent sirtuin inhibitor, nicotinamide (NAM). As discussed previously, glucose restriction in yeast does not change the NAD/NADH ratio, nor does it change the expression levels of Sir2; however dietary restriction and several other conditions of mild stress do induce the expression of the gene *pnc1* (Anderson et al 2003). Pnc1 protein is a nicotinamidase that converts NAM into nicotinic acid, which is subsequently converted to NAD through the NAD salvage pathway in yeast. When *pnc1* is deleted, DR-mediated lifespan extension in yeast is lost, while when Pnc1 is over-expressed, yeast lifespan is increased in a Sir2-dependant manner (Anderson et al 2003). This observation led to the model of dietary restriction causing an increase in Pnc1 expression, which leads to a removal of nicotinamide and thus activation of Sir2, resulting in an increase in lifespan. Expression of Pnc1 in nematodes does not increase lifespan; however it does endow protection from oxidative stress, and mutation of *pnc1* causes a reduction in lifespan (van der Horst 2007). In chapter two, our identification of a *Drosophila* ortholog of Pnc1, termed DNAAM (*Drosophila* nicotinamidase), allowed for the discovery that lifespan controlling properties of this gene are conserved in flies. Over-expression of DNAAM both ubiquitously and in the fly nervous system

resulted in significant increases in both mean and maximal lifespan, which required a functional Sir2 (Balan et al, chapter 2). Interestingly, conditions of oxidative stress, but not of dietary restriction resulted in increased production of DNAAM in Drosophila cells and ectopic DNAAM expression in human neuroblastoma cells resulted in resistance to oxidative damage that required mammalian SIRT1 (see chapter 2). This data provides evidence that Sir2 may also be involved in oxidative stress response, which was confirmed through analysis of oxidative stress response in flies expressing dSir2 (see Chapter 3).

In mammals, NAM catabolism requires the enzyme nicotinamide phosphoribosyltransferase (Nampt) with converts NAM into nicotinamide mononucleotide, which is then converted into NAD by the enzyme nicotinamide mononucleotide adenylytransferase (Nmnat) as part of the mammalian NAD salvage pathway. Nampt expression is regulated by nutrients and stress in human cells and rat tissues and plays a role in cellular stress resistance through regulation of NAD levels and the regulation of the mammalian sirtuins SIRT3 and SIRT4 in mitochondria (Yang et al 2007). Nampt expression increases cellular NAD levels during smooth muscle maturation, and over-expression of Nampt delays smooth muscle cell senescence in a SIRT1-dependent pathway (van der Veer JBC 2007). Nmnat has been shown to protect axons against injury and toxicity in both mice and *Drosophila*, possibly through increased production of NAD (Arakai, et al 2004, Zhang et al 2009). It has been shown recently that both Nampt and Nmnat expression regulates a variety of targets that are also regulated by SIRT1. Furthermore, Nmnat interacts with SIRT1 and is recruited to gene promoters to influence transcription, a mechanism for SIRT1 regulation that may be coupled

with NAD production and SIRT1 activation (Zhang et al 2009). Aside from its ability to regulate sirtuin activity, NAM has been linked to cellular and organism lifespan as a regulator of many other NAD consuming enzymes, including ADP ribosyl transferases, C-ADP-ribose synthetases, and poly-ADP-ribosyl transferases; all of which are inhibited by NAM (reviewed in Brenner 2006). NAM addition significantly shortens the lifespan of yeast and nematodes, and can alter lifespan in yeast through mechanisms independent of sirtuins (Bitterman et al 2002, van Der Horst et al 2007, Tsuchiya et al 2006). Interestingly, NAM appears to have a positive role for cell survival, as addition of NAM to cultured human fibroblast results in increased oxidative stress resistance and replicative lifespan (Hwang et al 2006, Helm et al 2006). Our analysis of DNAAM has shown that while it is a functional nicotinamidase and extends longevity in *Drosophila*, it does not increase oxidative stress resistance in adult flies (Chapter 3). Sir2, however, can both increase lifespan and increase resistance to oxidative stress in adult flies, indicating that these two genes have different effectors with regards to these two phenotypes. It may be that Sir2, but not DNAAM, is a broad regulator of lifespan under conditions of stress, including dietary restriction; while DNAAM is more required for maintenance of metabolism during fly aging through regulation of NAM.

Contemporary Aging Theory

The implications of our work with DNAAM and Sir2 in *Drosophila* to the various theories of how senescence occurs and is managed by organisms are a major focus of chapter 3. In order to fully understand how aging theory correlates senescence with changes in gene activity

and biochemical pathways, it is necessary to understand how experimental observations such as dietary restriction have contributed to the evolution of aging theory. Early aging theory was centered on the observation that ectotherms kept at warmer temperatures exhibited an increased metabolism than those maintained at cooler temperatures, and died at an earlier age. Similarly, invertebrates were shown to exhibit a large increase in lifespan when raised at cold temperatures (Loeb and Northrop 1917). Taken together with the observation that larger endotherms tend to live longer than more metabolically active small endotherms, a "rate-of-living" hypothesis was proposed (Pearl 1923) to describe lifespan as being dependent upon intrinsic metabolic rate. This "rate-of-living" hypothesis proposed that an animal begins life with a predetermined amount of metabolic energy that, when spent, results in death. Furthermore, the theory dictates that there is an inversely proportional ratio between rate of metabolism and lifespan; the faster the metabolic rate, the shorter the lifespan of the organism. More recent evidence offers mixed support for the rate of living hypothesis. In 1982 Cutler published a comprehensive analysis of lifespan and metabolic rate across 77 different mammalian species, and determined the average lifelong metabolic activity per unit mass for each species studied, termed the lifetime energy potential (LEP). When correlated with lifespan, it appeared that there was indeed an inverse relationship between shorter lived and more metabolically active animals (high LEP) versus longer lived animals with a lower LEP. However, the fact that LEP differed vastly among different mammalian species indicated that not all organisms begin with the same total metabolic energy when normalized for size. Furthermore, many species with a similar LEP exhibited vast differences in mean and maximum lifespan, showing that organisms with similar total metabolic energy and body size do not

always have a similar lifespan, as predicted by the theory. Particularly incongruous with the rate of living theory are small flying animals, such as birds and bats, who despite a high metabolism exhibit a robust lifespan compared with other animals of similar size, and humans, who have a lifespan far greater than many other large mammals with similar lifetime metabolisms.

The rate of living theory is contradicted by a wealth of data obtained from the evolutionarily diverse model organisms *Drosophila* and rodents (mice and rats). Research in *Drosophila* showed that breeding for longevity resulted in animals with enhanced lifespan, but with no difference in energy management from progenitor controls at a wide range of temperatures. The long-lived flies exhibited a 40% increase in lifespan and use approximately 40% more calories over their lifespan than controls of the same species and genetic background (Arking et al 1988). Because the LEP of the long-lived flies is higher, the rate of living predicts these animals to have a *shorter* lifespan, in direct contradiction to the observed data. In addition to a lack of consistency between rate of metabolism and lifespan, well characterized environmental manipulations that increase lifespan do not always result in a lowered metabolic rate. It determined that exposure of ectotherms to transient periods of high temperature will result in an increase in lifespan, despite the concomitant increase in metabolic rate during intermittent heat shocks (Lithgow and Miller 2008). A second example is exercise, which results in an increased metabolism (primarily in muscle tissues) via an increase in physical activity. Initial studies of exercise effects in rodents seemed to corroborate the rate of living theory, as extensive experimentally induced exercise resulted in a reduced lifespan versus

sedentary animals (Slonaker 1912). The results of this study were deemed incorrect, as many important variables of nutrition and pathogen exposure were not tightly controlled (Holloszy and Kohrt 1993). In more recent and fastidiously controlled studies, exercise trained rodents were found to have an increase in mean lifespan, or in both mean and maximum lifespan depending on the particular strain of animal tested (Holloszy 1993, Goodrick 1980). Regardless of these stain specific interactions, the trend clearly shows the benefit of periodically increased metabolic activity on mammalian lifespan. The benefit of exercise seems to be conserved in some invertebrates, as certain paradigms of induced physical activity in flies also results in increased lifespan (Piazza et al 2008). The most well characterized and conserved paradigm for lifespan extension is the implementation of the dietary restriction paradigm. In mice, DR results in an increase of both mean and maximum lifespan of up to 60%, but does not significantly alter metabolic rate when normalized to body weight, thus disproving the rate of living hypothesis (McCarter et. al 1996).

These and other similar observations resulted in a consensus among researchers in the latter half of the 20th century that the rate of living theory was not applicable in describing the etiology of aging. Metabolism was however, still strongly correlated with lifespan, and evidence was mounting that diet and the physiological response to stress played a profound role in lifespan regulation. Researchers began to examine cellular processes that could be influenced by alterations in metabolism but may contribute to mortality in a manner independent of metabolic rate. Such a mechanism may be affected by metabolic rate, but would induce mortality through an increase in cellular senescence, and likely initiate pathways

involved in the organism response to stress and cellular damage. One mechanism present in all eukaryotic cells that links metabolism to cellular damage in a time dependant manner is the production of reactive oxygen species (ROS). Eukaryotic cells derive the majority of their energy through the process of mitochondrial oxidative phosphorylation (OXPHOS), which generates ATP through the metabolism of carbohydrates and fats utilizing O_2 as an acceptor for free electrons that are oxidized by the electron transport chain (ETC). During this complex process atmospheric oxygen is utilized at several steps to accept electrons and form water while pumping hydrogen ions into the mitochondrial inner membrane to form a proton gradient used to drive the synthesis of ATP. This process is also responsible for the production of superoxide anion (O_2^-), which is a highly reactive molecule that can oxidize and damage molecules important to cellular function, or react with hydrogen peroxide to form even more damaging hydroxyl radicals (reviewed in Wallace 2008). Over the lifetime of an individual cell, ROS buildup can result in sufficient damage to the mitochondria or other cellular components (such as DNA) that a cell will begin to lose function and/or initiate apoptosis. Accordingly, ROS accumulation has been linked to many age related disorders, including neurodegenerative disease, heart disease, and cancer.

The study of ROS metabolism and connection to cellular senescence resulted in the presentation of the theory now termed the “Free radical (or ROS)” theory of aging by Denham Harman in 1956. Elegant in its simplicity, the theory states that aging is an effect of the accumulation of ROS over the lifetime of an organism. Eventually the accumulation of ROS induced damage results in the etiology of a terminal disease, or significant tissue malfunction

that cannot be righted by the intrinsic defenses of the organism. The ROS theory of aging is particularly compelling as it couples both cellular metabolism and organism stress response to longevity. Alterations in metabolism, such as excess calories, can increase the flux of electrons through the ETC, resulting in increased electron transfer to O_2, and thus increased production of ROS (Wallace 1999). Alternatively, more efficient metabolism, such as that exhibited in dietary restricted animals results in the reduction of excess electrons, and a concomitant reduction in the production of ROS (Weindruch and Walford 1988). This metabolic model of ROS production helps explain why obesity is associated with increased risk of age-related disease, while dietary restriction results in a decrease in age-related disease and increase in longevity (Wallace 2005).

Cells have evolved a variety of enzymes to manage ROS, which are often regulated by pathways that sense changes in metabolism or respond to an increase in environmental stress. Antioxidant enzymes help protect a cell from oxidative damage through direct detoxification of ROS, and include manganese superoxide dismutase (MnSOD), glutathione peroxidase (Gpx1), and catalase. Other enzymes such as uncoupling proteins (UCPs) help to control oxidative damage by increasing the efficient movement of electrons through the ETC and thus reduce the production of ROS (Cadenas et al 1999, Lee et al 2002). Enzymes such as insulin and glucagon regulate the production of ROS by increasing or decreasing the ratio of glycolysis versus oxidative phosphorylation, respectively. Insulin causes an increase in glycolysis and decrease in OXPHOS, which generates an abundance of reducing compounds such as NADH and FADH in the mitochondrial matrix, and leads to the increased production of ROS (Wallace MBA text).

Still other enzymes are responsible for increasing or decreasing the transcription of antioxidant enzymes based on changes in metabolism and the incidence of environmental stress. Any environment, enzyme or pathway that is capable of modulating these and other similar enzymes has the potential to augment the production and/or detoxification of ROS and thus modify the ability of an animal to respond to oxidative damage.

The primary evidence supporting the role of ROS in aging is the observation that ROS and oxidative damage do indeed increase with age in a diverse array of cell types and tissues across several model organisms (Bokov et al 2004). Secondly, exposure of model organisms to oxidative stress inducing compounds, and/or antioxidant gene mutation can all cause an increase in age associated mortality and disease. Considering this fact, the main hypotheses of the oxidative stress theory can be readily tested in experimental organisms. The main hypothesis is as follows: organisms with increased ROS production will exhibit increased mortality and incidence of age related disease versus organisms with decreased production of ROS. Testing of this hypothesis involves comparison of ROS production and damage between long-lived and shorter lived animals of the same species, and the analysis of the effects of gene mutations in pathways related to oxidative stress resistance and/or lifespan. Trends in data initially provided numerous examples of support for the ROS theory of aging. Recent observations however, including our own experimental data suggests that while there is often a correlation between oxidative stress resistance and enhanced longevity, there are many exceptions that disprove the ROS hypothesis as a universal phenomenon. Therefore the correlation between oxidative stress response and lifespan is contingent upon a complex

interaction between the genes and pathways that are being modified experimentally and the background genetics of the organism tested (see chapter 3).

The ROS theory of aging is supported by studies showing a correlation between extended lifespan with the increased resistance to oxidative damage or lower cellular levels of ROS, or observations of a decrease in lifespan of animals with reduced tolerance to oxidative stress or exhibiting increased ROS production. A powerful example that supports the ROS theory is the observation that dietary restricted animals have a greater ability to tolerate experimentally induced oxidative stress, and show a reduction in the production of intrinsic ROS relative to controls. As previously stated, dietary restriction is the most reliable and replicable environmental manipulation to extend the lifespan of a variety of organisms, and there is ample evidence that DR animals superior ROS management is evolutionarily conserved (reviewed in Merry 2004, Sinclair 2005, Bishop and Guarente 2007). DR mice display an age-related reduction in molecular oxidative damage to lipids, proteins, and nucleic acids perhaps owing to a decrease in mitochondrial ROS production, and are more tolerant to experimentally induced oxidative damage to both isolated cells and through feeding of ROS inducing chemical such as paraquat (Sohol et al 1994, Yu and Masoro 2002, Sanz et al 2006). DR rodents also exhibit changes in gene expression consistent with an increase in oxidative stress response pathways, such as increased antioxidant genes and augmentation of genes that modulate stress response elements (Lee et al 1999, Lee et al 2002). There is also a correlation between oxidative stress and longevity in *Drosophila* that are artificially selected for extended lifespan, however this is not observed in animals selected for oxidative stress resistance. We have

shown that flies selected for longevity (LA) display a reduction in ROS production and oxidative damage, as well as an increase in antioxidant defenses versus non selected controls (RA) (Vettraino et al 2001). Long-term selection for resistance to the oxidative stress inducing chemical paraquat (PQR F5-F23), results in flies with over 100% increases in oxidative stress resistance, however without concomitant increases in longevity (see Table 1) versus progenitor strain controls. While enhanced longevity endows oxidative stress resistance, oxidative stress resistance does not seem to enhance longevity in our model, and thus may not be causal to the enhanced lifespan of our LA strain (Table 1).

While enhanced oxidative stress resistance did not appear to cause extended longevity on our model, genetic analysis has shown that there may be a causal link between oxidative stress response and lifespan when the regulation of certain critical metabolic pathways is experimentally altered. In flies, the neurofibromatosis gene (NF1) stimulates adenlylcyclase, increasing the concentration of cyclic AMP (cAMP) which is known to modulate the efficiency of mitochondria and increase flux through OXPOHS complex I of the electron transport chain (Tong et al 2007). When NF1 is mutated, flies exhibit a reduction in lifespan, reduced tolerance to oxidative stress, and a doubling of mitochondrial ROS production (Tong et al 2007). When this gene is over-expressed, flies exhibit increased lifespan, oxidative stress resistance, and decreased ROS production. This contrast is proposed to occur as a result of cAMP regulation of mitochondrial efficiency, which strongly effects the production of ROS. Mutations in insulin signaling components can increase both stress resistance and longevity in a wide variety of organisms, including yeast, *C. elegans*, *Drosophila*, and mice (reviewed in Wallace 2008). As

mentioned previously, insulin is a powerful regulator of metabolism, causing a shift from OXPHOS to glycolyisis and resulting in more reducing components in the ETC of the mitochondria that can lead to the production of ROS. When the insulin ligand is itself mutated, or components of the insulin signaling pathway mutated to reduce signal output, FOXO transcription factors are activated, and up-regulate the transcription of genes involved in mitochondrial biogenesis, such as peroxisome-proliferation-activated receptor coactivator (PGC-1α), and antioxidant genes such as MnSOD and catalase (Furuyama et al 2003, Wallace 2005, Kelly and Scarpulla 2004). Thus down regulation of insulin dependent metabolism may increase lifespan through increased mitochondrial efficiency and increased antioxidant protection. Other gene mutations correlating longevity to oxidative stress resistance include the sirtuin family protein deacetlyase *Sir2*, the G-protein coupled receptor *Methuselah*, the proto-oncogene *p66shc*, the ROS detoxification gene *SOD1*, and the stress response gene *HSP22* (Morrow et al 2004, Migliaccio et al 1999, Fabrizio et al 2001). It has also been shown in mice that dietary supplementation of antioxidants, such as lipoic acid and coenzyme Q10, can also moderate transcription of key elements of the oxidative stress response and elicit phenotypes of reduced aging rate (Lee et al 2004). Continued examination of characterized longevity and stress response genes and canonical pathways that effect lifespan and oxidative stress management will continue to add to this growing list of proposed stress resistance/longevity determining factors.

Despite strong evidence supporting the oxidative stress theory, recent studies review and highlight the numerous exceptions which disprove a correlation of ROS production and

management with lifespan (Perez et al 2009, Sanz et al 2010). In Perez et al. researchers explore the effects of both mutation and over-expression of an impressive array of antioxidant enzymes on lifespan in mice. The genes included in their analysis include *Sod1, Sod2, Gpx1*, and *Gpx4*; which code for enzymes critically important for the detoxification of superoxide and hydrogen peroxide. Interestingly, only *Sod1-/-* mice display a decrease in lifespan, despite increased oxidative damage and/or a reduced oxidative stress tolerance phenotype being present in each mutant genotype. Importantly, over-expression of individual antioxidant enzymes did not increase lifespan even though data indicated an overall increase in oxidative stress resistance. *Drosophila* expressing the powerful plant and fungal antioxidant enzyme AOX showed a strong age-related decrease in mitochondrial ROS (mtROS) production without any effect on lifespan (Sanz et al 2010). Furthermore, flies mutant for the peroxiredoxin gene dj-1β exhibit an increased production of mtROS and exhibit an *increased* in lifespan of both sexes versus controls (Sanz et al 2010). This direct contradiction of the oxidative stress theory has also been observed in flies expressing both MnSOD and catalase in the mitochondria exhibit an increase in oxidative stress resistance, but a robust decrease in lifespan (Bayne, et al 2005). Finally, as mentioned, our analysis of *Drosophila* strains selected for paraquat resistance (Table 1) shows that the acquisition of significant resistance to oxidative stress is not accompanied by an extended longevity (Vetraino et al., 2001). Thus oxidative stress resistance and longevity do not always occur together in both *Drosophila* and mice; therefore the ROS theory is in need of modification to reflect these exceptions.

A second theory that expands on the idea of oxidative stress reduction and increased longevity is the multiple stress response theory or multiplex theory of aging (reviewed in Miller 2009). The multiplex theory postulates that changes in lifespan result from a conserved stress response network that manages several forms of stress and induces longevity genes during that response. The multiplex hypothesis is strongly supported by work in *C. elegans*; particularly with the insulin signaling hypomorphic mutant *daf-2*. *Daf-2* worms show a large increase in lifespan (Kenyon et al 1993), along with a concomitant increase in resistance to thermal stress (Gems et al 1998), oxidative stress (Vanfleteren 1993), UV radiation (Murakami and Johnson 1996), and bacterial infection (Garsin et al 2003). Microarray analysis details the up-regulation of a wide variety of stress response genes as a result of *daf-2* mutation, which likely mediates the observed multiplex response of these organisms (Fisher and Lithgow 2006). Outside of worms, however, there are discrepancies with the multiplex theory in the observation that there is not always a correlation between stress response and longevity in model organisms. Certain forms of stress resistance have been shown to correlate with increased longevity, but also with an increased susceptibility to other forms of stress. In *Drosophila*, for example, when insulin-like peptides are eliminated, flies exhibit an increase in longevity, oxidative stress resistance, and starvation resistance; but show a *decrease* in tolerance to thermal stress (Broughton et al 2005). Similarly, while over-expression of the antioxidant enzyme MnSOD increases lifespan in *Drosophila*, it does not significantly increase tolerance to oxidative stress and also *reduces* thermo-tolerance (Sun et al 2002). Therefore, in *Drosophila*, stress resistance and longevity are not always linked, and furthermore increases in one form of stress resistance can correlate with a reduction in a second form of stress resistance.

The effect of dietary restriction can also be considered a form of environmental stress, and DR mediated affectation of mortality rate is very different than the effect of temperature reduction on lifespan. Despite both manipulations being capable of increasing lifespan, the induction of dietary restriction can reverse mortality rate while temperature reduction slows the rate of increased mortality, and both manipulations have different effects on biomarkers of aging (Jacobson et al 2010). Thus, these different forms of environmental stress do not appear to elicit the same physiological response, as predicted by the multiplex theory. It is possible that because of their ectothermic life histories flies may have evolved a disparate response to temperature that would not be necessary in endothermic animals. Currently, analysis of the multiplex theory in the mammalian models is in a fairly nascent stage. Recent data reports that fibroblast cells derived from long lived mice are resistant to many, but *not all* forms of cellular stress, and that resistance appears to be congenital in these animals (Murakami S, et al 2003, Harper et al 2007, Salmon et al 2005). The fact that not all forms of stresses applied were protected against in these models presents a problem to the multiplex assumption that all stressor elicit the same coordinated cellular response. Furthermore the lack of data from whole organisms has hampered a more complete analysis of this theory in higher organisms. Our work analyzing the effect of artificial over-expression of longevity and non-longevity genes on oxidative stress response in chapter 3 sought to understand if the ROS theory or multiplex theory provides a plausible explanation for the longevity effects measured under conditions of gene induction and provides evidence for necessitated augmentation of these theories to account for different genes activating different pathways toward longevity that may depend entirely on the genetic background of the organism or strain tested.

Chapter 2

Life Span Extension and Neuronal Cell Protection by *Drosophila* Nicotinamidase

Abstract

The life span of model organisms can be modulated by environmental conditions that influence cellular metabolism, oxidation, or DNA integrity. The yeast nicotinamidase gene *pnc1* was identified as a key transcriptional target and mediator of calorie restriction and stress-induced life span extension. PNC1 is thought to exert its effect on yeast life span by modulating cellular nicotinamide and NAD levels, resulting in increased activity of Sir2 family class III histone deacetylases. In *Caenorhabditis elegans*, knockdown of a *pnc1* homolog was shown recently to shorten the worm life span, whereas its over-expression increased survival under conditions of oxidative stress. The function and regulation of nicotinamidases in higher organisms has not been determined. Here, we report the identification and biochemical characterization of the *Drosophila* nicotinamidase, DNAAM, and demonstrate that its over-expression significantly increases median and maximal fly life span. The life span extension was reversed in Sir2 mutant flies, suggesting Sir2 dependence. Testing for physiological effectors of DNAAM in *Drosophila* S2 cells, we identified oxidative stress as a primary regulator, both at the transcription level and protein activity. In contrast to the yeast model, stress factors such as high osmolarity and heat shock, calorie restriction, or inhibitors of TOR and phosphatidylinositol 3-kinase pathways do not appear to regulate DNAAM in S2 cells. Interestingly, the expression of DNAAM in human

neuronal cells conferred protection from oxidative stress-induced cell death in a sirtuin-dependent manner. Together, our findings establish a life span extending the ability of nicotinamidase in flies and offer a role for nicotinamide-modulating genes in oxidative stress regulated pathways influencing longevity and neuronal cell survival.

Introduction

Genetic manipulations and environmental conditions have been shown to modulate life span in various experimental model systems (Sinclair 2005, Guarente and Kenyon 2000). The environmental conditions include restricted calorie and nutrient availability that affect cellular metabolism as well as stress conditions such as heat and osmotic shock. Accordingly, the genetic manipulations are documented in stress response proteins and in metabolic proteins such as those along the insulin signaling pathway (Guarente and Kenyon 2000, Tatar et al 2003). In the yeast *Saccharomyces cerevisiae*, several of these experimental alternations have been found to affect the replicative life span. Recent studies identified the pyrazinamidase/nicotinamidase gene *pnc1* as a key effecter of calorie restriction and mild stress-induced life span extension. These conditions induce increased *pnc1* transcription and activity (Gallo et al 2004, Anderson et al 2003, Bitterman et al 2002). In addition, over-expression of PNC1 was shown to be sufficient for extending the replicative life span of yeast.

In yeast, PNC1 functions in the NAD salvage pathway by converting nicotinamide to nicotinic acid (see Fig. 1*A*) (Yang et al 2006, Revollo et al 2004, Rongvaux et al 2003). Nicotinamide is a component of vitamin B3/niacin and serves as a precursor in NAD biosynthesis (Suave 2008, Csiszar et al 2005). NAD serves as a coenzyme in reversible redox

reactions associated with cellular metabolism in all living cells. Recently, NAD and nicotinamide have emerged as regulators of a class of enzymes known as "NAD consumers" that have been linked to cellular stress resistance, life span, and various diseases (Bordone and Guarente 2005, Belenky et al 2007, Bogan and Brenner 2008). These enzymes include ADP-ribosyl transferases, poly-ADP-ribosyl polymerases, cADP-ribose synthetases, and Sir2 (silent information regulator 2) protein deacetylases. Reactions performed by these enzymes can rapidly deplete cellular NAD and generate nicotinamide, which acts as a potent feedback inhibitor of the NAD consumers. NAD depletion and nicotinamide buildup is ameliorated by salvage enzymes such as PNC1 that convert nicotinamide to metabolites that can be recycled back to NAD (Berger et al 2004). Increasing evidence suggests that nicotinamide recycling is essential for both maintenance of intracellular NAD and regulation of NAD consumers in response to various internal and external stimuli (Rongvaux et al 2003, Anderson et al 2003, Rogina and Helfand 2004).

The life span extending functions of *pnc1* in yeast are thought to be mediated at least in part by increasing the activity of the NAD-dependent protein deacetylase Sir2, and *pnc1* is considered the key regulator of Sir2 in the yeast response to calorie restriction and stress (Anderson et al 2003, Tissenbaum and Guarente 2001). PNC1 is thought to increase Sir2 function through two complementing mechanisms: 1) by increasing the NAD/NADH ratio, providing higher concentration of the NAD cofactor, and 2) by decreasing the concentration of nicotinamide, which acts as a noncompetitive feedback inhibitor of Sir2. It is important to note that although increased Sir2 activity has been shown to extend the life span of several model

organisms (Kaeberlein et al 1999, Haigis and Guarente 2006, Longo and Kennedy 2006), it is not yet established whether all of the life span-extending functions of PNC1 can be attributed to increased Sir2 activity.

Yeast Sir2 is a prototype NAD-dependent histone/protein deacetylase conserved through evolution (Michishita et al 2005 Guarente 2007, Lagouge et al 2006). Mammals express at least seven Sir2 orthologs, designated sirtuins (Sirt1–7), varying in cellular expression and function (Baur et al 2006). Mammalian sirtuins have been shown to deacetylate and regulate a large array of substrates including p53, FOXO, tubulin, and metabolic proteins such as PGC-1, PPAR, GDH, and acetyl-CoA synthetase (Yang et al 2006, Mishishita et al 2005, Kaeberlein and Rabinovitch 2006, van der Horst et al 2007, Wang and Pichersky 2007). Thus, sirtuins have a significant potential to impact metabolic pathways involving glucose homeostasis, the insulin/IGF-1 signaling pathway, and stress response pathways associated with DNA damage and oxidative stress (Sinclair 2005). Pointing to a possible role of sirtuins in mammalian aging and metabolism, two independent studies have demonstrated recently that oral administration of resveratrol (a natural compound enriched in grapes and identified as a direct activator of sirtuins) can reverse the pathophysiologic effects of high fat diet and restore life span (Hunt et al 2007, Revollo et al 2007). However, it remains to be determined to what extent these beneficial effects of resveratrol result from its ability to activate sirtuins (Yang et al 2007). *Pnc1* homologs have been reported recently in *Caenorhabditis elegans* and Arabidopsis. A recent study (van der Horst et al 2007*)* identified a *C. elegans* homolog of *pnc1* and demonstrated that knockdown of the gene decreases the adult worm life span. Although increasing *pnc1* dosage

did not result in increased adult life span, it did confer increased survival under oxidative stress. The increased survival was reduced in worms treated with Sir2 RNA interference, suggesting that in worms the effects ofPNC1 are also mediated at least in part through Sir2. In Arabidopsis, at least two nicotinamidase genes have been identified (AtNIC1 and NIC2), and null alleles have been associated with increased sensitivity to salt, abscisic acid, and DNA damaging agents (Chong et al 2004, Kang et al 2003).

Although PNC1 appears the primary enzyme that metabolizes nicotinamide in yeast, Arabidopsis, and low metazoans, higher organisms and mammals evolved a somewhat different NAD salvage pathway (see Fig. 1*A*). The functional counterpart of yeast PNC1 is proposed to be nicotinamide phosphoribosyltransferase (Nampt/PEBF/visfatin), which converts nicotinamide into nicotinamide mononucleotide instead of nicotinic acid, with nicotinamide mononucleotide being subsequently converted to NAD. Nampt function has been correlated with insulin regulation, cell survival, and life span and has also been shown to affect the activity of mammalian sirtuins, including the mammalian ortholog of yeast Sir2, SIRT1 (Yang et al 2006, Revollo et al 2004, Busso et al 2008). Nampt appears to function both intracellularly and as a secreted enzyme; however, our current knowledge of its exact regulation and function is limited (Bordone and Guarente 2005, Du et al 2001). Nevertheless, the nicotinamide-Sir2 link seems conserved through evolution, because mammalian sirtuins are also inhibited by nicotinamide. Thus, nicotinamidase orthologs, through affecting the activity of sirtuins and other NAD consumers, could prove key players in physiological pathways controlling cellular metabolism, cell death and survival, and ultimately aging.

In the present study, we describe the identification and biochemical characterization of the *Drosophila* nicotinamidase DNAAM, and demonstrate life span-extending properties of the gene. The nicotinamidase activity of DNAAM was cell type autonomous and displayed comparable activity when purified from mammalian or *Drosophila* cell lines. In contrast to yeast, DNAAM expression and activity in *Drosophila* cells was not affected by calorie and nutrient availability or by inhibitors of the PI3K3 or TOR pathways. Rather, DNAAM was found to be responsive primarily to oxidative stress and anisomycin. Low oxidative stress increased DNAAM mRNA expression by up to 6-fold and also increased protein expression and cellular nicotinamidase activity. Importantly, transgenic flies over-expressing DNAAM exhibited an increase in both the mean and maximal life span of up to 30%. The increased life span was reversed in Sir2 mutant flies, suggesting a role of sir2 in DNAAM-induced life span extension. Interestingly, DNAAM was found to be functional in mammalian cells, and its expression in human neuronal cells conferred resistance to oxidative stress-induced cell death in a manner dependent on sirtuin activity. Our results establish a life span extending the ability of nicotinamidase in flies and suggest a role for nicotinamide-modulating genes in oxidative stress pathways influencing longevity and neuronal cell survival.

Materials and Methods

DNA Constructs and Antibodies. DNAAM was PCR-amplified from a *Drosophila* gene collection clone LD05707 and subcloned into pAc 5.1, pUAST, pExchange 5A, or pEGFP vectors as Acc65I-BstEII, Acc65I-Acc65I, or SacII-XhoI fragment, respectively. pEGFP-Sirt1 was a kind gift from Dr. Izumi Horikawa. V5 epitope antibodies were from Invitrogen. DNAAM antibodies were

generated by PickCell Laboratories (Amsterdam, The Netherlands) against the last 20 carboxylterminal DNAAM residues. Crude serums from two immunized rabbits were tested for DNAAM reactivity and were affinity-purified using the immunizing peptide and a SulfoLink kit (Pierce). Preimmune serum from the above rabbits served as a control antibody as indicated in the figure legends.

Tissue Culture, Transfection, and Cell Death Assay. Embryonic *Drosophila* S2 cells, mammalian COS-7 cells, and human neuroblastoma SH-SY5Y cells were maintained in their appropriate growth media. S2 cells were transfected using CellFectin (Invitrogen), COS-7 cells using Lipofectamine (Invitrogen), and SH-SY5Y cells using lipofection and lipofectamine. For neuronal cell death assay, 60–70% confluent cells expressing the indicated vectors were treated with 100 or 300 M 6-(2-hydroxy-1-methyl-2-nitrosohydrazino)-*N*-methyl-1-hexanamine (NOC-9) or sodium nitroprusside, and cell death was analyzed using trypan blue dye exclusion 24 h post-treatment, and cell apoptosis was analyzed using a deoxynucleotidyltransferase- mediated dUTP nick end labeling assay as previously described (Chong et al 2004). The mean survival was determined by counting eight randomly selected non-overlapping fields with each containing 10–30 cells (viable and nonviable). Each experiment was replicated 4–6 times with comparable results. In experiments using EGFP-tagged proteins, images were acquired with “blinded” assessment with a Leitz DMIRB microscope (Leica) and a Fuji/Nikon Super CCD (6.1 megapixels), and cell death was analyzed in all cells or was analyzed separately for EGFP-positive and -negative cells. In experiments using sirtinol, the cells were treated with varying concentrations of sirtinol 1 hour prior to exposure to NOC-9.

Nicotinamidase/Pyrazinamidase Assay. Cellular and whole fly protein extracts were prepared in extraction buffer containing 50 mM Tris-Cl, pH 7.5, 100 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, 1mM EDTA, 1mM EGTA, 2mM Na3VO4, 50 mM glycerophosphate, and a protease inhibitor mixture (GE Healthcare, Piscataway, NJ).DNAAM for the assay was immunopurified from 1 mg of protein extracts using DNAAM or V5 antibodies conjugated to protein A beads. As a control, preimmune serum was used for the DNAAM antibody, and nontransgenic fly extracts or nontransfected cell extracts were used for the V5 immunopurifications. The beads were washed twice with extraction buffer, twice with extraction buffer containing 500 mM LiCl, and twice with nicotinamidase reaction buffer containing 5mM Tris-Cl, pH 7.5, 150mM NaCl, and 1mM MgCl2. For the assay, the beads were incubated in reaction buffer alone or in reaction buffer containing 4mM nicotinamide or pyrazinamide for 0 min or for 90 min as indicated in the figure legends at 30 °C in a rotating mixer. Following the incubation, the mixture was spun down, and the supernatant was collected and analyzed for ammonia content using ammonia detection kit (Wako Chemicals, Richmond, VA) according to the manufacturer's instructions. The amount of the nicotinamidase used in the assay and the reaction conditions were set in a way that the final ammonia readings were in a linear range, *i.e.* final *A*630 readings were in the range of 0.1–1. In all experiments, the *A*630 readings in control immunoprecipitates, 0-min incubations, and nicotinamide-omitted samples were consistently below 0.01.

DNAAM Expression Analysis. For analyzing effects of culture conditions on DNAAM expression, S2 cells were maintained in serum-free complete growth medium (*Drosophila* SFM medium;

Invitrogen) or were cultured in medium lacking proteins (DS2 medium; CellGrow) for growth factor depravation. For nutrient depravation, the SFM and DS2 media were diluted 1:4 with phosphate-buffered saline as indicated in the figure legends. For assaying stress effects, the cells were grown in complete medium and treated with stress factors as indicated in the figure legends. DNAAM expression was analyzed using real time PCR for mRNA and Western blotting for protein expression. Real time PCR was performed according to established methods using 107 S2 cells. ToTALLY RNA kit (Ambion, Austin, TX) and RNeasy (Qiagen) were used forRNAextraction and purification, and Applied Biosystems kits (Roche Applied Science) were used for primer design/synthesis, reverse transcription, and the real time PCR. For standardization, DNAAM real time PCR results were normalized against *Drosophila* ribosomal protein L32 using a standard probe from Applied Biosystems. Each experiment was performed in duplicate, and the real time PCR was done in triplicate. The fold change in RNA expression was calculated using the *C*t method. The DNAAM expression in S2 cells growing in complete medium was taken as the standard point for calculating fold change.

Genetic Crosses, Longevity Assays, and Statistical Analysis. The UAS-Gal4 system was used to drive over-expression of DNAAM. Over-expression was driven with both the ubiquitous *tub*-Gal4 driver and the pan-neural *elav*-Gal4 driver. pUAS-DNAAM-V5 DNA was injected into w1118 embryos (Duke university), and the resulting positive transformants were identified and chromosomal insertions balanced using standard techniques. Transformants were crossed directly to *tub*-Gal4 and or *elav*-Gal4 when their insertion was on a different chromosome from the Gal4 driver. This allowed for obtaining flies heterozygous for the UAS-DNAAM insertion on

one chromosome and for the Gal4 driver on another chromosome. F1 test flies of the genotypes (DNAAM33/+, *tub*-Gal4/+; DNAAM42/+, *tub*-Gal4/+; DNAAM31/+, *tub*-Gal4/+; and *elav*-Gal4/+, DNAAM42/+) were selected by absence of balancers. Genetically matched wild type controls lacking both driver and UAS-transgene were generated by self-crossing F1 test flies. When the DNAAM transgene and Gal4 driver were on the same chromosome, lines were first out-crossed to w1118 flies to obtain DNAAM33/+, DNAAM31/+, and *elav*-Gal4/+ flies, and these lines were crossed to collect test flies (DNAAM33/*elav*-Gal4 and DNAAM31/*elav*-Gal4) based on eye color and matched wild type controls by the absence of eye color. To over-express DNAAM in a Sir2 mutant, we used $Sir2^{4.5}$/CyO, $Sir2^{5.26}$/CyO (obtained from Stephen Helfand), DNAAM42/TM3, and *elav*-Gal4/TM3 to generate two parental stocks: $Sir2^{4.5}$/CyO, *elav*-Gal4/TM3; and $Sir2^{5.26}$/CyO, DNAAM42/TM3. These flies were crossed to generate flies over-expressing DNAAM in a Sir2 mutant background ($Sir2^{4.5}$/ $Sir2^{5.26}$, DNAAM42/*elav*-Gal4). To control for *elav*-Gal4 or UASDNAAM42 insertion sites, these parental stocks were crossed to the opposite Sir2 allele, *i.e.* to generate ($Sir2^{4.5}$/ $Sir2^{5.26}$, *elav*-Gal4/+; and $Sir2^{4.5}$/ $Sir2^{5.26}$, DNAAM42/+, respectively). The Sir2 mutant control was obtained by directly crossing $Sir2^{4.5}$/CyO to $Sir2^{5.26}$/CyO to generate $Sir2^{4.5}$/ $Sir2^{5.26}$ flies. Flies over-expressing DNAAM42 in neurons and their genetically matched controls were generated as described above. All of the fly stocks were maintained on standard cornmeal molasses medium. Test and control flies were verified with PCR analysis for transgenes/mutations and Western blotting for DNAAM over-expression. For the longevity experiments, the flies were mated and raised on sucrose medium (Luckinbill et al 1984). Virgin male and female flies were separated into aliquots of 20/vial and transferred into fresh vials every 2 days while scoring for viability. Recording of deceased flies

began after one initial food transfer to avoid anesthetization effects. Deceased flies were recorded and replaced with marker flies to maintain population density. Chi square tests compared the proportion of flies surviving between test and control flies to assign significance to the curves. Median survival was compared using Kruskal-Wallis tests. Mean and maximal survival of the top 10 and 20% were calculated for males and females separately using a one-way analysis of variance and orthogonal contrasts. All of the analyses were done using SPSS, version 13.5.

Results

Identification and Biochemical Characterization of Drosophila Nicotinamidase, DNAAM—To identify PNC1 homologs in higher organisms, we used the yeast PNC1 protein sequence to blast search the Swiss-Prot and GeneBank data bases, identifying two open reading frames in *C. elegans* (accession number NP 499876 and NP 001023531) and one in *Drosophila melanogaster* (accession number NP 732446). Alignment of the *Drosophila* protein, which we designate DNAAM (*Drosophila* nicotinamide amidase), with yeast PNC1 and PNCA protein from *Pyrococcus horikoshii* shows a moderate overall homology however, the homology is significantly higher when looking at regions in PNCA regarded as critical for catalytic activity (Araki et al 2004) suggesting functional conservation. A DNA clone obtained from the *Drosophila* gene collection containing the full-length DNAAM cDNA (LD05707) was verified for sequence accuracy and subcloned with a carboxyl-terminal V5-His6-epitope tag into the *Drosophila* expression vector pAc 5.1 for expression in *Drosophila* Schneider2 (S2) cells and the pUAST vector for P-element mediated germ-line transformation (Brand and Perrimon 1993).

DNAAM protein expression was examined in S2 cells transfected with pAc 5.1-V5-His6-DNAAM or pUAST-V5-His6-DNAAM cotransfected with pMET-Gal4 that encodes the yeast Gal4 transcription factor needed for expression from the pUAST vector (Fig. 1*B*). The tagged protein in S2 cells migrated as a 48-kDa band efficiently purified by immunoprecipitation using V5 antibodies.

To examine the enzymatic activity of DNAAM, the tagged protein was immunopurified using V5 antibodies and subjected to a nicotinamidase activity assay developed in this study by modifying a previously described calorimetric assay (Chaykin 1969) (Fig. 1, *C* and *D*). The assay measures nicotinamidase activity using either nicotinamide or pyrazinamide as a substrate and determining ammonia release as the reaction end product. Nicotinamidase activities measured in this assay had low background readings even after 90-min incubations in the absence of a substrate or the enzyme (V5 immunoprecipitate from mock transfected cells was used as a negative control). These experiments established that DNAAM encodes a *bona fide* nicotinamidase and that DNAAM does not distinguish between nicotinamide and pyrazinamide as a substrate, similarly to the bacterial PNCA. To test whether DNAAM functions autonomously or whether it requires specific cofactors present exclusively in *Drosophila* cells, we expressed DNAAM in mammalian COS-7 cells and compared its nicotinamidase activity with that of DNAAM expressed in *Drosophila* S2 cells (Fig. 1*E*). COS-7 cells expressed comparable protein levels and nicotinamidase activity as S2 cells, indicating that DNAAM does not require *Drosophila* cell-specific cofactors for expression or activity.

DNAAM Expression and Activity Are Regulated by Oxidative Stress—PNC1 in yeast has been demonstrated to be regulated by calorie and nutrient availability as well as by mild stress conditions such as heat shock and osmotic stress (Anderson et al 2003). To examine the regulation of DNAAM in *Drosophila* S2 cells, we measured the expression and activity of DNAAM under varying environmental conditions (Figs. 2 and 3). Using real time PCR experiments, we identified primarily two factors that significantly affected DNAAM mRNA expression: hydrogen peroxide and anisomycin (Fig. 2*A*). Interestingly, various other stress factors, such as osmotic and heat shock, high salt, or UV irradiation did not significantly affect DNAAM expression levels (Fig. 2, *A* and *B*), although they induced comparable cell death to oxidative stress (data not shown). These results suggest a unique regulation of DNAAM by pathways sensitive to oxidative stress and anisomycin. The effects of hydrogen peroxide on DNAAM mRNA expression were dose- and time-dependent, reaching up to a 5-fold increase at 10 μM and following 8–16 h of treatment (Fig. 2*C*). Anisomycin treatment resulted in a higher increase, reaching up to 20-fold (Fig. 2, *A* and *B*). Importantly, we did not observe any affect of nutrient restriction or compounds that inhibit signaling molecules along the insulin/IGF-1 pathway such as PI3K inhibitors or TOR inhibitors on DNAAM expression (Fig. 2*A*). These results indicate that DNAAM regulation in *Drosophila* cells does not follow the same pattern as in yeast, at least with regards to dietary restrictions and responses to high salt and osmotic shock.

To confirm the results obtained with the mRNA experiments at the protein level, we developed antibodies against a carboxylterminal DNAAM peptide and examined DNAAM protein expression in S2 cells grown under various media and stress conditions (Fig. 3*A*).

Similarly to what we observed with DNAAM mRNA expression, only oxidative stress significantly affected DNAAM protein expression, inducing up to a 3-fold increase. As anticipated from its function as a protein synthesis inhibitor, the ability of anisomycin to induce DNAAM mRNA expression was not reproduced at the protein level. These experiments also pointed out that DNAAM is not a highly stable protein, showing a half-life of approximately 8 hours (data not shown). Finally, the activity of DNAAM in S2 cells exposed to various growth conditions was determined by immunopurifying endogenous DNAAM and assaying for nicotinamidase activity (Fig. 3*B*). The increased activity observed with oxidative stress correlated with increased DNAAM expression, suggesting that the differences in activity reflected increased DNAAM protein expression rather than changes in specific activity. Confirming this notion, we did not observe any effects of oxidative stress on DNAAM activity when it was expressed from an expression vector or when the activity was standardized for DNAAM protein recovery (data not shown). Combined, these results indicate that oxidative stress regulates DNAAM at the transcriptional level, increasing its mRNA and protein expression and thus its cellular nicotinamidase activity.

Transgenic Flies Overexpressing DNAAM Exhibit Increased Nicotinamidase Activity—As noted above, PNC1 over-expression extends yeast replicative life span. We hypothesized that DNAAM may have similar effect in *Drosophila*. To test this hypothesis, 10 independent germ-line transformants carrying a pUAST-DNAAM vector were generated and crossed with *tub*-Gal4 driver flies to achieve ubiquitous expression of DNAAM via the UAS-Gal4 system. These flies expressed varying amounts of DNAAM protein (Fig. 4*A*), consistent with different insertion

sites. The transformants were grouped as low (*lanes 3* and *5*), medium (*lanes 1*, *2*, *4*, and *6–8*), or high (*lanes 9* and *10*) expressers. The high expressing DNAAM transgenic lines exhibited up to a 5-fold increase in DNAAM protein expression when compared with the endogenous protein level (Fig. 4*B*). The DNAAM antibody stained a doublet band in fly extracts, suggesting alternative splicing or a post-translational modification of DNAAM. Importantly, the level of DNAAM expression in the different transformants directly correlated with increased nicotinamidase activity in these flies (Fig. 4, *C* and *D*). The difference in activity seen when using V5 and DNAAM antibodies reflected the difference in their immunoprecipitation efficiencies (data not shown). As expected, nicotinamidase activity was not detected in control preimmune serum immunoprecipitates from transgenic flies or from V5 immunoprecipitates from control, w1118 flies. Similar to PNC1 overexpression in yeast, DNAAM overexpressing flies showed, in an initial analysis, increased NAD/NADH levels, resulting mainly from decreased NADH levels (data not shown). These experiments confirm that over-expression of DNAAM increases the cellular nicotinamidase activity in adult flies and that this expression potentially affects NAD metabolism.

DNAAM Overexpression Extends Drosophila Life Span—To determine the effect of nicotinamidase over-expression on fly life span, low, middle, and high DNAAM-expressing flies using the *tub*-Gal4 or an *elav*-Gal4 driver (allows expression of the transgene exclusively in post-mitotic neurons) were analyzed for life span and compared with genetically matching w^{1118} control flies not expressing DNAAM (Fig. 5, data not shown). Up to 200 flies were used in each group of male and female flies, which were collected as virgins and analyzed separately to avoid

mating effects. Low expressing lines such as DNAAM33 had a small but significant increase in life span compared with their controls (Fig. 5, *A* and *B*). Importantly, the higher expressing DNAAM lines such as DNAAM31 or DNAAM42 displayed a more robust increase in life span as compared with their respective matching controls (Fig. 5, *C*–*F*). The life span extension was observed both in males and females and reached up to a 30% increase in the mean life span and in the maximal life span calculated for the top 20% survivors in each group (Fig. 5. *G*–*J*). Statistical analyses verified that these increases were significant, with most of the experiments reaching *p* values below 0.005. Comparable results were obtained with other DNAAM transformants (data not shown). The fact that multiple UAS-DNAAM insertions exhibit extended life span using three different driver lines strongly argues that the over-expression of DNAAM, and not insertion site effects, is responsible for the observed life span extension. In support, no increase in life span was measured in adult flies carrying only the UAS-DNAAM transgene, arguing against effects of heterosis (data not shown). Importantly, the increased life span observed using *elav*-Gal4 driver lines that over-express DNAAM strictly in neuronal tissues suggests that increased DNAAM activity in neuronal tissues is sufficient to affect the life span of adult flies. This result is in agreement with previous studies showing that Sir2 over-expression in neuronal tissues is sufficient to increase fly life span (Rogina and Helfand 2004) and with studies linking NAD production to neuroprotection. We cannot yet conclude whether this effect is mediated exclusively by affecting the neuronal tissue or involves secondary effects mediated by the neuronal cells, *e.g.* secretion of specific hormones.

DNAAM-induced Longevity Is Reversed in Sir2 Null Mutants— Because *pnc1*-induced longevity in yeast is dependent on Sir2, we sought to determine whether DNAAM induced longevity has a similar requirement in *Drosophila*. Sir2 null flies were generated by breeding flies carrying two independent dSir2 excisions, $Sir2^{4.5}$ and $Sir2^{5.26}$. The transheterozygote adults are viable and reported to exhibit a life span comparable with control animals (Newman et al 2002). DNAAM was over-expressed in this background by generating lines that carried $Sir2^{4.5}$ on the second chromosome and the *elav*-Gal4 driver on the third chromosome and crossing to a second line carrying the $Sir2^{5.26}$ allele on the second chromosome and the high expressing DNAAM42 transgene on the third chromosome. Progeny of this cross over-express DNAAM in neurons while being deficient for Sir2. Parallel control crosses were used to test for potential background effects of *elav*-Gal4 and DNAAM chromosomes or their transgene insertion sites (see Methods). In male flies, over-expression of DNAAM resulted in a significant ($p < 0.001$) increase of 23% (56.9 days *versus* 46.35 days) in mean life span *versus* genetically matched control flies (Fig. 6, *left panel*). This was comparable with the increase in life span observed using the *elav*-Gal4 driver on the second chromosome (Fig. 5*E*). In agreement with the earlier studies, the mean life span of $Sir2^{4.5}$/ $Sir2^{5.26}$ flies did not differ significantly from matched wild type control (46.67 days *versus* 46.35 days). Importantly, DNAAM over-expression in the Sir2 mutant background failed to extend life span, with the mean life span being similar to control animals (46.83 *versus* 46.35 days). Life span of control flies harboring only the UAS-DNAAM transgene or the *elav*-Gal4 driver in a Sir2 mutant background was also similar to controls. Thus, in adult males, the extension of life span mediated by DNAAM over-expression is completely abolished if the Sir2 gene is mutated. Similar results were observed in female flies

(Fig. 6, *right panel*). Over-expression of DNAAM resulted in a significant ($p < 0.002$) increase of 17% (56.83 *versus* 48.48 days) in the mean life span *versus* matched controls. As with male flies, there was no significant change in female mean life span in $Sir2^{4.5}$/ $Sir2^{5.26}$ transheterozygotes *versus* control flies (47.22 *versus* 48.48 days). DNAAM over-expression in Sir2 mutants did not extend life span, and in fact, these flies exhibited a significant ($p < 0.001$) decrease in mean life span when compared with either matched wild type controls or Sir2 mutant flies (40.35 *versus* 48.48 and 47.22 days, respectively). The reduced life span appears to reflect an additive negative effect of the two transgenic third chromosomes on fly life span. Female flies carrying either the UAS-DNAAM insertion or the *elav*-Gal4 driver insertion in a Sir2 mutant background displayed a small reduction in mean life span (43.86 and 44.76 days *versus* 47.22), which when combined appear to have an additive effect on female life span. Why this is observed in females only is unclear, but as virgin females continue to produce eggs, it may reflect a potential trade-off between life span and fecundity, a phenomenon observed with several other gene mutations (Partridge et al 2005). Nevertheless, as in males, pan-neural over-expression of DNAAM increases adult female life span as long as the Sir2 gene is functional.

DNAAM Expression Protects Human Neuronal Cells from Oxidative Stress-induced Cell Death—The ability of neuronal restricted DNAAM over-expression to affect fly life span and the finding that oxidative stress was the main regulator of DNAAM led us to hypothesize that DNAAM might hold a role in oxidative stress resistance in neuronal cells. This hypothesis was also prompted by previous studies showing that activation of the mammalian homolog of Sir2, SIRT1, has a neuronal protective function (Wang et al 2005, Parker et al 2005, Araki et al 2004)

and that SIRT1 expression may be linked to nitric oxide production (Nisoli et al 2005). As demonstrated in Fig. 1*E*, DNAAM expresses well in mammalian COS-7 cells and displays comparable nicotinamidase activity levels as when expressed in *Drosophila* cells, suggesting that DNAAM could be functional also in mammalian cells. Expression of DNAAM in human SH-SY5Y neuroblastoma cells markedly inhibited cell death and apoptosis induced by treating the cells with the reactive oxygen-generating agent NOC-9 (Fig. 7) or a nonrelated nitric oxide donor, sodium nitroprusside (data not shown). The inhibition of cell death and apoptosis were not observed in control cells expressing an empty vector. These experiments indicated that increased cellular nicotinamidase activity, similarly to SIRT1 activation, has a neuronal protective function. To examine whether the protective effects of DNAAM over-expression were related to sirtuin activation, cells were treated with the sirtuin inhibitor sirtinol 1 h prior to exposure to NOC-9 (Fig. 7, *E* and *F*). These experiments showed that sirtinol treatment abolished the protective effects of DNAAM on neuronal cells in a similar manner to its ability to abolish the protective effects of SIRT1 over-expression, suggesting sirtuin involvement in the protective effects of DNAAM. Finally, to test whether DNAAM affected only the transfected cells or also had an effect on adjoining, nontransfected cells, we constructed a GFP-DNAAM expression vector and compared cell death in GFP-positive and GFP-negative cells from the same culture dish (Fig. 7, *G–J*). GFP-negative cells analyzed from samples transfected with GFP-DNAAM had similar cell death rates as cells from samples transfected with a GFP control vector, whereas the GFP-DNAAM and GFP Sirt1-positive cells showed marked cell death resistance. Thus, the results showed that at least *in vitro*, DNAAM over-expression exclusively affects the cells over-expressing the gene but not adjoining cells.

Discussion

The results presented here identify the *Drosophila* nicotinamidase, DNAAM, as an oxidative stress-regulated gene that when over-expressed, significantly extends adult fly life span and confers resistance to oxidative stress in human neuronal cells. Both the extended longevity and neuroprotection were dependent on Sir2/sirtuin function. The conserved family of NAD-dependent protein deacetylases that exhibit homology to the yeast Sir2 have been shown to affect a variety of cellular functions ranging from response to stress and calorie restriction to extending life span in *Drosophila* and nematodes and promoting mammalian neuronal cell survival (Guarente 2007, Anekonda and Reddy 2006, Blander and Guarente 2004). In addition, small molecule activators of sirtuins have been demonstrated recently to affect mammalian aging and health under conditions of a high fat diet (Lagouge et al 2006, Baur et al 2006). The function of Sir2 deacetylases, including vertebrate sirtuins, is negatively regulated by nicotinamide (Anderson et al 2003, Bitterman et al 2002), a by-product feedback inhibitor in deacetylation reactions involving NAD-dependent deacetylases and a metabolite in the nucleotide salvage pathway. In this context, nicotinamidases can be viewed as master regulators of proteins that are regulated by nicotinamide such as sirtuins, and therefore, manipulation of their function could affect a large variety of cellular functions. Our demonstration of a *Drosophila* nicotinamidase and its role in longevity and neuronal protection support this view.

The analysis of DNAAM regulation in *Drosophila* S2 cells shows that both the gene and its function are regulated primarily by oxidative stress, but not by several other stress factors, growth conditions limiting calorie and nutrient availability or by pharmacological agents that block signaling along the insulin/IGF-1 receptor pathway. These findings are significantly different from the observations made in yeast showing PNC1 regulation by calorie restriction and several stress factors (5). In addition, although a recent study showed PNC1 regulation downstream of TOR through the yeast transcription factors MSN2/4 (Medvedik et al 2007), we did not observe changes in DNAAM expression in response to the TOR inhibitor rapamycin. Thus, it is possible that the yeast, as a single cell organism, combines stress and nutrient sensing pathways in same response elements, whereas in higher organisms there is more separation between these pathways. The insulin/IGF-1 pathway, through activation of the PI3K-AKT-FOXO pathway, has emerged as a key regulator of life span in response to calorie availability in various organisms including *Drosophila*, nematodes and to some extent, mammals (Barthel et al 2005). Because this pathway is not conserved in yeast, it is possible that in higher organisms there has been a specialization of the nicotinamidase pathway to respond to oxidative stress, whereas the PI3K-AKT-FOXO pathway became the primary responder to calorie restriction. Interestingly, these two pathways seem to cross-talk in higher organisms, and the FOXO transcription factors are deacetylation targets of sirtuins (Wang and Tissenbaum 2006, Arden 2004).

Over-expression of the nicotinamidase gene extended life span in *Drosophila* similarly to its ability to increase the replicative life span in yeast (Anderson et al 2003). The increased life

span appeared both in females and males and correlated with the level of DNAAM over-expression; it was not observed in matched controls lacking the driver or the transgene. Moreover, the increased life span was dependent on Sir2 levels because life span extension was completely reversed when DNAAM was over-expressed in a Sir2 null mutant. The observed effect of Sir2 mutation on *Drosophila* life span extension by DNAAM and of sirtinol on mammalian cell death protection points to critical role of sirtuins in these functions of DNAAM. However, the question of whether all these effects of DNAAM are mediated by sirtuins or whether they involve other targets needs to be further evaluated.

In a recent study it was demonstrated that knockdown of the *C. elegans* homolog of *pnc1* significantly shortens the life span of adult worms, establishing the role of PNC1 also in worm life span regulation (van der Horst et al 2007). In addition, over-expression of PNC1 conferred resistance to oxidative stress in a Sir2-dependent manner, which is consistent with our observation of neuronal protection in mammalian cells. However, the authors did not observe life span extension in worms over-expressing the PNC1 homolog. The lack of observed effect of PNC1 over-expression on life span extension could be a result of an inherent difference between worms and flies or the result of insufficient over-expression of functional protein. In the transgenic worms, PNC1 mRNA expression levels were increased up to 5-fold, but there was no indication of the protein levels. In our adult flies, the degree of life span extension correlated well with the level of protein over-expression, and maximum extension was observed when protein levels (not just mRNA levels) increased up to 5-fold. Thus, it is

possible that the level of PNC1 expression in worms was not robust enough to observe an effect on life span.

Our and other's bioinformatics approaches did not identify any genes in mammals with significant homology to yeast, bacterial, or lower metazoan nicotinamidases, leading to the view that the NAD salvage pathway in mammals is different from the one in yeast, worms, and flies (Fig. 1*A*). Nampt, the enzyme that converts nicotinamide into nicotinamide mononucleotide is presumed to be the functional counterpart of yeast PNC1. However, we would like to note that because of the low conservation of nicotinamidases from yeast to fly, our inability to identify an apparent nicotinamidase homolog in mammals does not explicitly exclude its existence. Regardless of the presence of a *de facto* nicotinamidase in mammals, our data provide strong evidence that increased nicotinamide clearance in cells provides positive effects on organism life span and cellular response to oxidative stress. This suggests that manipulation of nicotinamide metabolism through genetic approaches or pharmacological agents in vertebrates could yield similar beneficial results.

Finally, although our results clearly implicate Sir2 activity in life span extension and neuronal protection when DNAAM is over-expressed, it remains to be determined whether nicotinamidase activation or activation of other nicotinamide modulating enzymes, such as Nampt, work exclusively through sirtuins. In this regard, a recent study suggested that the effects of nicotinamide on yeast life span are not exclusively mediated by Sir2 or other yeast sirtuins (Tsuchiya et al 2006). This question also relates to the overall beneficial properties of direct sirtuin activators such as resveratrol in comparison with nicotinamide modulators, which

may provide their benefits both through activation of sirtuins and modulation of other as of yet unidentified targets.

Chapter 3

Oxidative Stress Response in *Drosophila* Expressing the Longevity Genes Sir2 and the nicotinamidase DNAAM.

Abstract

The oxidative stress theory of aging predicts that organisms with an increased tolerance to oxidative damage will have an increased longevity, while organisms with decreased tolerance will exhibit early mortality. Oxidative damage that accumulates in cells during normal aging contributes to a variety of diseases and pathologies, and can result in an increase in age specific mortality. Evidence is mounting, however, that many genetic manipulations that promote resistance to oxidative stress do not correlate with an increase in lifespan. Furthermore, many biochemical and genetic pathways involved in lifespan regulation do not have any known role in oxidative stress response. Here we report the oxidative stress responses of adult *Drosophila* that are engineered to over-express *Sir2* or the nicotinamidase *DNAAM* gene. Both of these gene manipulations are known to increase adult lifespan; however only *Sir2* endows strong, reproducible resistance to two different forms of acute oxidative stress. *Sir2* is required for normal oxidative stress resistance, as mutant animals have virtually no tolerance to dietary paraquat and a reduced tolerance to hydrogen peroxide. In order to determine the efficacy of our experimental system, we tested two inert genes, *GFP* and *LacZ*, for the effects of over-

expression on oxidative stress response. Interestingly, in male flies, small but significant increases in paraquat resistance could be observed when supposedly inert GFP constructs were expressed. This data highlights the important contribution of background genetics and/or expression system artifacts in eliciting stress response phenotypes. Thus, more comprehensive methodologies for controlling gene expression experiments should be required to confirm small effects on stress response and lifespan. Taken together, our data suggests that a correlation between oxidative stress and increased longevity is gene or gene-pathway dependent, and pathways independent of oxidative stress response can contribute to increased lifespan in a manner that may be dependent on the pre-existing genetic background. These findings have implications for our theoretical understanding of aging.

Introduction

The inexorable decline in function of cells and tissues that occurs during normal aging is one of the most vexing and important problems in molecular medicine. Despite efforts among researchers in diverse areas of cell and organism biology, there are few theories that formulate a consensus etiology for age-associated mortality. The original oxidative stress theory of aging was put forth in 1956 by D. Harman as a first approach to an integrative theory of aging, and described the aging phenotype as being a result of the accumulation of reactive oxygen species (ROS). The following decades of research made clear that the mitochondria played a major role

in metabolism and the generation of oxidative stress, and this insight resulted in the mitochondrial theory of aging being introduced as a logical successor to the original theory, (Harman 1972). Even while undergoing further progressive modifications, the oxidative stress theory has for over 50 years been one of the most referenced and tested theories to explain how aging contributes to increased mortality. The theory postulates that over time, cellular metabolism results in a build-up of potentially toxic reactive oxygen species (ROS). This results in an increase in oxidative damage to cells and tissues, thereby compromising their function, and leading to the aging phenotype (Sohol and Weindrich 1996). Two major hypotheses of the oxidative stress theory are 1) that organisms with increased ROS production will exhibit increased mortality and age related disease; and 2) that organisms with superior protection from ROS and/or a decrease in ROS production will exhibit a reduction in age associated mortality and disease. Ample evidence supports a role for ROS production in several age-related diseases; including neurodegenerative disease, cardiovascular disease, diabetes, and cancer (Abdulwahid et al 2010, Kaneto et al 2010, Halliwell et al 2010). Exposure of model organisms to acute and/or chronic oxidative stress inducing compounds can drastically decrease lifespan, as can mutations in genes that manage and detoxify ROS (Kirby et al 2002, Shigenaga et al 1994, Bonilla et al 2006). Furthermore, oxidative damage and mitochondrial dysfunction increases with advanced age in a wide variety of cell types and tissues across several model organisms (reviewed in Bokov 2004), providing correlative evidence for the hypothesis that increased oxidative damage has a causal role in the onset and progression of cellular and tissue loss of function during senescence.

There is also a wealth of evidence supporting the oxidative stress theory prediction that reduced ROS production and/or increased ROS tolerance correlates with increased longevity. The most convincing of this data involves model organisms that are long-lived (due to either genetic or environmental manipulations) being concomitantly protected from oxidative stress when compared with shorter-lived controls. The most reliable and replicable environmental manipulation to extend lifespan is the implementation of calorie restriction (CR) (McCay et al 1935), or as it is now called, dietary restriction (DR). This measured reduction in calorie or nutrient intake can increase longevity in a multitude of organisms and genetic backgrounds and has been extensively reviewed over the last decade (Sinclair 2005, Bishop and Guarente 2007, Fontana 2009). Dietary restricted mice display a reduction in molecular oxidative damage and are more tolerant to oxidative damage, perhaps owing to a decrease in mitochondrial ROS production (Sohol and Weindrich 1996, Barja et al 2002, Sanz et al 2006, Gredilla and Barja 2005). Dietary restricted rodents also exhibit changes in gene expression consistent with an increase in oxidative stress response pathways (Lee et al 1999, Lee et al 2002). Genetic mutation or expression alterations in pathways that are involved in the response to dietary restriction are also capable of eliciting both increased lifespan and increased resistance to oxidative stress. Mutations in insulin signaling, for example, which have been shown to be down-regulated under conditions of DR, affect changes in oxidative stress response in yeast, *C. elegans*, *Drosophila*, and mice (Honda and Honda 1999 , Holzenberger et al 2003, Clancey et al 2002). Similarly, increased expression or activation of the protein deacetylase *Sir2* has been suggested to act as a mimetic of DR, and has been shown to increase oxidative stress resistance and lifespan in both *C. elegans* and *Drosophila* (see results) (tissenbaum and guarente 2001, van

der horst et al 2007, Rogina and Helfand 2004). Other gene mutations correlating longevity to oxidative stress resistance that may activate pathways independent of the DR response include the G-protein coupled receptor *Methuselah*, the proto-oncogene $p66^{shc}$, the ROS detoxification enzyme *SOD1*, and the stress response gene *HSP22* (Migliaccio et al 1999, Morrow et al 2004). Newly characterized genes and the re-examination of canonical pathways for effects on lifespan and oxidative stress response will continue to add to the growing list of proposed stress resistance and longevity determining factors.

Despite strong evidence supporting the oxidative stress theory, recent studies review and highlight the numerous exceptions in which ROS correlation with lifespan is either not observed, or is in direct opposition to the predicted theory. (Perez et al 2009, Sanz et al 2010). Recent work has examined the effects of both mutation and over-expression of an impressive array of antioxidant enzymes on lifespan in mice (Perez et al 2009). The genes included in their analysis include *Sod1, Sod2, Gpx1*, and *Gpx4*; which each code for enzymes critically important for the detoxification of superoxide and hydrogen peroxide. Interestingly, only *Sod1-/-* mice display a decrease in lifespan, despite increased oxidative damage and/or reduced oxidative stress tolerance in each mutant genotype. Importantly, over-expression of these individual antioxidant enzymes did not increase lifespan even though data indicated either an overall increase in oxidative stress tolerance or reduction in ROS production. A similar report in *Drosophila* examined the stress response and longevity of flies expressing the plant and fungal antioxidant enzyme *alternative oxidiase* (AOX). AOX flies showed a strong age-related decrease in mitochondrial ROS (mtROS) production without any effect on lifespan. Furthermore, they

show that flies mutant for a second gene, *dj-1β* , display both an increased production of mtROS, and a significant *increase* in lifespan in both sexes. A direct contradiction of the oxidative stress theory has also been observed in flies over-expressing *MnSOD* and *catalase* in the mitochondria, which exhibit an increase in oxidative stress resistance, but a robust decrease in lifespan (Bayne et al 2005).

A very informative illustration of the contrasting data regarding the relationship of oxidative stress and longevity lies in two studies of our own laboratory using flies bred either for longevity or for oxidative stress resistance. When normal-lived Ra strain flies are artificially selected for extended longevity, they display a reduction in ROS production and oxidative damage, as well as an increase in antioxidant gene expression (Arking et al 1996, Dudas and Arking 1995, Vettriano et al 2001). However, when the same normal-lived Ra strain flies are artificially selected for resistance to the oxidative stress-inducing chemical paraquat, they exhibit a four-fold increase in paraquat resistance, but no significant increase in median or maximum longevity (Vetraino et al 2001). Other data presented therein led to the observation that selection for extended longevity led to a 75% increase in CuZnSOD gene and enzyme activity and a 7% decrease in P450 enzyme activities, while selection for paraquat resistance led to a 105% increase in P450 enzyme activities and a 15% decrease in CuZnSOD enzyme protein and activity levels. This data suggests that components of the oxidative stress response are regulated differentially under disparate selection conditions, and, most importantly, longevity is not always dependant on oxidative stress resistance. As such, this interpretation

casts doubt on the tight association between longevity and oxidative stress resistance intrinsic to the oxidative stress theory of aging.

These and other similar studies have generated serious contention over the validity of the oxidative stress theory of aging. A possible explanation for the discrepancies in data that support or contradict the ROS theory is that any correlation between oxidative stress resistance and lifespan is dependent on the genetic background of the organism and strain tested. Thus, some genetic manipulations may increase oxidative stress resistance concomitant with longevity, while others may increase lifespan through mechanisms independent of oxidative stress tolerance, depending on their interaction with strain specific genetic predispositions. In particular, strain dependant differences in the ability to tolerate ROS damage may dictate whether or not specific genes and pathways can engender longevity through oxidative stress resistance. In any event, such putative explanations of apparently contradictory data imply that the ROS hypothesis as stated does not constitute a full explanation of the aging phenotype, as other variables must be involved. We sought to test this hypothesis by performing different types of oxidative stress resistance experiments on alleles of two genes known to regulate longevity in *Drosophila*, and closely examined the contribution of our expression system and genetic background by utilizing the "inert" constructs GFP and LacZ as models of presumably inactive background genes. A disproof of our hypothesis, and thus support for the oxidative stress theory of aging, would involve the demonstration that both longevity genes correlate robustly with longevity on different types of oxidative stressors. The contrary result would

indicate that such a single stressor theory of aging was not sufficient to explain the observed data.

Short generation time, amicable genetics, and well-defined paradigms for analysis make *Drosophila* an ideal model to test the correlation between longevity and oxidative stress resistance. For this experiment we have chosen two genes known to increase fly longevity; the nicotinamidase *DNAAM,* and the protein deacetylase *Sir2*. *DNAAM* is the *Drosophila* ortholog of yeast and *C. elegans Pnc1*, which codes for an enzyme that converts nicotinamide to nicotinic acid, a precursor for NAD synthesis (Anderson et al 2003). In *S. cerivisiea* and *Drosophila*, over-expression of DNAAM results in an increase in lifespan, while in *C. elegans* increased PNC-1 expression increases resistance to oxidative stress, but does not increase lifespan (Anderson et al 2003, Balan and Miller et al 2008, van der horst et al 2007). The increased lifespan endowed by DNAAM requires the NAD-dependant protein deacetlyase Sir2, a potent regulator of longevity and stress response in an impressive variety of organisms and cell types. Sir2 expression increases longevity in *S. cerevisiae, C. elegans*, and *Drosophila,* and as discussed, plays a prominent role in mediating cellular stress response (Lin et al 2000, Guarente and Tissenbaum 2001, Rogina and Helfand 2004). Despite a requirement of Sir2 for DNAAM dependant lifespan extension, our data shows that artificial expression of Sir2, but not DNAAM, can increase tolerance to multiple forms of oxidative stress in *Drosophila*. By carefully monitoring the genetic background, we also show that significant changes in stress response can be elicited by generating different chromosomal combinations in otherwise isogenized animals. Taken together, our data shows that not all longevity genes also increase oxidative

stress resistance. It is therefore likely that longevity genes correlate with distinct and divergent stress response pathways, each of which can contribute to organism longevity. Furthermore, the contribution of oxidative stress resistance to longevity is likely mediated in large part by strain specific genetic background and the intrinsic differences in stress tolerance between diverse organisms.

Materials and Methods

Stocks and isogenization:

The UAS-Sir2^{DsRed} stock was kindly provided by Dr. Allan Spradling. The UAS-DNAAM^{31} and UAS-DNAAM^{42} stocks were generated as previously described (Balan and Miller et al 2008). The La stock was generated as previously described (Arking, 1986). All other stocks were obtained from the Bloomington Stock center (Indiana). All UAS transgenic stocks and Gal4 drivers were out-crossed to an isogenic w^{1118} stock (Indiana) for a minimum of 5 generations to "isogenize" them to our in house w^{1118} genetic background. The Sir2$^{2A7\text{-}11}$ stock was not out-crossed; however this *Sir2* precise excision stock was originally generated in the w^{1118} background. Out-crossed stocks were balanced to CyO-marked balancer chromosomes for insertions on the second chromosome and to TM3 marked balancer chromosomes for insertions on the third chromosomes. Both these balancer stocks were similarly isogenized to our w^{1118} background. The resulting balanced and isogenized stocks were confirmed via PCR

analysis (data not shown). Stocks are routinely maintained as vial populations and amplified in bottles in preparation for experimental crosses.

Generation of test and control flies:

For over-expression experiments, UAS transgenic males were crossed to Gal4 virgin females. Two alleles were tested for *DNAAM* overexpression, and one for *Sir2* over-expression. All three were also tested for background effects and for over-expression effects. UAS-GFP and UAS-LacZ were crossed to the Gal4 drivers. Ubiquitous over-expression was elicited using the Tubulin-Gal4 driver and pan-neuronal expression was elicited using the Elav-Gal4 driver. Controls for UAS transgenes were generated by out-crossing UAS male flies to w^{1118} virgin female flies, while controls for Gal4 drivers were generated by out-crossing Gal4 virgin females to w^{1118} males. In this way the sex directionality of the over-expression test crosses was recapitulated by the control flies. For analysis of *Sir2* mutation effects, the $Sir2^{2A7\text{-}11}$ stock was tested as both a homozygous null and a heterozygous null (generated by out-crossing males to w1118 females), and compared to w^{1118} flies. La flies exhibit a paraquat resistant phenotype (Arking et al 1991) while flies deficient in bsk2 flies are paraquat sensitive (Karpac et al 2009), and these two strains were used in each assay as a positive and negative control respectively. All flies were collected as virgins and separated by sex to avoid the effects of mating. Male and female flies were separated into aliquots of 25 and reared in vials of standard cornmeal molasses media for 10 days, being placed on fresh vials every two days. Deaths during rearing recorded and the total number of flies entering the oxidative stress assays was recorded after the transfer to drug containing vials on day 10.

Paraquat assays:

Paraquat assays were performed in two replicates. In the first set of experiments, alleles of various genes were tested independently for the effects of over-expression on paraquat resistance. In the second experiment, alleles of each individual test with significant and/or potent effects were combined into a single assay. For each test allele and control lines, an average of over 200 flies was analyzed per sex in each assay. Flies at 10 days age were transferred to vials containing 5 Whatman 2.3cm grade 3 filter discs saturated with 15mM paraquat in a 5% sucrose solution. Flies were monitored every 24 hours and deaths recorded. Mortality data was plotted and analyzed for significance using Graphpad Prizm. Results of the first set of independent experiments were compared with the combined assay to determine gene-specific effects versus background genetic effects. Sexes were analyzed separately.

Hydrogen Peroxide:

Flies were reared as described above, and placed on a solution of 2% hydrogen peroxide in 5% sucrose following the same protocol as described for the paraquat assays. Only the significant alleles (those tested in the second paraquat assay) were subjected to hydrogen peroxide treatment. Flies were monitored every 24 hours after exposure, deaths recorded, and mortality differences measured for statistical differences.

RESULTS

Paraquat dosage, experimental controls, and criteria for validating effects:

In order to find a working concentration of paraquat that could resolve changes in oxidative stress response, w^{1118} flies were collected as described in the methods and exposed to concentrations ranging from 0-40mM in 5% sucrose. Male and female flies exposed to 15mM paraquat exhibited a standard S-shaped dose response curve with a median lifespan of 2-3 days (data not shown). Concentrations either above or below 15mM were either too dilute to eliminate the possibility of desiccation effects, measured to occur after 5-6 days, or killed the animals within 24 hours (data not shown). In order to introduce positive and negative controls for the paraquat experiments we sought genotypes that had been reported as paraquat resistant or paraquat sensitive. We obtained two reported paraquat resistant strains; La and *mth*, and two paraquat sensitive strains; *bsk2* and *Dj-1β*. La is a strain that was selected for longevity and measured to have enhanced paraquat resistance (Arking et al 1986, Dudas and Arking 1995); *mth* is a mutation in a G-protein coupled receptor associated with enhanced longevity and stress resistance; bsk^2 is a mutation in the JNK signaling pathway; and *Dj-1β* is a mutation associated with early onset Parkinson's disease (Lavara-Culebras and Paricio 2007). When compared with our w^{1118} background stock, La males and females exhibited a strong increase in paraquat resistance, whereas only mth females had a small increase in resistance. Both bsk^2 and *Dj-1β* male and female flies were more susceptible to paraquat than w^{1118}

controls (Supplemental Fig 1). Due to the increased fecundity of *bsk*^{2} flies compared to *Dj-1β* flies, we chose *bsk*^{2} as our negative control (paraquat sensitive) and the La flies for our positive control (paraquat resistant); and these genotypes were run in each experiment. In each experiment we also ran internal controls which are separate heterozygotes of each transgene and driver, which are used to test for gene dependant changes in paraquat resistance and monitor the effect of different background chromosome combinations on oxidative stress response.

We used very large population sizes in our assays, and so we were able to observe the usual standard for statistical significance ($p < .05$) with very small changes in mortality between different survival curves. We therefore decided that our level of acceptable significance should be more stringent, and decided that we would require a significance of $p < .01$ before we would consider any effect as valid. We also stipulated that any significant increase in paraquat resistance would have to be replicated in both sets of experiments. Furthermore, for each experiment, we decided that any measured gene-dependant increases in paraquat resistance would have to be greater than the longest lived internal control from *both* experiments (with the opposite criteria for paraquat sensitivity) for the characterization of that gene as a stress resistance element. In this way we sought to control for any variation that may be a result of experimental variance and not gene expression. Finally, we added a positive (paraquat resistant, La) and negative (paraquat sensitive, *bsk2*) control to each experiment, with the rationale that gene-dependant effects on paraquat resistance can be further validated if they

are compared with previously verified control strains known to affect oxidative stress response.

DNAAM does not increase paraquat resistance

In the first round of DNAAM experiments, we selected two different transgenic insertions for analysis, *UAS-DNAAM*^{31} and *UAS-DNAAM*^{42}, both of which are associated with extended longevity (Balan and Miller et al 2008). The *DNAAM*^{31} allele was shown to increase lifespan in both sexes under conditions of neuronal expression using the *elav-Gal4* driver, and ubiquitous expression using the *tub-Gal4* driver (Balan and Miller et al.). In our first experiment we found that only in male flies did *DNAAM*^{31} expression elicit paraquat resistance, and did so in both patterns of expression (Figures 1, 2). In female flies there was no increase in paraquat resistance measured in either tissue pattern when *DNAAM*^{31} was expressed (Figures 1-2). It is plausible that there could be sex specific DNAAM effects that result in male, but not female, flies exhibiting paraquat resistance; as sex specific effects on longevity and oxidative stress response have been reported for a variety of genes (Nuzdhin et al 1997, Tower 2006). However, when the *DNAAM*^{42} allele was expressed using either neuronal or ubiquitous drivers; we did not measure any significant changes in survival on paraquat versus controls in either males or females (data not shown). We have previously shown that when *DNAAM*^{42} is expressed in the nervous system using the *elav* driver, male and female flies exhibit a robust

increase in both mean and maximum lifespan; thus this observation highlights a disconnect between paraquat resistance and increased longevity.

We reasoned that it was possible that the *DNAAM*^{42} insertion may have a transgene insertion site that reduces paraquat resistance, and that the effect of the *DNAAM*^{31} insertion required further analysis. When the analysis of the *DNAAM*^{31} allele was repeated in the second assay, male paraquat resistance appeared to be replicated (Fig 1,2). However, when we superimposed the results of both experiments together to reveal experimental variance, there was vanishing significance under conditions of neuronal expression and overlap in the longevity curves of control and experimental flies under conditions of ubiquitous expression (Fig1C, 2C). Because of this measured variance between experimental replicates, and because the *DNAAM*^{42} insertion showed no effect on paraquat resistance when expressed under control of either driver, we determined that the effect observed in *DNAAM*^{31} males is *not* due to expression of the DNAAM construct. This data also detailed that it was necessary to further validate our experimental system by investigating the effects of our over-expression system and variation in chromosomal background before the analysis of our second gene, *Sir2*. Importantly, our results show clearly that DNAAM-associated lifespan extension does not correlate with an increase in paraquat resistance. This provides evidence that DNAAM induced lifespan extension in *Drosophila* is likely not mediated by a congenital increase in oxidative stress resistance and therefore this result does not validate the ROS hypothesis. We explored this possibility further by challenging DNAAM flies with a second form of oxidative stress,

hydrogen peroxide, which corroborates our hypothesis of ROS independent mechanism of lifespan extension in these animals (see below).

GFP/LACZ expression reveals gene independent effects on paraquat resistance

In order to investigate the role of background genetic effects and UAS driven over-expression on paraquat resistance, we analyzed the effect of two constructs that are hypothetically inert toward oxidative stress response. For this experiment we chose *UAS-GFP* and *UAS-LacZ*, that are both non-endogenous constructs, and are homozygous viable and therefore do not have lethal insertions. The *UAS-GFP* insertion is on the second chromosome and the *UAS-LacZ* insertion is on the third chromosomes, allowing us to track effects that are chromosome dependant. To the best of our knowledge, expression of non-endogenous constructs has never been examined for effects on stress response or longevity, and may have the potential to uncover changes in lifespan that are gene independent. Because these constructs have no relevant biological function in *Drosophila*, changes in stress response upon induction of these genes would reveal the effect of our *UAS-Gal4* over-expression system itself, or possibly the effect of various chromosomal combinations on stress response. In the initial round of experiments, neuronal and ubiquitous *LacZ*-expression had no effect on paraquat resistance in either sex; a result that was confirmed by the second assay (data not shown). This result was similar to the data obtained with *UAS-DNAAM*[42].Considering that both constructs

have insertions on the third chromosome further validates our conclusion that DNAAM does not increase paraquat resistance in male flies.

Interestingly, our analysis of the *UAS-GFP* construct, shed light on the effects we measured for our second chromosomal insertion construct *DNAAM*^{31}. Both of these insertions are on the second chromosome, and both are capable of eliciting small effects on male, but not female paraquat resistance. In male flies, neuronal expression of *GFP* resulted in a significant increase in paraquat resistance in only one of our two replicates, and had no effect on female survival (Supplemental figure 3). However, ubiquitous over-expression of the *GFP* construct resulted in a similar increase in paraquat resistance as was measured by *DNAAM*^{31} in both replicates (Fig 2). As with the *DNAAM* experiment, this increase could be attributed to experimental variance, as overlap of the two experiments showed that for both sexes and both expression patterns, control replicates are not always significantly different from over-expression for paraquat resistance (Fig 3). Importantly, this result indicated that the effect we measured in *DNAAM*^{31}*-elav* and *DNAAM*^{31}*-tub* male flies was the result of background genetics and not a result of *DNAAM* expression. We rationalize that our heterozygous controls may have genetic elements introduced during out-crossing to w^{1118} that are detrimental to paraquat resistance. Trans-heterozygous *UAS-Gal4* flies that do not possess a w^{1118} second chromosome would ameliorate these effects, or this effect is reduced via unknown metabolic effects possibly arising from the over-expression system itself. This set of experiments was critically important for developing our criteria for gene-dependant effects versus effects that are due to background genetics or over-expression. Because our *UAS-Sir2* construct is also on the second

chromosome, this analysis allowed us to better understand how to interpret the results of our next experiment.

Sir2 expression increased resistance to dietary paraquat

As indicated by our GFP experiment, insertions on the second chromosome need to meet certain criteria before they can be considered to have real gene-dependant effects on paraquat resistance. Our *Sir2* over-expression stock, *UAS-Sir2*^{DsRed} is a transgenic construct introduced into the second chromosome via p element transposition, as with *UAS-DNAAM*[31] and *UAS-GFP*. As previously mentioned, *Sir2* over-expression increases the lifespan of a variety of organisms, including *Drosophila* (Lin et al 2000, Rogina and Helfand 2004, Guarente and Tissenbaum 2001), however its effects on fly lifespan under conditions of oxidative stress response is unknown. Cell culture data suggests that mammalian *Sir2* (*Sirt1*) is involved in the cellular response to oxidative stress, and promotes cell survival by deacetylating the tumor suppressor p53, and thus inhibiting *p53* mediated apoptosis (Luo et al 2001, Vaziri et al 2001), Our own analysis has shown that *Sirt1* activity is required for normal resistance to oxidative stress in human neuroblastoma cells, and that over-expression of *Sirt1* can protect these neurons from oxidative stress induced apoptosis (Balan and Miller et al 2008). Taken together, this and other data suggests that *Drosophila Sir2* is a good candidate gene to regulate lifespan under conditions of oxidative stress.

When *Sir2* is expressed in neurons, female flies exhibit a strong increase in resistance to paraquat versus their transgene and driver controls (Fig. 4, Supplemental Fig. 3). The *Sir2* effect is reproducible, highly significant, and considerably potent; as these females consistently outlive even the paraquat resistant La female controls (Supplemental Fig. 3.) Because we never observed any consistent and significant increases in female lifespan on paraquat in any of our previous experiments, and considering the magnitude of the *Sir2* effect, we conclude that *Sir2* neuronal expression does indeed protect female flies from oxidative stress. The effect of neuronal expression in *Sir2* in male flies is more difficult to interpret. In both replicates small and significant increases in survival are observed, and unlike our *DNAAM* and *GFP* expressing males, these increases are significantly greater than controls from each replicate (Fig. 4). However, because the magnitude of increase is similar to that observed in other experimental lines with insertions on the second chromosome, the effect may be the result of genetic background. Thus the effect of neuronal *Sir2* expression in males remains elusive. When *Sir2* is expressed ubiquitously, there is a profound increase in paraquat resistance in *both* sexes (Fig 4, Supplemental Fig 3). This *Sir2* dependent increase is especially profound in male flies, and in both sexes results in a lifespan increase that supersedes the La positive control (Supplemental Fig.3). Taken together with our results from nervous system expression, we conclude that *Sir2* is protective against paraquat induced acute oxidative stress in adult *Drosophila*.

Sir2 null mutation increases paraquat sensitivity

Our observation that *Sir2* expression results in paraquat resistance raised the question of what role endogenous *Sir2* plays in normal oxidative stress resistance. In *C.elegans*, reduction of *Sir2* using RNAi results in a decrease in paraquat resistance, and addition of the sirtuin inhibitor NAM also decreases resistance (van der horst et al 2007). Mutation of *Sir2* causes a decrease in the replicative lifespan of yeast, however it has been shown have no effect on the lifespan of flies (Guarente and Tissenbaum 2001, Newman et al 2002). In order to address this question in *Drosophila*, we took advantage of the $Sir2^{2A711}$ strain, a precise excision of the *Sir2* gene locus in the w^{1118} background that results in a homozygous viable *Sir2* null mutant (Furuyama et al 2004). In both experimental replicates, male *Sir2* null flies were extremely susceptible to paraquat when compared to heterozygous *Sir2-/+* animals, with over 90% of individuals perishing before 24 hours (Fig. 5). A similar result was observed in female flies, with the majority of *Sir2* null animals perishing before 48 hours (Fig. 5). In male flies, *Sir2* null animals were significantly more sensitive to paraquat than bsk^2 negative control animals while male *Sir2* null flies were either more sensitive or not significantly different from bsk^2 animals depending on the replicate (Supplemental Fig 4). Thus, endogenous *Sir2* expression is absolutely required for normal oxidative stress resistance. Taken together with our over-expression data, this highlights the importance of *Sir2* as a regulator of oxidative stress response in *Drosophila*, which may contribute to the observed effects of over-expression on lifespan (see discussion).

Sir2 helps regulate the response to hydrogen peroxide

In addition to dietary paraquat, we measured the lifespan of flies of the above genotypes on a 2% solution of hydrogen peroxide. This experiment represents a secondary measure of oxidative stress response, as hydrogen peroxide is metabolized by the peroxidases and catalases (reviewed in Zamocky et al 2008) while paraquat is throught to be metabolized primarily by the enzymes by NADPH-cytochrome c reductase and NADH quinone oxidoreductase in the mitochondria (Shimada et al 1998). Exposure to hydrogen peroxide accelerates cellular senescence (Furukawa et al 2007) and in adult Drosophila is associated with an increase in mortality as well as changes in locomotor activity (Bayne et al 2005, Grover et al 2009). We observed flies of all genotypes exhibiting more erratic flight behavior following exposure to H_2O_2, and after 48 hours flies of both sexes exhibited lethargic behavior and reduced negative geotactic climbing. Consistent with differing mechanisms of toxicity from paraquat, La and *bsk* flies exhibited a markedly different pattern of survival on H_2O_2, with *bsk* flies actually out-living La flies (data not shown).

After analyzing all genes for survival, only nervous system expression of *Sir2* protects against H_2O_2 mortality (Fig 5, data not shown). Small effects were measured with ubiquitous *Sir2* expression; however due to the background genetic effects observed in our paraquat experiments; it is possible these effects are not Sir2 dependant. The fact that neuronal, but not ubiquitous expression of *Sir2* is protective against hydrogen peroxide provides evidence for an acute toxicity of this chemical to neurons, which may explain the observed changes in locomotor activity. Similar to our observation with paraquat, mutation of *Sir2* also resulted in a

reduced tolerance to hydrogen peroxide; however heterozygous *Sir2 +/-* flies exhibited a slightly higher resistance than other control strains (Figure 5). Because this observation was not consistent with our paraquat experiments, it is possible that *Sir2* may have both positive and negative effects on toxicity depending on the acute cellular concentration. Additionally, because none of the other transgenes tested, including DNAAM, GFP, and LacZ exhibited any effect on hydrogen peroxide, we believe that the background effects that result in paraquat resistance in male flies belong to a different pathway of stress resistance and highlight that diverse pathways of ROS management occur in *Drosophila*.

DISCUSSION

Denham Harmon proposed the first incarnation of the oxidative stress theory of aging 20 years before the first gene sequences were deduced, and almost 50 years before the discovery that *Sir2* over-expression can increase yeast lifespan (Harmon 1956, Kaeberlein et al 1999). It is a testimony to Dr. Harmon's remarkable insight that despite vast advances in molecular biology, his theory remains still a fertile source of ideas and counter-arguments in the modern field of biological gerontology. The advent of molecular genetics has provided a significant amount of support for Dr. Harmon's theory; however, as our data clearly shows, it is the exceptions that disprove the rule. That the longevity-promoting gene *Sir2*, but not *DNAAM*, promotes oxidative stress resistance adds to a growing array of data that suggests the validity

of the ROS hypothesis depends on which genes or pathways are involved. Thus some genes may increase both longevity and oxidative stress resistance, while others increase one independently of the other; indicating that there are pathways outside of oxidative stress that can determine longevity. This raises the question as to what contribution to longevity does oxidative stress have? The answer may depend on the genetic background of the organism, a hypothesis that is corroborated by our experiment. Certain strains may have inherent sensitivity to ROS production, such as our w^{1118} flies, and amelioration of this sensitivity may contribute to the observed Sir2 dependent longevity. Other strains, however, may engender extended longevity through genetic and epigenetic modifier effects throughout the genome that are not present in our w^{1118} stock, and will not be affected by increased Sir2 expression. This follows from the fact that different strains of the same species that are reproductively isolated will accumulate various genetic and epigenetic modifications over time that will result in different lifespan and stress responses, and effect the correlation between these two traits.

The ability of epigenetic effects and trans-acting elements, such as those introduced during transgenic modification, to effect the expression of genes and affect phenotypes including lifespan is well documented (Simonsen et al 2007; Bhadra et al 1997). Thus in any analysis of lifespan and stress response it is essential to monitor the range of these effects as closely as possible. We have done so by adding flies with different genetic backgrounds (La and bsk^2) as positive and negative controls, and closely monitored the effect of all transgenic elements with trans-heterozygous; controls. Furthermore we have monitored the effect of our expression system in our GFP and LacZ expression experiments. Without these controls, we

believe any assessment of gene dependant effects must be considered with caution; and even when these controls are instituted, it is very likely that any significant effects may be strain specific. With all of this in mind, it is perhaps easier to imagine how a gene such as *Sir2*, which can regulate both transcription factors and chromatin architecture, could have a profound enough effect to modify longevity and stress response in certain strains of *Drosophila*.

From a survey of the literature, it is apparent that the contribution of our chosen longevity genes to lifespan and oxidative stress management in other experimental models is a complex and occasionally contradictory story. To begin with the nicotinamidase gene *DNAAM*, the biochemical activity of the gene is simple; to convert nicotinamide (NAM) into nicotinic acid. The result of this activity however, is complex; as this conversion is proposed to increase NAD synthesis and affect the activity of a variety of enzymes that are inhibited by NAM. Enzymes affected by NAM levels belong to a diverse class of proteins known as NAD consumers, and include ADP-ribosyl transferases (ARTs), cADP-ribose sythetases, poly-ADP-robosyl polymerases (PARPs), and sirtuin deacetylases. In each case these enzymes catabolize NAD and release NAM, which acts as a potent inhibitor of their activity (Brenner 2006). NAM salvage by enzymes such as DNAAM appears to be of paramount importance for maintaining intracellular NAD and in regulating the activity of NAD consuming enzymes (Bogan et al 2008, Berger et al, Ziegler et al 2004). Interestingly, NAM has both positive and negative effects on lifespan and stress response, depending on the experimental model. NAM has a negative effect on survival of both yeast and *C. elegans*, and interferes with DR-mediated yeast lifespan extension (van der Horst et al 2007, Tsuchiya et al 2006). Over-expression of the *DNAAM* ortholog *PNC1* in yeast

increases lifespan (Anderson et al 2003) and increases tolerance to dietary paraquat in *C. elegans* (van der horst), both of which requires a working copy of *Sir2* to facilitate these effects. However there is a partial rescue of oxidative stress induced mortality in PNC-1 expressing worms in the absence of Sir2, and the effect of exogenous NAM on yeast lifespan is independent of sirtuins (van der Horst et al 2007, Tsuchiya et al 2006)

Our previous data shows that *Sir2* is also required in *Drosophila* to facilitate the longevity associated with nicotinamidase expression (Balan and Miller et al 2008). There is however, no conclusive proof that DNAAM is working through Sir2 to extend fly lifespan, and it may be that Sir2 deficient animals are simply incapable of lifespan extension from any mechanisms that require Sir2 activity for their regulation and/or effect. A potential second mediator of oxidative stress response and longevity that could be affected by NAM levels is the enzyme poly-(ADP-ribose) polymerase-1. PARP-1 is involved in DNA damage repair and important for maintenance of DNA integrity (Burkle et al 2004, Tong et al 2001). PARP-1 has been shown to be protective against physiologically relevant levels of oxidative stress in cortical neurons (Diaz-Hernandez et al 2007). Despite the observed negative effects of NAM on yeast and worm survival, in many cell types of higher eukaryotes NAM has a positive effect on cell survival and stress resistance. In human fibroblasts, application of NAM extends replicative lifespan, reduces mitochondrial activity, and decreases the production of ROS (Kang et al 2006). NAM has been shown to protect neuronal cells from anoxia and nitric oxide exposure, pancreatic cells from free radical exposure, and human β-cells from hydrogen peroxide induced necrosis (Kallmann et al 1992, Chong et al 2002, Lin et al 2001). This apparent contradiction

could arise from both the different pathway of NAM management observed in mammals and the observed difference in normal concentrations of NAM present in mammalian systems versus lower eukaryotes (Revollo et al 2004).

Regardless of the data from other organisms and cell models, our work in *Drosophila* clearly shows that there is a decoupling between the longevity associated with DNAAM activity and a congenital resistance to oxidative stress. Thus, the ROS theory does not appear to account for DNAAM associated longevity. Of critical importance to this conclusion is the ROS theory hypothesis that the effect of acute oxidative stress and the chronic accumulation of age associated oxidative stress on mortality will *both* be reduced in long lived animals. It is possible that normal ROS management in *Drosophila* declines with age, contributes to mortality, and may be ameliorated by *DNAAM* induction. This is certainly not a trivial hypothesis, as we believe that it is possible that the response of adult flies to acute oxidative stress is not an accurate reflection of the response of aged flies to endogenous damage. It may be that the positive effect of DNAAM expression and concomitant reduction of NAM on lifespan is outweighed by the cellular requirement for NAM to combat the acute oxidative damage we are administering in our experiments. In this case DNAAM may play both positive and negative roles in oxidative stress response, depending on the strength and duration of exposure. It would be very informative to test DNAAM expressing flies of advanced age for their ability to withstand oxidative stress. However, the ROS theory states that oxidative damage is the sole cause of aging, and it is reasonable to infer therefore that long lived animals will be able to tolerate acute oxidative damage better than shorter lived controls; which is not the case for

DNAAM flies. Our data suggests that pathways outside of oxidative stress response are at least partially responsible for the longevity of DNAAM expressing *Drosophila*, and that the ROS theory does not adequately resolve this phenotype.

The effect which Sir2 expression has on lifespan and stress response is more unilaterally positive than the observed contradictory effects of NAM metabolism. *Sir2* has been proposed to act as a key regulator of dietary restriction (DR), a phenomenon in which a reduction in available dietary components (such as calories, amino acids, or proteins) resulting in an increase in longevity that is conserved in vastly divergent species (reviewed in Guarente and Bordonne 2005, Sinclair 2005, Guarente and Picard 2005). The importance of *Sir2* as a regulator of DR was discovered in yeast, in which the aging phenotype is associated with the accumulation of toxic extrachromosomal rDNA circles (Sinclair and Guarente 1997). DR-mediated lifespan extension in yeast is achieved by a reduction in glucose availability or the mutation of a component in glucose metabolism, and requires *Sir2*, which is proposed to mediate the effects of DR by reducing the formation of toxic rDNA circles. Interestingly, Sir2 is also involved in mediating the effects of DR in organisms in which rDNA circles do not contribute to aging, including *Drosophila* and mice. In DR flies, there is a two fold increase in Sir2 mRNA levels, and DR-mediated lifespan extension is abrogated in flies mutant for *Sir2* (Rogina et al 2002, Rogina and Helfand 2004). Similarly, DR induces the expression of the mammalian ortholog of Sir2, SIRT1, in liver, fat, brain, and kidney tissues in rats and white adipose tissue (WAT) in mice (Cohen et al 2004, Nisoli et al 2005). *SIRT1* appears to be required for many of the effects of DR in rodents, including the reduction of WAT, increased physical

activity, and changes in energy metabolism and endocrine signaling (Nisoli et al 2005, Bioley et al 2008, Chen et al. 2005, Cohen et al 2009).

The effect of Sir2 on lifespan is not limited to mediating the effects of DR; in yeast *Sir2* mutation results in a decrease in lifespan, and in *C.elegans* a decrease in stress resistance, including paraquat-induced oxidative stress (Kaeberlein et al 1999, Tissenbaum and Guarente 2001, van der Horst 2007). In *Drosophila, Sir2* mutation has been reported to have moderate negative effects on lifespan; or no significant effect at all. This is possibly owing to strain specific differences and/or redundancy in the genome as there are five sirtuins in *Drosophila* (Newman et al 2002, Astrom et al 2003). Importantly, artificially increasing Sir2 in yeast, *C. elegans*, and *Drosophila* results in an increase in lifespan; showing that the ability of Sir2 to increase lifespan has been conserved through evolution (Kaeberlein et al 1999, Wang and Tissenbaum 2006, Rogina and Helfand 2004). The mechanism by which Sir2 increases longevity in higher eukaryotes appears to be tightly linked to stress resistance. In nematodes, Sir2 is proposed to increase lifespan by increasing stress resistance, as heat stress promotes a physical interaction between Sir2 and the forkhead transcription factory *DAF-16*, which is required for Sir2 mediated lifespan extension. Furthermore, mutation of *Sir2* results in increased susceptibility to many environmental stresses, including oxidative stress (Wang and Tissenbaum 2006).

Our data shows that this requirement of *Sir2* for oxidative stress resistance is conserved in *Drosophila*, and that over-expression of Sir2 can increase resistance to multiple forms of oxidative stress. In mammalian cells, SIRT1 has been shown to protect against cellular oxidative

stress and DNA damage induced apoptosis via interaction and deacetylation of p53 and various FOXO forkhead transcription factors (Luo et al 2001, Vaziri et al 2001, Kitamura et al 2005, Brunet et al 2004). Adding to the correlation of *Sir2* with lifespan and stress regulation are data on additional sirtuin family members in yeast (4), *C. elegans* (4), *Drosophila* (5) and mammals (6). These other sirtuins have been studied extensively in mammalian systems, and have proposed roles in both longevity and stress response that may in some cases overlap with Sir2, and in others may have evolved disparate functions to accommodate the complexity of vertebrate physiology and tissue organization.

An interesting observation is that DR retards both the age dependant increase in molecular oxidative damage and production of mitochondrial ROS in rodents (Bolkov et al 2004, Sohal et al 1994). Because of the connection between Sir2 and the DR response, it has been suggested that Sir2 may act as a mimetic of DR, and thus engender the positive effects of DR on stress response. Hydrogen peroxide treatment causes an increase in nuclear localization of SIRT1, dependent upon phosphorylization by the mitogen activiated kinase JNK1, which initiates the deacetylation of histone H3 (Nasrin et al 2009). Furthermore, treatment with H_2O_2 causes a depletion in NAD+ levels and results in an accumulation of acetylated p53, likely resulting from a reduction in SIRT1 activity (Furukawa and Oikawa 2007). Taken together with our data, this suggests that Sir2 responds directly to conditions of oxidative stress, as well as dietary restriction. Because oxidative stress is not a direct cause of DR, or vice versa, it is possible that Sir2 is at the nexus of the cellular and/or organism response to several stresses; a hypothesis made more tempting by the knowledge that DR is itself a form of non-lethal stress.

The model of multiple independent forms of stress eliciting a conserved and integrated genetic response has been termed "multiplex stress resistance". In the multiplex theory, organisms have evolved conserved and correlated damage control pathways that when activated result in both longevity and resistance to several forms of stress (reviewed in Miller 2009). Thus events that result in non-lethal stress will affect pathways that positively regulate longevity through an increased protection from *all* forms of lethal stress. Because Sir2 is intimately tied to the response to DR, and can protect against both oxidative and DNA damage, it is plausible that it is acting along a multiplex axis to regulate longevity in a more comprehensive manner than through oxidative stress resistance alone. This could explain why DNAAM is capable of inducing longevity but not oxidative stress response in *Drosophila*, while Sir2 can induce both. DNAAM may not be capable of initiating the multiplex response, but could provide longevity through stress-independent pathways, or via stress response pathways that bifurcate and are downstream of the multiplex response. To test this hypothesis Sir2 and DNAAM expressing flies will have to be assessed for resistance to more forms of stress than simply oxidative stress. The multiplex theory, like the ROS theory, hypothesizes that long lived organism will have enhanced stress responses that are engendered throughout the life history. Because of our variable results with *DNAAM* and *Sir2* when correlating lifespan to oxidative stress response, we believe the multiplex theory suffers from the same over-simplification as the ROS theory; however does encompass the literature on stress response and longevity more so than the ROS theory.

The multiplex hypothesis is strongly supported by work in *C. elegans*; particularly with the insulin signaling hypomorphic mutant *daf-2*. *Daf-2* worms show a large increase in lifespan (Kenyon et al 1993), along with a concomitant increase in resistance to thermal stress (Gems et al 1998), oxidative stress (Vanfleteren et al 1993), UV radiation (Murakami and Johnson 1996), and bacterial infection (Garsin et al 2003). Microarray analysis details the up-regulation of a wide variety of stress response genes as a result of *daf-2* mutation, which likely mediates the observed multiplex response of these organisms (Fisher and Lithgow 2006). Outside of worms, however, there are discrepancies with the multiplex theory in the observation of a correlated and conserved stress response in long-lived animals. In *Drosophila*, when insulin-like peptides (DILPS) are eliminated flies exhibit an increase in longevity, oxidative stress resistance, and starvation resistance; but show a *decrease* in tolerance to thermal stress (Broughton et al 2005). This has been proposed to arise from the different specificities of the seven known DILPS in mediating stress response (Broughton et al 2005). Similarly, when the antioxidant enzyme MnSOD is over-expressed in *Drosophila*, the resulting lifespan extension does not correlate with increase tolerance to oxidative stress and also reduces thermotolerance (Sun et al 2007). Thus, in *Drosophila*, multiple stress resistance and longevity are not always linked, and furthermore increases in one form of stress resistance can correlate with a reduction in a second form of stress resistance.

Analysis of mutiplex theory in mammalian models is in a fairly nascent stage. Recent data from cell culture experiments report that fibroblast cells derived from long lived mice are resistant to many, but *not all* forms of cellular stress, and importantly the observed resistance is

congenital in these animals (Murakami et al 2003, Harper et al 2007, Salmon et al 2005). The fact that not all forms of stresses applied were protected against in these models presents a problem to the multiplex assumption that all stressors elicit the same coordinated cellular response. Furthermore the lack of data from whole organisms has hampered a more complete analysis of this theory in higher organisms. It is certain that the multiplex theory is in need of added complexity to accommodate for different forms of stress and the effects of one form of stress on the tolerance of others. For example, it has been well established that DR mice may have increased tolerance to oxidative damage, but are not more resistant to thermal stress or starvation stress, simply owing to physiological changes in fat deposition and available energy stores associated with reduced food intake.

Because both the ROS theory and multiplex theory predict a correlation between oxidative stress response and lifespan, data that disproves this correlation highlights the requirement for aging theory modification. Recent studies in both *Drosophila* and mice have proven there is not always increased oxidative stress resistance in long lived animals, and that increased oxidative stress resistance does not always correlate with longevity. The most encompassing analysis of this phenomenon in mice was undertaken in Arlan Richardson's laboratory, during which several antioxidant genes were either mutated or over-expressed and resulting animals analyzed for markers of oxidative stress resistance and recorded for lifespan (Perez et al 2009). The experimenters failed to find any correlation between increased oxidative stress response and longevity, and observed a direct contradiction to the ROS theory in mutants for the glutathione peroxidase (*Gpx4*) and Methionine sulfoxide reductase-A (*msrA*).

Similar observations were made by Howard Jacob's group in *Drosophila*, who report that the long lived strain Oregon R (OR) exhibit an increased lifespan and oxidative stress response compared to the short lived Dahomey (DAH) strain; however breeding OR mitochondrial genes into DAH flies increases female longevity independent of ROS protection (Sanz et al 2010). A similar approach was undertaken in our own laboratory, and revealed that breeding long-lived LA strain mitochondria into normal-lived RA flies enhanced their longevity (Jung et al 2007). These data shows that it is possible to increase longevity by selective strain interbreeding, and that this can occur without simultaneously eliciting enhanced oxidative stress response.

Indeed genetic background appears to play an extremely important role when correlating stress response and longevity. For example, transgenic *Drosophila* expressing CuZnSOD or Catalase has been independently reported to increase longevity or have no effect on longevity (Orr and Sohol 1994, Orr and Sohol 2003) which appears to depend on the background genetics and/or chromosomal insertion position of the transgenic elements. Specifically, in long lived flies, expression of these genes is not capable of altering longevity despite differences in ROS management (Orr et al 2003). Importantly, an effort to correlate genetic background to lifespan undertaken in Daniel Promislow's laboratory resulted in the observation of significant variance in the ability of CuZnSOD to alter lifespan between 10 different strains of *Drosophila* for both sexes (Spencer et al 2003). In addition to oxidative stress response varying significantly in different backgrounds of *Drosophila*, it has been shown recently that dietary restriction response is also largely dependent on genetic background in mice. In the particular experiment 41 recombinant mice strains generated to study alcohol

sensitivity were placed on ad libitum (AL) and dietary restriction diets and monitored for lifespan. Interestingly, researchers observed a 2-3 fold difference in lifespan in median lifespan under AL conditions and a 6-10 fold variation in DR strains (Liao et al 2010). Importantly, DR actually *shortened* lifespan in more strains than those for which it increased lifespan. This study details the important contribution of genetic and epigenetic variation resulting from selective inbreeding to both normal lifespan (AL) and organism response to experimental manipulations such as DR.

Perhaps of equal importance to genetic background is the contribution of different stresses to mortality depending upon the life history, which can be altered significantly by breeding and rearing conditions. In yeast, deletion of CuZnSOD will shorten lifespan under aerobic conditions, but not under anerobic conditions (Longo et al 1996). In the previously discussed experiment on the contribution of antioxidant enzymes to longevity in mice, a 35% difference in the lifespan of genetically identical control animals was observed between two different laboratories, entirely owing to differences in breeding and rearing conditions (Perez et al 2009, Moskovitz et al 2001). Thus differences in rearing techniques resulted in suboptimal lifespan in controls animals in one laboratory that profoundly affected analysis of antioxidant genes on lifespan. Rearing conditions resulted in a much shorter-lived control animal, and mutation effects that further reduced this lifespan were likely the resulted from exacerbation of the husbandry conditions rather than oxidative stress. This highlights the need for stringency in generating equivalent and optimal conditions of rearing that need to be met in all laboratories that study a particular model organism. Taken together, different genetic backgrounds and

rearing conditions has a profound influence on which cellular and organism stresses are seminal to age associated mortality, and must be strongly considered in any analysis of lifespan and extended longevity.

Our data in GFP expressing animals show that it is possible to measure significant changes in oxidative stress response with different chromosomal combinations in otherwise genetically isogenic individuals (Fig 3, supplemental figure 2). While we have shown that this increase may be attributed to experimental variance, it is nonetheless replicable in independent experimental trials, and may result from genetic or epigenetic modifications inherent to the *Drosophila* stock maintenance technique of breeding small isolated populations over several generations. The contribution of these changes to lifespan will be a focus of further experimentation. Our previous and current data has shown that our background w^{1118} stock is deficient in both longevity and ROS response versus longer lived wild type strains. It is reasonable to assume that any manipulation that increases oxidative stress resistance will play a larger role in augmenting the lifespan of w^{1118} flies due to their intrinsic susceptibility to this form of stress. Our Sir2 and DNAAM data show that there are at least two pathways for increased longevity in this strain, which may both result from the predisposed susceptibility of these animals to different forms of stress that contribute to increased age dependant mortality.

We hypothesize that the correlation between stress resistance and mortality is intimately tied to the differences in genetic background between diverse strains of the same organism. Thus different genes and pathways will have profound differences in their ability to modify lifespan and stress response depending on the strain specific susceptibility to various

types of age associated damage. We believe that the spectrum of genetic backgrounds in *Drosophila* (and all organisms) ranging from stress sensitive to resistant for a variety of potentially lethal stressors, will exhibit complex interactions between specific gene pathways and their ability to ameliorative stress induced mortality and/or increase lifespan. Additionally, genetic mutation and epigenetic variation between laboratory strains will likely mask or obscure the fidelity of many proposed longevity genes to affect aging phenotypes in human populations; thus the identification of pathways that affect longevity in a broad spectrum of strains and species is of critical importance. Aging theory must accommodate for diverse etiologies of mortality that are dependent upon both the background genetics of the same organism and the diversity in life histories of different organisms. Such an effort will be necessary to identify regulators of human senescence and produce viable targets for the prevention of age associated disease.

Chapter 4

Discussion/Future considerations

There are multiple pathways that lead to an extended longevity, but the two of the major ones are those that involve non-lethal stress-inducing environmental manipulations or by environmental or genetic activation of pathways that regulate the response to dietary restriction. The current model of DR mediated lifespan extension in *Drosophila* requires the activation of the protein deacetylase Sir2 (Guarente and Kenyon 2000, Sinclair 2004), which affects a plethora of gene transcription events that en masse result in extended longevity. We have shown that the increased expression of the *Drosophila* nicotinamidase, DNAAM, extends lifespan in both male and female adult flies when expressed ubiquitously or in the nervous system alone. DNAAM shows both phenotypic and functional conservation with its yeast ortholog PNC1, suggesting that nicotinamide (NAM) metabolism may affect lifespan in a variety of organisms (Anderson et al 2003). In nematodes, exogenous NAM results in a decrease in lifespan; however increased PNC1 does not result in lifespan extension (van der Horst 2007). Interestingly, while PNC1 in yeast is up-regulated by a variety of stresses including dietary restriction, we only found induction of DNAAM expression in *Drosophila* cells under conditions of oxidative stress. Oddly, when assayed for longevity on the oxidative stress inducing chemical paraquat or hydrogen peroxide, DNAAM expressing flies did not show an increased resistance when compared with control animals. This contradiction between the longevity data of *Drosophila* and *C. elegans* may result from a different management of ROS between the two organisms, and/or in differences in the ability of NAM to regulate lifespan. In *C. elegans*, PNC1

expression does not increase lifespan, but does increase resistance to oxidative stress; and NAM addition results in not only a decrease in lifespan, but an increased susceptibility to oxidative stress (van der Horst 2007). A functional *C elegans* Sir2 (Sir2.1) is only partially required for PNC1 to increase resistance to oxidative stress, as there is significant PNC1 induced enhancement of this phenotype in Sir2.1 deficient worms. Importantly, NAM can reduce lifespan and abrogate DR mediated longevity in yeast even when all sirtuin family members are mutated (Tsuchiya et al 2005). Taken together, this data supports a role of NAM and nicotinamidase genes in stress response and lifespan regulation that is not solely dependent on Sir2.

There are several models of NAM regulation that may explain why nicotinamidase expression increases lifespan but not oxidative stress response in *Drosophila*, yet has the opposite effect in *C. elegans*. Perhaps most important to any conjecture is the very different effect of experimentally exogenous NAM on lifespan of the two model organisms. In contrast to nematodes, the addition of NAM to *Drosophila* culture media has no negative effect on lifespan, and in fact has been shown to improve outputs of functional senescence in flies with mutations that model Parkinson's disease (Jia et al 2008). In mammalian cell culture models, NAM has been shown to provide protection from oxidative stress, and increase cell replicative lifespan (Lin et al 2001, Hwang et al 2006, Jia et al 2008,). Thus there are differences in NAM effects that may correlate with both increased physiological complexity and evolutionary divergence in pathways that metabolize NAM. In both *Drosophila* and mammals, there is at least one other enzyme in the pathway between nicotinamide and NAD+ synthesis,

nicotinamide mononucleotide adenylyl transferase (Nmnat1). Nmnat1 is involved in both NAD+ synthesis, and in axonal protection, which has been extensively studied in the Wallerian degeneration slow (Wlds) strain of mice. Wlds mice express a fusion protein containing Nmnat1, which is critical for axonal protection from a variety of insults including axotomy, chemotherapeutic agents, or genetic mutations (Sasaki et al 2009). The enzymatic function of Nmnat is required for axonal protection, and expression of Drosophila Nmnat can provide similar protection when expressed in mammalian neurons (Sasaki et al 2009).

Our data shows that expression of DNAAM in mammalian neurons engenders protection from oxidative stress induced apoptosis. Taken together with the fact that neuronal expression of DNAAM is sufficient for lifespan extension in flies, this highlights an important role of neuronal protection in *Drosophila* lifespan. Sir2 over-expression in neurons is also sufficient to extend lifespan in flies, and thus a pathway involving NAM metabolism and sirtuin signaling may evoke neuronal protection that can affect lifespan in eukaryotes with a more complex nervous system than nematodes (Rogina and Helfand 2004). Thus, a possible reason that PNC1 cannot increase lifespan in *C. elegans* is that effect of NAM and/or NAM regulation may be broader in nematodes, which exhibit a less complex nervous system and lack some elements of regulation such as Nmnat. Excessive NAM may be toxic in all nematode cells, which is ameliorated by increased PNC1 expression; however without additional regulation by enzymes such as Nmnat, this effect is insufficient to induce lifespan extension. In this model NAM may have evolved a neural protective ability based on its ability to affect the enzymatic activity of Nmnat and perhaps other diverse enzymes that require Nmnat activity or NAM. It

has been shown that Nmnat can interact with SIRT1 in mammalian cells to regulate targets of transcription, which may account for its cell protective abilities (Zhang et al 2009).

The importance of Sir2 to longevity and stress response phenotypes is shown by the fact that DNAAM induced lifespan extension in *Drosophila* and PNC1 induced oxidative stress resistance in *C.elegans* both require a functional Sir2. Similarly DNAAM induced oxidative stress resistance in mammalian cells is abrogated in the presence of the sirtuin inhibitor sirtinol. While elevated PNC1 cannot extend lifespan in *C. elegans*, increased Sir2 expression has been shown to increase worm lifespan (Tissenbaum et al 2001). Interestingly, while PNC1 deficiency reduces the lifespan of nematodes, Sir2 mutation has been shown to consistently reduce lifespan only under conditions of enhanced environmental stress (van der horst et al 2007, Berdichevsky et al 2006, Wang and Tissenbaum 2006). In *C. elegans*, lifespan and stress response are tightly linked, as mutations that increase stress response also increase nematode lifespan, while in *Drosophila* this correlation is not always observed (reviewed in Lithgow and Miller 2007). We have shown that in *Drosophila,* Sir2 expression, but not DNAAM expression enhances resistance to oxidative stress, while in nematodes both genes can enhance oxidative stress resistance. It is therefore probable that, in both organisms, Sir2 contributes to both longevity and stress response; however our evidence suggests that regulation of Sir2 through pathways independent of increased nicotinamidase activity is required for oxidative stress resistance. It is possible that the positive effects of DNAAM on do occur through Sir2 activation; however when challenged with oxidative stress, pathways that require endogenous NAM levels are negatively affected and thus Sir2 protection lost. This could occur if increased

Sir2 protects cells from oxidative stress by increased interaction with an enzyme such as Namnt1 that may require normal NAM levels. This could provide for a scenario where long term reduction of NAM by DNAAM is beneficial to flies through a chronic increase in Sir2 activity, but this effect is only positive when there is not a situation of acute oxidative stress that would require normal levels of NAM for tolerance. Because DNAAM expression does not reduce tolerance to oxidative stress, there may be an amalgamation of positive and negative effects that cancel each other with regards to survival on paraquat.

In nematodes, it has been shown that NAM increases the toxicity of paraquat; thus PNC1 may increase oxidative stress response simply by removal of NAM, rather than through induction of enzymes such as Sir2. This could explain why oxidative stress response, but not lifespan is increased in PNC1 expressing worms. It has been demonstrated that PNC1 knockdown in nematodes does shorten the life span of adult worms, establishing the role of PNC1 also in worm life span regulation. It is possible that the inability of nematode PNC1 over-expression to extend lifespan could be a result of insufficient levels of expression. The degree of life span extension induced by DNAAM correlated well with the level of protein over-expression, and low levels of expression resulted in very small increases in lifespan that would not have been significant with a smaller sample size. Thus, it is possible that the level of PNC1 over-expression in worms was not robust enough to observe an effect on lifespan, perhaps owing to inadequate Sir2 activation. A definitive role of Sir2 in mediating the effects of DNAAM has not been observed, as we have only shown that DNAAM induced longevity requires Sir2, and thus Sir2 mutation is epistatic to DNAAM longevity. In yeast there is experimental data

showing that increased PNC1 expression results in lifespan extension mediated by a PNC1 induced increase in Sir2 activity (Anderson et al 2001). However, in *C.elegans*, while the removal of NAM by PNC1 can increase oxidative stress resistance, there is either insufficient activation of Sir2 to affect changes in transcription that mediate longevity, and/or affects that are entirely independent of Sir2.

Because both the extended longevity and neuroprotection endowed by DNAAM were dependent on Sir2/sirtuin function, there are likely targets of nicotinamidases that require Sir2 for either activation or transcriptional regulation. There are many NAD consuming enzymes that are inhibited by NAM, and thus could be regulated by nicotinamidases; however manipulation of their function may depend on Sir2 for transcriptional regulation. Data supports that there are pathways of longevity independent of Sir2 activation, however data also supports that Sir2 acts as a master regulator of lifespan; thus it may be required for the modifications of these pathways to affect longevity. The number of transcription factors that are affected by Sir2, in addition to the ability of Sir2 to deacetlyate histones has led to the characterization of hundreds of genes whose expression is modulated by changes in Sir2 activity (Oberdoroerffer et al 2008). Many of these genes are in pathways of stress resistance, and could be downstream targets that are regulated by nicotinamidase activity. Our demonstration that DNAAM can increase longevity and neuronal protection in a manner similar to Sir2, but does not have the same effect on oxidative stress resistance; supports a view that nicotinamidase genes affect these phenotypes at least in part through a mechanism independent of Sir2.

The question of whether the effects of D-NAAM and other nicotinamidases on lifespan and stress response are mediated by sirtuins or whether they involve other targets needs to be further evaluated. Several experiments will be necessary In order to address these hypotheses, which can be performed in *Drosophila* utilizing some of the same stocks already used in our analysis, and others that are currently being generated and characterized. Of critical importance is the generation and testing of a DNAAM null mutant or RNAi knockdown construct that has proven efficacy. Currently we have created several lines with either partial or complete removal of the DNAAM locus. Unfortunately, when the DNAAM locus is partially removed (via p element mobilization of the 5' sequence), only a small portion of the 5'UTR is deleted, and we did not measure a significant reduction in mRNA levels. It may be necessary to repeat this experiment using different p element insertions that map to the coding sequence as they become available. When the entire locus is removed using FRT mediated gene excision from the specialized Exelixis collection, three other genes are simultaneously lost, which would make analysis of DNAAM specific effects difficult, and further exacerbated by the fact that the excision is not homozygous viable. It would be possible to insert the missing gene via homologous recombination or p element insertion, which could allow for DNAAM specific effects to be monitored. Most promising are lines with p element insertion of UAS-DNAAM RNAi constructs, which when activated in the presence of Gal4 drive expression of DNAAM double strand RNA, which should mediate substantial knockdown of protein levels. Currently these lines need to be assayed for DNAAM mRNA levels, protein levels, or reduced nicotinamidase activity.

Once a viable DNAAM mutant is isolated, reduction in DNAAM can be assayed for effects on lifespan and oxidative stress response. PNC1 deletion reduces lifespan in both yeast and *C. elegans*, which suggests that there is some conservation of NAM in regulating lifespan, however these strong negative effects of NAM on lifespan appears to not have been conserved in flies. In particular the contrast in over-expression effects on oxidative stress and lifespan observed between *Drosophila* and *C. elegans* points to a divergence in NAM regulation of oxidative stress management and possibly mechanisms of lifespan regulation as well. Because *Drosophila*, but not *C. elgans*, has the Nmnat1 gene which can metabolize NAM derivatives, there may be a second pathway of metabolism that results in redundancy with DNAAM. Our bioinformatics approach did not identify any genes in mammals with significant homology to DNAAM or PNC1, and in mammals the enzyme responsible for NAM metabolism is nicotinamide phosphoribosyltransferase (Nampt) which converts nicotinamide into nicotinamide mononucleotide, the substrate for mammalian Nmnat1. Drosophila represents an organism where both a nicotinamidase (DNAAM) and nicotinamide mononucleotide adenylylreansferase (Nmnat) are present; however the ortholog of Nampt is missing. Thus flies may represent a point of evolutionary divergence between lower and higher eukaryotes in NAM metabolism, signaling effects, and lifespan regulation. This divergence may have partitioned different roles for NAM in lifespan and stress response based on tissue specific expression of enzymes that regulate its metabolism and mediate its effect on NAD+ consuming enzymes like Sir2.

In order to elucidate changes in gene activity that may contribute to DNAAM induced lifespan or stress response versus those that accompany Sir2 induction, it would be necessary to perform quantitative assays for expression or activity of known targets, or utilize microarrays for gene expression. This would allow us to determine if DNAAM induced lifespan is mediated by Sir2, or involves other pathways, and provide information as to how Sir2 endows both longevity and stress resistance. There are many known targets of Sir2 deacetlyation, including p53, Foxo, and histone lysine residues, many of which have commercially available antibodies that can allow discrimination between acetylated and deacetlyated forms. Using the Sir2 over-expression construct, it would be possible to elucidate which of these can be measured to have an increase in deacetylation, and then ask whether or not DNAAM over-expression can induce similar changes. If DNAAM expression up-regulated Sir2 target deacetylation, but this does not occur in DNAAM expressing flies that are deficient for Sir2 we would have definitive proof that DNAAM can activate Sir2. Once these targets are elucidated, it would be possible to determine their effects on lifespan and stress response, and thus provide pathways for which Sir2 modifies these phenotypes.

Work in this vein has already identified *Drosophila* p53 as a potential mediator of Sir2 dependant lifespan extension. (Bauer et al 2009). Sir2 interacts with *Drosophila* p53 (Dmp53), and can deacetlyate Dmp53 derived peptides, and furthermore while both dominant negative p53 and Sir2 expression lead to lifespan extension, in combination the two do not have an additive effect (Bauer et al 2009). It is possible that DNAAM expression or activation could up-regulate this pathway by providing relief of Sir2 inhibition, which could be tested using p53

acetylation as an output. There are many other NAD consuming enzymes that in theory could mediate DNAAM longevity, including the enzyme poly-(ADP-ribose) polymerase- (PARP-1) which is involved in DNA damage repair and important for maintenance of DNA integrity (Burkle et al 2004, Tong et al 2001). Monitoring changes in expression or activity of PARP-1, other sirtuins, and other NAD consumers, may allow us to identify pathways outside of Sir2 that are up-regulated in DNAAM expressing flies. Microarray analysis of DNAAM over-expressing, DNAAM mutant, Sir2 over-expressing, and Sir2 mutant fly lysates, when compared with genetically matched controls would assist greatly in our understanding of how DNAAM and Sir2 affect lifespan and stress response.

An alternative mechanism for DNAAM longevity that correlates with Sir2 but is independent of NAM effects could exist through modification of the NAD+/NADH ratio. While it has been shown that the main activation of Sir2 by PNC1 in yeast is through the removal of NAM by conversion to nicotinic acid, this resulting product is a substrate for NAD synthesis (Anderson et al 2003). It has been shown that DR mediated lifespan requires NAD, which may be increased by a shift toward respiration resulting in an increase in the NAD/NADH ratio, which could be mediated by increased salvage through PNC1 (Lin et al 2004). In both yeast and C. elegans, excess NAD resulting from gene mutations or media addition respectively result in an increase in lifespan that correlates with increased Sir2 activity (Lin et al 2002, Lin et al 2004, Hashimoto er al 2010). It is suggested that in vertebrates, NAM inhibition of Sir2 is expected to be much less important than in lower eukaryotes due to much lower concentrations of NAM (Catz et al 2005). Indeed vertebrate Nampt, which performs a similar function as DNAAM, has

been shown to activate Sir2 through NAD synthesis rather than NAM removal (Revollo et al 2004).

A recent theory that ties changes in NAD/NADH metabolism to aging is the Epigenetic Oxidative Redox Shift theory (EORS). EORS hypothesizes that aging results from a shift in redox metabolism toward oxidation proposed to occur by epigenetic modifications that result from reduced physical activity and insulin sensitivity, which both correlate with advancing age (Brewer 2010). In this hypothesis, despite the higher ratio of NAD/NADH resulting from the oxidizing environment, there is a concomitant increase in the requirement for NAD to facilitate glycolysis in favor of oxidative phosphorylation as a generator of energy. This results in an increase in expression of plasma membrane and cytosolic oxidoreductases that are much less efficient than mitochondrial oxidoreductase (which is reduced in activity as OXPHOS declines), and generate large numbers of ROS in effort to regenerate the necessary NAD (Brewer 2010). This could provide for a scenario in which Sir2 activity should be up-regulated during normal aging, which would be triggered by an increase in the NAD/NADH ratio, but is not due to this requirement of NAD for glycolysis. If DNAAM up-regulation is capable of providing more substrate for NAD synthesis, the increased need to regenerate NAD through oxidation could be ameliorated, resulting in less ROS production, and perhaps providing more available cofactor for enzymes like Sir2. Careful monitoring of the NAD/NADH ratio in adult flies that express DNAAM or are DNAAM deficient, along with analysis of Nmnat1 expressing or mutant flies could provide insight as to how the NAD/NADH ratio is affected by aging and its contribution to lifespan and Sir2 regulation.

Our data provide strong evidence that increased nicotinamide clearance in cells provides positive effects on organism life span, and in mammalian neurons, endows cellular resistance to oxidative stress. However, the fact that DNAAM expressing flies do not exhibit increased resistance to multiple forms of oxidative stress suggests that the observed ability of DNAAM to protect mammalian neurons is either not universal to all cell types, or does not occur in flies. Importantly this data highlights a disconnect between oxidative stress resistance and longevity in DNAAM transgenic flies. Alternatively, Sir2 expressing flies have been previously shown to be long lived, and we have shown that these animals have a congenital resistance to oxidative stress. The ROS theory of aging predicts that ROS are the sole cause of aging, and that animals with extended longevity will have a concomitant increase in tolerance to oxidative stress. Thus our data refutes the ROS hypothesis because only one of our two longevity genes (Sir2) fits this hypothesis. It is possible however, that normal ROS management in *Drosophila* declines with age, contributes to mortality, and may be ameliorated by DNAAM induction. As our experiment is a measure of acute oxidative stress response, it does not account for this possibility. It may be that a positive effect of DNAAM expression on endogenous ROS management is outweighed by the cellular requirement for NAM to combat the acute oxidative damage we are administering in our experiments. In this case DNAAM may play both positive and negative roles in oxidative stress response, depending on the strength and duration of exposure.

In order to prove that DNAAM is not capable of increasing tolerance to oxidative stress, then DNAAM flies of advanced age for their ability will need to be tested for their ability to

withstand oxidative stress, perhaps using several different concentrations of paraquat. Alternatively, lysates from DNAAM flies will need to be monitored for endogenous level of ROS, oxidative damage, or expression of antioxidant enzymes through the fly life span to determine if chronic endogenous oxidative stress response is altered. However, as previously discussed, and considering the ability of Sir2 (and many other previously identified enzymes) to endow congenital oxidative stress resistance that is observable in our experimental paradigm, we believe that DNAAM induced longevity occurs largely in the absence of increased oxidative stress response. This theory is supported by observations that many elements of pathways involved in oxidative stress response do not regulate lifespan, and that lifespan extension can occur in the absence of increase oxidative resistance.

Our data adds to a wealth of recent data that supports the postulation that the ROS theory does not adequately account for the diversity of etiologies that contribute to mortality or increased longevity. Accordingly, alternate theories or variations on the ROS hypothesis are emerging to account for the ability of some genes and pathways of longevity to increase oxidative stress resistance and others that do not. The most reliable and replicable environmental manipulation to extend lifespan is the implementation of dietary restriction (DR) (McCay et al 1935). DR has been shown to both increase lifespan in rodents, and reduces both the age dependant increase in molecular oxidative damage and production of mitochondrial ROS in rodents (Bolkov et al 2004, Sohal et al 1994). Over the last decade enormous effort aimed at understanding this phenomenon has led to the discovery of several genes and pathways that appear to mediate the effects of DR, which includes the Sir2 deacetylase (Sinclair

2005, Bishop and Guarente 2007, Fontana 2009). Increased expression or activation of the protein deacetylase Sir2 has been shown to mediate the effects of DR in yeast and Drosophila, and has been shown to increase oxidative stress resistance and lifespan in both C. elegans (Lin et al 2000, Tissenbaum and Guarente 2001, van der horst et al 2007, Rogina and Helfand 2004,). In rodents, DR induces the expression of the mammalian ortholog SIRT1 in several tissues important for the regulation of metabolism and endocrine signaling, including liver, fat, and brain, (Cohen et al 2004, Nisoli et al 2005). Furthermore many functional outputs of DR do not occur in rodents deficient in SIRT1, including the reduction of WAT, increased physical activity, and changes in energy metabolism and endocrine signaling (Bioley et al 2008, Chen et al 2005, Cohen et al 2009). Because of the connection between Sir2 and the DR response, it has been suggested that Sir2 may act as a mimetic of DR, and thus engender the positive effects of DR on stress response.

Our data has correlated *Drosophila* Sir2 to both longevity regulation through its requirement for DNAAM induced lifespan extension, and to oxidative stress resistance as s both are required for normal oxidative stress response and can increase resistance when over-expressed. Taken together with the above observations, it seems that the Sir2 pathway does support the ROS hypothesis; however this may only be part of the story. DR is itself considered to be a non-lethal stress, and Sir2 is not only tied to the response to DR, but also protects cells from DNA damage, pro-apoptotic gene activity, and irradiation, and has several proposed neuronal protective functions outside of ROS management (Luo et al 2001, Vasari et al 2001, Greer and Brunet 2005, Cohen et al 2004, Chen et al 2005). Thus, Sir2 appears to mediate

lifespan extension through mechanisms outside of oxidative stress response alone. The "multiplex stress resistance theory" has evolved from the ROS hypothesis to reflect the observation that some genes and pathways affect multiple forms of stress, each of which may contribute to lifespan extension. In multiplex theory, organisms have evolved conserved and correlated damage control pathways that when activated non-lethal stress result in both longevity and an increased protection from all forms of lethal stress (reviewed in Miller 2009). It is therefore plausible that Sir2 is acting at the nexus of a multiplex axis to regulate longevity in a more comprehensive manner by up-regulating stress response genes in response to various environmental or cellular stresses, with the end result being increased longevity through a combinatorial response. Among Sir2 targets that are involved in regulating stress response are p53 and members of the Foxo family of transcription factors, which results in a decrease in apoptotic gene activity and an increase in many stress response genes (Vaziri et al 2001, Brunet et al 2004, van der horst 2007).

DNAAM on the other hand, is capable of inducing longevity in the absence of any observed increase in oxidative stress response. As discussed, there is evidence that PNC1 can increase oxidative stress response in yeast; however this does not accompany increased lifespan. If DNAAM and PNC1 are affecting lifespan and stress response through Sir2 activation alone, as has been shown in yeast, it is uncertain why there are disparate results between DNAAM and Sir2 over-expression in both flies and nematodes. A possible explanation could be provided by the multiplex theory, if DNAAM is not capable of fully initiating the multiplex response, but may affect pathways that are downstream of the multiplex response that can

affect lifespan. In this model DNAAM may have effects that are downstream of Sir2, which would regulate multiplex upstream of the nicotinamidase. This does not seem likely, as there is no evidence to suggest that Sir2 activity regulates DNAAM or PNC1 expression or activity, but rather evidence that nicotinamidases can regulate Sir2. Regardless, if DNAAM initiates a component of multiplex this element would have to be determined to link longevity to a stress response.

To test this hypothesis DNAAM expressing flies will have to be assessed for resistance to more forms of stress than simply oxidative stress. Even if this is proven to be the case, there are problems with the multiplex theory in interpreting ours and others data. First of which is the evidence that would place DNAAM upstream of Sir2, and thus capable of initiating multiplex resistance itself. If this does occur, and as we have hypothesized NAM has positive effects on oxidative stress resistance, then DNAAM would be initiating both positive effects through Sir2 activation, and negative effects through removal of NAM. By increasing Sir2 activity without manipulating NAM levels, any negative effects of DNAAM induction on oxidative stress would be bypassed. This does not corroborate the theory of a broad stress response mechanism that causes an increase in all stress responses, but rather that there is up-regulation of some stress response pathways at the expense of others, and complex interactions between elements that are involved in these pathways.

As a result of these concerns, we believe the multiplex theory suffers from the same over-simplification as the ROS theory, but does encompass stress response and longevity data more so than the ROS theory. Unfortunately, both the ROS theory and multiplex theory predict

a correlation between increased oxidative stress response and longevity. Studies in both Drosophila and mice have proven that this is not always observed, and that there are in fact cases in which a direct contradiction of this hypothesis is observed. Our own data in *Drosophila* has shown that is possible to separate an identical wild type progenitor strain (termed Ra) and select for either longevity or oxidative stress resistance through breeding for long lived or for paraquat resistant animals (Vettraino et al 2001). After multiple rounds of selection, long lived animals (La) exhibit a 50% increase in mean lifespan, and 40% increase in paraquat resistance. In contrast, however, paraquat bred animals (PQR F7) exhibit over 100% increases in paraquat resistance without any change in lifespan. Furthermore, it appears that longevity associated with the La animals endows oxidative stress resistance through a different pathway than the oxidative stress resistance of PQR flies, with CuZn SOD and catalase being strongly up-regulated in these animals while P450 cytochrome oxidase is up-regulated in PQR flies. This data suggests that extended lifespan may be causal for oxidative stress resistance, but that oxidative stress response is not a cause of extended longevity. Thus the ROS hypothesis that increased tolerance of oxidative stress is a cause of extended lifespan is disproven. It rather seems that longevity may endow resistance to oxidative and perhaps other stresses. Our data on divergent pathways of oxidative stress resistance in La and PQR animals contradicts the multiplex hypothesis of conserved stress responses as there appear to be different pathways that initiate the same form of stress resistance.

Despite the reported ability of artificial expression or mutation of antioxidant enzymes to contribute to lifespan in both mice and Drosophila, recent evidence suggests that these

observations may have been the result of strain specific effects or differences in rearing and/or breeding conditions. An encompassing analysis of this phenomenon in mice was undertaken in Arlan Richardson's laboratory, during which several antioxidant genes, including Mn-superoxide dismutase, Cn/Zn superoxide dismutase, Glutathionine peroxidases, and Methionine sulfoxide reductase, were mutated and analyzed for lifespan and oxidative stress resistance (Perez et al 2009). Despite data showing a reduced tolerance to oxidative stress in these mutants, there was no negative effect on lifespan, and in fact mutants for the glutathione peroxidase (Gpx4) exhibited a significant increase in mean lifespan. When a subset of these antioxidant genes was selected for over-expression analysis, transgenic animals exhibited cellular protection from oxidative stress without any concomitant increase in lifespan. Data obtained in this study was in contradiction to reports from a second laboratory, which appeared to result from a 35% difference in the lifespan of genetically identical control animals. Thus differences in rearing techniques resulted in suboptimal lifespan in controls animals that affected analysis of antioxidant genes on lifespan (Perez et al, 2009 Moskovitz et al 2001). In one laboratory rearing conditions resulted in a much shorter-lived control animal, and mutation effects that further reduced this lifespan were likely the result of the husbandry conditions rather than oxidative stress. This highlights the need for stringency in generating equivalent and optimal conditions of rearing that need to be met in all laboratories that study a particular model organism. This would allow for the analysis of gene dependant effects on lifespan and stress responses that are not obscured by anomalies in experimental technique. Furthermore, the genetic background control for mutation and transgenic analysis should be closely monitored

for lifespan and stress response variations between experimental replicates and independent laboratories to better elucidate how breeding and rearing influence these phenotypes.

Data in *Drosophila* has also shown there is a lack of consistent evidence for the ROS theory by showing a lack of correlation between lifespan and oxidative stress response. Flies expressing the plant and fungal antioxidant enzyme alternative oxidiase (AOX) showed a strong age-related decrease in mitochondrial ROS (mtROS) production without any resulting effect on lifespan (Sanz et al 2010). In the same study, flies mutant for a second gene, dj-1β, display both an increased production of mtROS, and a significant increase in lifespan in both sexes. This direct contradiction of the oxidative stress theory has also been observed in flies over-expressing antioxidant enzymes MnSOD and catalase in the mitochondria, which exhibit an increase in oxidative stress resistance, but a robust decrease in lifespan (Bayne et al 2005). Alternatively, when MnSOD is over-expressed ubiquitously in Drosophila, a resulting lifespan extension has been observed, but does not correlate with an increased tolerance to oxidative stress and reduces thermotolerance (Curtis et al to Tower 2007). Observations were made by Howard Jacob's group in Drosophila, who report that the long lived strain Oregon R (OR) exhibit an increased lifespan and oxidative stress response compared to the short lived Dahomey (DAH) strain. The selective breeding of OR mitochondrial genes into DAH flies increases female longevity independent of ROS protection (Sanz et al 2010). This data shows that it is possible to increase longevity by selective strain interbreeding without simultaneously eliciting enhanced oxidative stress response, suggesting pathways to longevity independent of ROS protection. Taken together this and our previously mentioned data highlights that there are pathways of

longevity independent of oxidative stress response, and that the phenotypes of longevity and stress resistance can result from elements that appear to operate through divergent pathways.

Work in *Drosophila* has also uncovered a large component of background genetics on lifespan and stress response, resulting in large variance in gene dependant effects likely owing to strain specific differences in epigenetic factors and mutations that vary as a result of breeding conditions and husbandry. It was reported that transgenic flies expressing CuZnSOD or catalase have increased longevity; however this result was subsequently reversed by the same group of researchers who later reported no effect of these genes on longevity (Orr and Sohol 1994, Orr et al 2003). They reasoned that their initial result was an artifact of the background genetics and/or chromosomal insertion position of the transgenic elements, which has been shown to affect many phenotypes including lifespan (Kaiser et al 1997,). Thus the flies in their initial study were short lived, and when the causal genetic elements were removed, the experimenters were no longer capable of altering longevity despite differences in ROS management (Orr et al 2003). The effect of genetic background on longevity and oxidative stress response has been further explored by efforts in Daniel Promislow's laboratory. In these experiments, 10 different strains were used to test the effect of of CuZnSOD expression on lifespan of *Drosophila*. This comparative effort resulted in the observation of significant variance in the ability of transgenic expression to alter lifespan in both sexes (Spencer et al 2003). This data suggests that it is necessary to determine if Sir2 and DNAAM induced lifespan extension is universal in flies. Our background strain, w1118, is known to have a significantly shorter lifespan than most wild type and other mutant backgrounds, and it is possible that

expression of our two transgenes may not increase the lifespan in *Drosophila* that are longer lived. The w1118 strain presumably has genetic differences that negatively affect normal lifespan, which may be ameliorated by Sir2 or DNAAM expression; however in other strains the pathways affected by these genes may already be optimal and thus not contribute to lifespan regulation in wild type animals. The difference in the strain-specific effectiveness of these pathways may logically be attributed to differences in the strains' background genetics.

The presumed ability of dietary restriction to increase lifespan across organisms with different genetic backgrounds has been challenged by a recent study in mice. In the particular experiment, 41 recombinant mice strains that were previously generated and separated by inbreeding to study the effects of genetic variation on alcohol sensitivity (Bennett et al 2006) were placed on ad libitum (AL) and dietary restriction diets and monitored for lifespan. Interestingly, researchers observed a 2-3 fold difference in median lifespan under AL conditions and a 6-10 fold variation in DR strains (Liao et al 2010). Importantly, DR actually shortened lifespan in more strains than those for which it increased lifespan. This study details the important contribution of genetic and epigenetic variation resulting from selective inbreeding to both normal lifespan (AL) and organism response to experimental manipulations such as DR. Dietary restriction drastically alters the life history of an animal, and is likely able to lengthen or shorten lifespan based on how genetic variation interacts with specific environmental situations. As previously noted, husbandry conditions can have large effects on laboratory organism lifespan, and manipulations like DR or oxidative stress induction, designed to affect stress response, will likely result in even more divergent strain specific effects. Different

genetic backgrounds and rearing conditions will likely change which cellular and organism stresses are most significantly associated with age associated mortality, and must be strongly considered in any analysis of lifespan, stress response and the characterization of extended longevity phenotypes.

Our data in GFP expressing animals show that it is possible to measure significant changes in oxidative stress response with different chromosomal combinations in otherwise genetically isogenous individuals While we have shown that this increase may be attributed to experimental variance, it is nonetheless replicable in independent experimental trials, and may result from genetic or epigenetic modifications inherent to the *Drosophila* stock maintenance technique of breeding small isolated populations over several generations. Our previous and current data has shown that our background w1118 stock is deficient in both longevity and ROS response versus longer lived wild type strains. It is therefore reasonable to assume that any manipulation that increases oxidative stress resistance will play a larger role in augmenting the lifespan of w1118 flies due to their intrinsic susceptibility to this form of stress. Our Sir2 and DNAAM data show that there are at least two pathways for increased longevity in this strain, which may both result from the predisposed susceptibility of these animals to different forms of stress that contribute to increased age dependant mortality. We thus hypothesize that the correlation between stress resistance and mortality is intimately tied to the differences in genetic background between diverse strains of the same organism. Thus different genes and pathways will have profound differences in their ability to modify lifespan and stress response depending on the strain- specific susceptibility to various types of age associated damage.

We hypothesize that the spectrum of genetic backgrounds in *Drosophila* (and all organisms) ranges from stress sensitive to resistant for a variety of potentially lethal stressors. Thus measurement of lifespan and stress response will unveil complex interactions between specific gene pathways and their ability to ameliorative stress induced mortality and/or increase lifespan that will vary as a result of genetic variation. Genetic mutations and epigenetic variation that becomes fixed in interbred laboratory strains has and will likely continue to mask or obscure the fidelity of many proposed longevity genes. It is of paramount importance for the field of aging research to begin implementation of stringent strain maintenance and husbandry paradigms that can detect real gene dependant effects with the most reliable efficacy and reproducibility. Furthermore, laboratories should begin to utilize multiple strains when testing for the effect of single genetic elements on complex phenotypes such as aging and oxidative stress response.

Our research and that of other laboratories has shown that Sir2 and DNAAM can regulate lifespan in *Drosophila*, and can increase longevity when artificially expressed, possibly through the ability to mimic the effects of dietary restriction. We have shown that the contribution of oxidative stress resistance to enhanced longevity differs with regards to Sir2 or DNAAM transgene expression, providing evidence that pathways outside of those hypothesized by the ROS theory are important for longevity regulation. It remains to be determined how nicotinamidase activation or activation of other nicotinamide modulating enzymes, such as Nampt and Nmnat, integrate the effects of NAM on lifespan and stress response in Drosophila, and if they modulate lifespan through Sir2 or through other pathways or both. Finally, we have

shown that small but significant effects on oxidative stress response can be elicited by so-called "inert" transgenic elements, and thus transgenic protocols and strain interbreeding can change stress response in otherwise genetically isogenous animals. Aging theory must accommodate for diverse etiologies of mortality that are dependent upon both the background genetics of the same organism and the diversity in life histories of different organisms. Such an effort will be necessary to identify regulators of human senescence and produce viable targets for the prevention of age associated disease. While the study of individual genes through mutation or over-expression is a useful tool to identify effectors of longevity and stress response, it is becoming clear that results can vary greatly between organisms, genetic backgrounds, and laboratory conditions.

In light of these findings, it is our opinion that there should be a renewed interest in using forward genetics to reveal conserved mechanisms that underlie longevity in model organisms. *Drosophila* would be an ideal model for this line of research, as there are dozens of available strains of both wild type and mutant origin that have been previously used in longevity and stress response experiments. We propose using selection for longevity and various forms of stress resistance on several diverse strains to select for populations that are long lived or stress resistant versus their progenitor, which should occur via the same or similar mechanisms that are responsible for strain specific effects. Because of the relatively short generation time and well established protocols for stress induction and longevity determination flies are well suited a rapid matriculation through this process. Once generated, it would be possible to compare long lived or stress resistant animals with their progenitor strain to

examine the etiology of the phenotype for each strain. Overlap between different strains in longevity or stress response elements would indicate a conserved pathway that has a high probability of relevancy to these phenotypes, while elements that are modulated in some, but not other strains would indicate pathways that mediate strain specific responses. Both of these subsets would be useful in determining the efficacy of gene dependant results in other model organisms. In order to explore the connection between stress response and longevity, long lived animals would be monitored for increased stress resistance, and stress resistant animals would be monitored for lifespan effects. This would allow for both the identification of which stress pathways are involved in longevity in all genetic backgrounds, or which contribute to strain specific lifespan regulation. This would assist in the determination of how genetic background interacts with the complex phenotype of aging, and provide targets for further study in vertebrates that have a high probability of successful longevity enhancement and/or conserved pathways of stress response.

Tellos

Appendix A: Table from Chapter 1

Table 1. Effects of Selection for PQ Resistance on the Adult Survival Characteristics of the Population

Strain & Gen.	Selected For	PQ Resistance LT_{50} (h)*	% Early Survival†	Life Span (d) Mean	Median	Max.	*n*
Ra, F138	nonselected con.	50.9 ± 1.3	91	41	42	77	250
La F75	long-lived con.	83.1 ± 1.3	95	80	73	109	240
PQR F5	PQ resist.	89.6 ± 2.6	96	41	39	73	455
PQR F7	PQ resist.	104.8 ± 2.9	97	42	40	61	99
PQR F16	PQ resist.	149.4 ± 2.8	97	47	46	77	482
PQR F23	PQ resist.	155.2 ± 5.6	95	47	46	79	874

Note: PQ = paraquat; LT_{50} = mean lethal time.

*PQ resistance is mean ± standard error of the mean.

†As measured at day 20.

Appendix B: Figures from Chapter 2

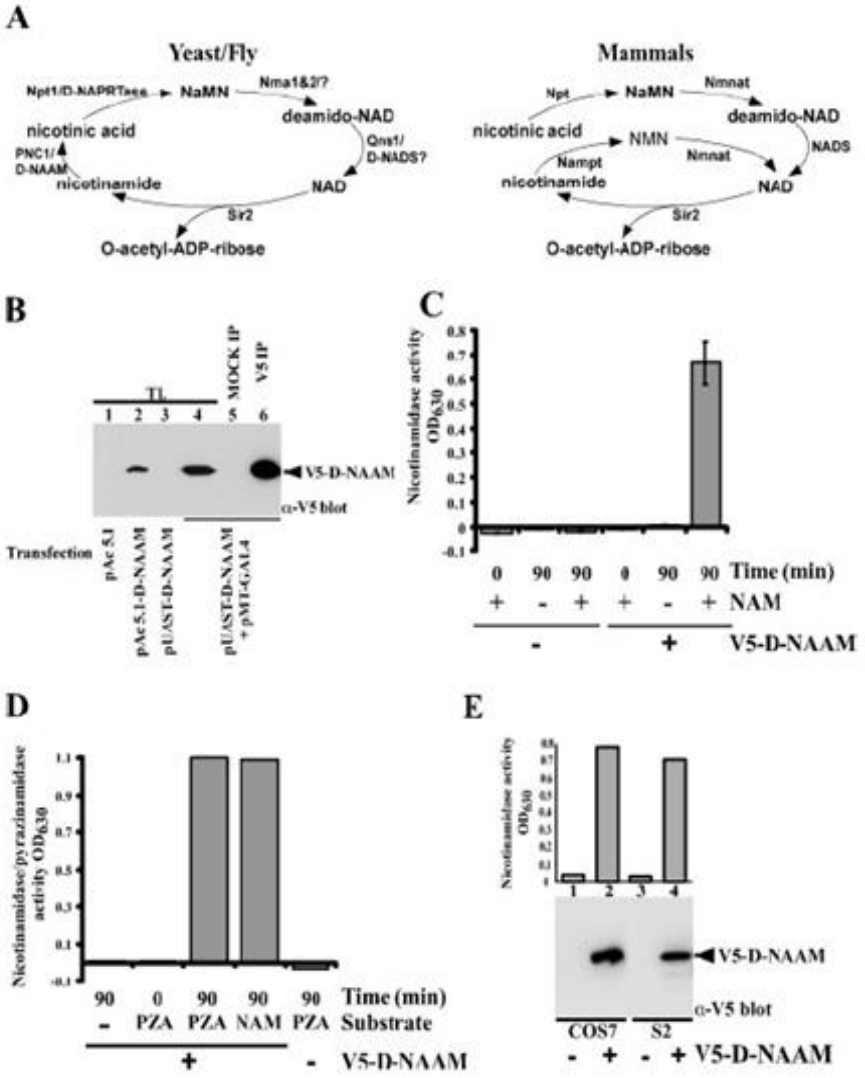


FIGURE 1. **The *Drosophila* homolog of yeast PNC1 encodes an active nicotinamidase.** *A*, comparison of the NAD^+ salvage pathway between mammals and yeast/flies. The nomenclature used is as in Rongvaux *et al.* (9) and Revollo *et al.* (8): *PNC1*, nicotinamidase; *Npt1*, nicotinic acid phosphoribosyltransferase; *Nma1&2*, nicotinic acid mononucleotide adenylyltransferase 1 and 2; *Qns1*, NAD synthetase; *Nmnat*, nicotinic acid mononucleotide adenylyltransferase; *NADS*, NAD synthetase; *NaMN*, nicotinic acid mononucleotide; *NMN*, nicotinamide mononucleotide. D-NAAM is our designation for *Drosophila* nicotinamidase. *B*, *Drosophila* S2 cells were transfected with carboxyl-terminally tagged V5-His-D-NAAM using the indicated expression vectors, and D-NAAM expression was determined in whole cell lysates (*TL*) or in V5 immunoprecipitates by V5 immunoblotting. *C* and *D*, V5 immunoprecipitates from S2 cells expressing a control vector or pAc 5.1-V5-His-D-NAAM were assayed for amidase activity using nicotinamide (*NAM*, *C*) or pyrazinamide (*PZA*, *D*) as a substrate. The activity is provided in A_{630} units corresponding to released ammonia. *E*, *Drosophila* S2 and mammalian COS-7 cells were transfected with control or pAc 5.1-V5-His-D-NAAM (S2 cells) or pExchange 5A-V5-His-D-NAAM (COS-7 cells) and nicotinamidase activity in V5 immunoprecipitates was analyzed as in *C* (*top panel*). D-NAAM recovery was determined using V5 immunoblotting (*bottom panel*).

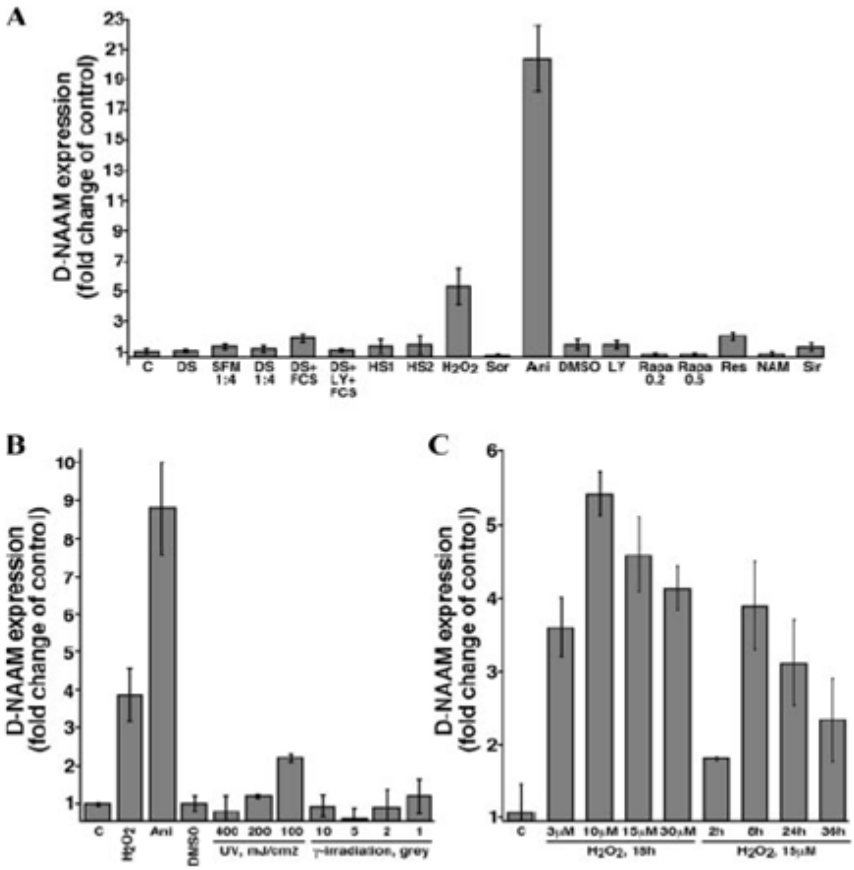


FIGURE 2. **D-NAAM mRNA expression is regulated by oxidative stress.** *A*, *Drosophila* S2 cells were cultured under the indicated growth conditions, and the expression of D-NAAM mRNA was analyzed using real time PCR. The data are presented as fold change in D-NAAM expression after standardizing to ribosomal protein L32. The treatments were as follows: *C*, control, complete SFM media; *DS*, DS2 media (media lacking protein factor additives); *SFM 1:4*, SFM medium diluted 1:4 with phosphate-buffered saline (nutrient deficient media), 18 h; *DS 1:4*, DS medium diluted 1:4 with phosphate-buffered saline, 18 h; *FCS*, 10% fetal calf serum, 4 h; *LY*, 10 μM LY294002, 18 h (PI3K inhibitor); *HS1*, heat shock, 2 h at 37 °C and 24 h recovery; *HS2*, heat shock, 2 h at 37 °C; *H_2O_2*, 15 μM, 18 h; *Sor*, 0.5 M sorbitol, 18 h; *Ani*, 10 μg/ml anisomycin, 18 h; *DMSO*, 0.1% Me_2SO, 18 h (control); *Rapa*, rapamycin, 0.2 or 0.5 μg/ml, 18 h; *Res*, 100 μM resveratrol, 18 h; *NAM*, 40 mM nicotinamide, 18 h; *Sir*, 50 μM sirtinol, 18 h. *B*, *Drosophila* S2 cells growing in complete SFM medium were treated as indicated or cells were irradiated and left to recover for 24 h. The cells were analyzed for changes in D-NAAM mRNA expression as in *A*. *C*, *Drosophila* S2 cells were treated with the indicated concentrations of H_2O_2 for the indicated times, and the D-NAAM mRNA levels were analyzed as in *A*.

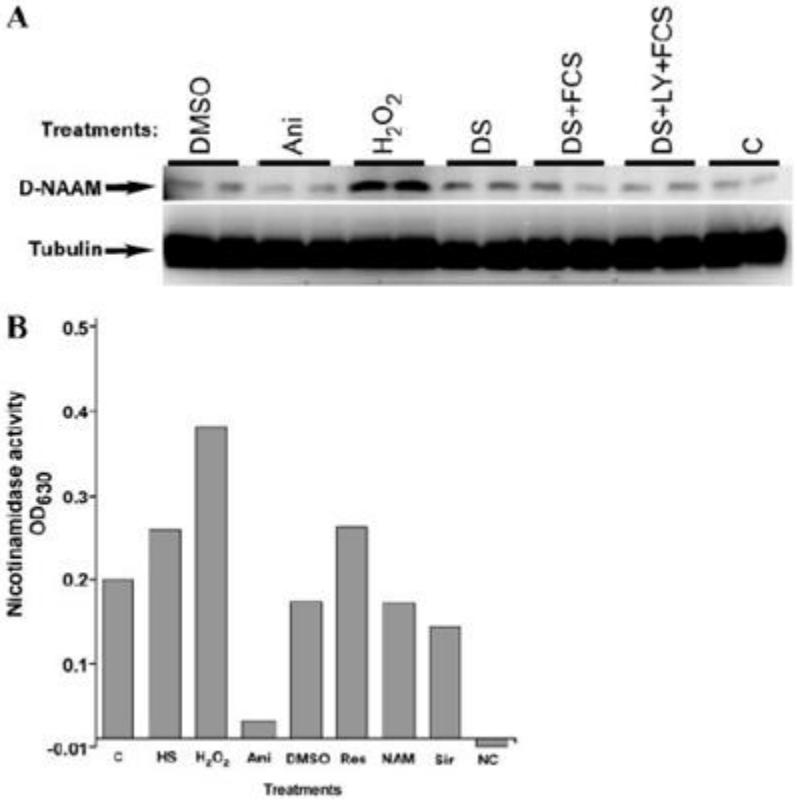


FIGURE 3. **Oxidative stress enhances D-NAAM protein expression and activity.** *A*, *Drosophila* S2 cells were cultured under the indicted growth condition (as in Fig. 2*A*) for 18 h, and total cell lysates were analyzed for D-NAAM protein expression using a carboxyl-terminal D-NAAM peptide antibody (*top panel*). Tubulin immunoblotting was used to confirm protein loading (*bottom panel*). Cell treatments and the protein expression analysis were performed in duplicate. *DMSO*, dimethyl sulfoxide. *B*, D-NAAM was immunopurified from S2 cells cultured under the indicated conditions using the carboxyl-terminal D-NAAM antibody and assayed for nicotinamidase activity as in Fig. 1. *NC*, negative control, no substrate (NAM) was added in the nicotinamidase assay. The experiment was performed in duplicate and is representative of three independent experiments. The treatments were as follows: *C*, control; *HS*, heat shock, 2 h at 37 °C; *H_2O_2*, 15 μM, 18 h; *Ani*, 10 μg/ml anisomycin, 18 h; *DMSO*, 0.1% Me_2SO, 18 h (control); *Res*, 100 μM resveratrol, 18 h; *NAM*, 40 mM nicotinamide, 18 h; *Sir*, 50 μM sirtinol, 18 h.

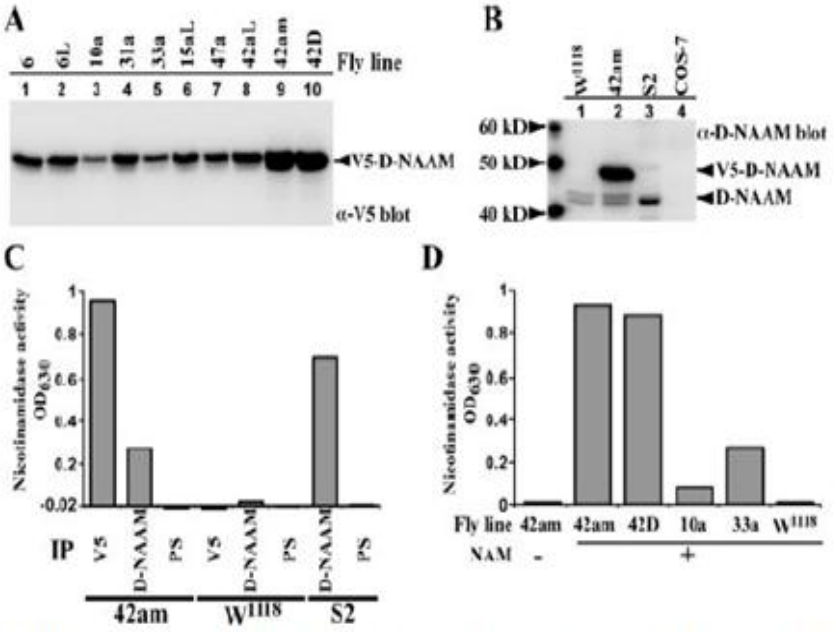


FIGURE 4. **Increased nicotinamidase activity in transgenic *Drosophila* lines overexpressing D-NAAM.** *A*, expression of V5-D-NAAM protein in pUAST-D-NAAM transgenic lines in tubulin-Gal4 driver background was analyzed using V5 immunoblotting. *B*, D-NAAM protein expression in control w^{1118} flies, a high expressing D-NAAM transgenic line 42am (tubulin-Gal4 driver), S2 cells, and control COS-7 cells were analyzed using a carboxyl-terminal D-NAAM antibody. *C* and *D*, nicotinamidase activity in protein extracts of the indicated fly lines or S2 cells was analyzed by immunoprecipitating (*IP*) D-NAAM using V5 or a carboxyl-terminal D-NAAM antibody and assaying amidase activity as in Fig. 2*B*. Preimmune serum for the D-NAAM antibody (*PS*) was used as a control antibody. The difference seen in activity between V5 and D-NAAM immunoprecipitates reflects the difference in immunoprecipitation efficiencies (data not shown). Note that the low activity seen in the w^{1118} sample is a result of assay linearity limitations (the total amount of protein to be used for the immunoprecipitation was determined in a way to allow remaining in the linear range of the assay for the high expressing D-NAAM lines). *NAM*, nicotinamide.

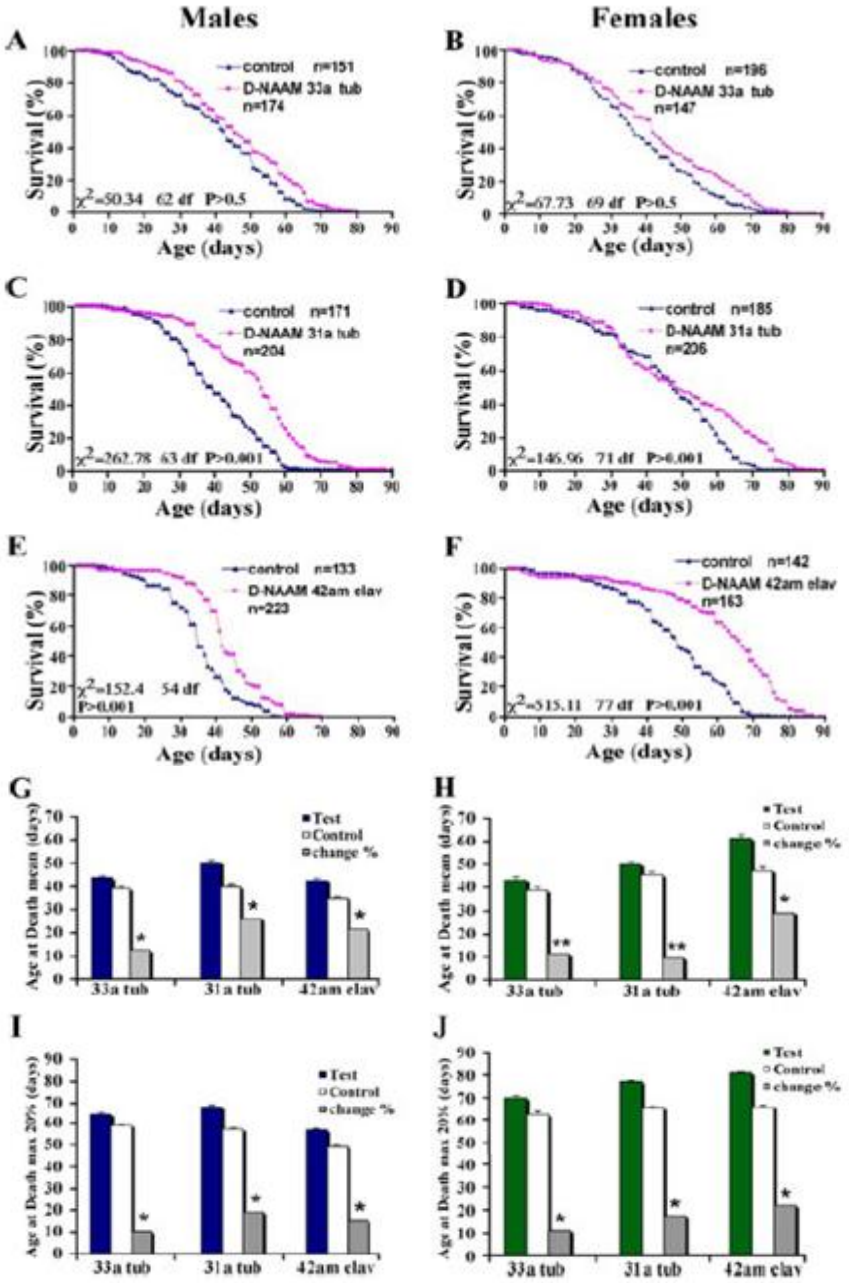


FIGURE 5. **D-NAAM overexpression extends *Drosophila* life span.** *A–F*, survival curves for male (*A*, *C*, and *E*) and female (*B*, *D*, and *F*) adult flies expressing low (D-NAAM^{33}; *A* and *B*) or middle (D-NAAM^{31}; *C* and *D*) D-NAAM protein levels ubiquitously via the tubulin-Gal4 driver or of a high expressing line expressing D-NAAM strictly in neurons via the *elav*-Gal4 driver (D-NAAM^{42}; *E* and *F*; see Fig. 4*A* for D-NAAM expression levels in the specific lines). A genetically matching control line was used for each test line. Indicated are the *n* values for each experiment and the statistical parameter values. See supplemental Table S2 for the complete longevity analysis. *G–J*, mean (*G* and *H*) and the maximal life span of the top 20% survivors of test and control flies (*I* and *J*). The *gray bars* represent the percentage of change between the matching control and the test line for each pair. The *single asterisk* denotes a *p* value <0.005, and *two asterisks* denote a *p* value <0.05.

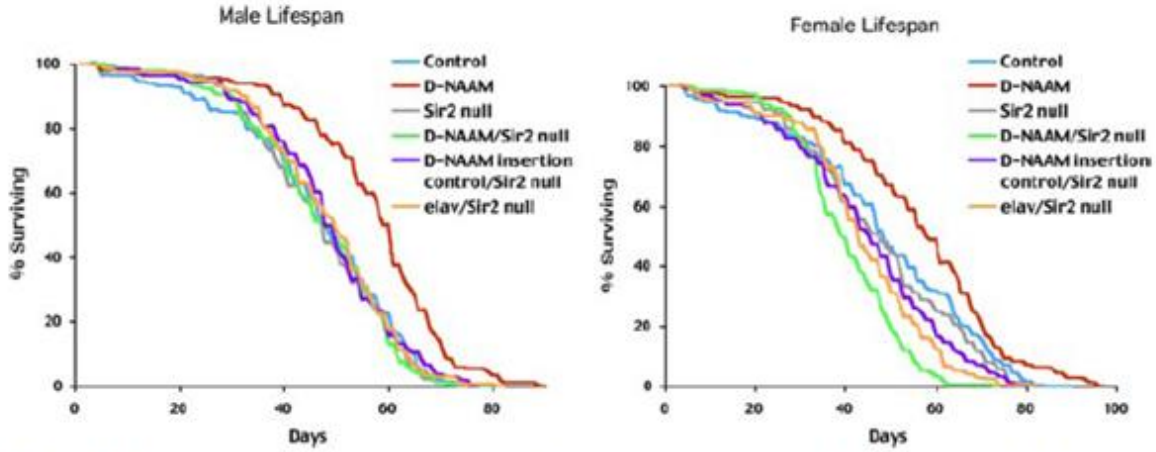


FIGURE 6. **D-NAAM-induced longevity requires Sir2.** Survival curves of male (*left panel*) and female (*right panel*) adult flies expressing D-NAAM^{42} using the *elav*-Gal4 driver in control and Sir2 null background. Genotypes: wild type control: w^{1118}; D-NAAM overexpression: w^{1118};UAS-D-NAAM^{42}/*elav*-Gal4; Sir2 null: w^{1118}; Sir2$^{4.5}$/Sir2$^{5.26}$; D-NAAM overexpression in Sir2 null: w^{1118};Sir2$^{4.5}$/Sir2$^{5.26}$;UAS-D-NAAM^{42}/*elav*-Gal4; D-NAAM insertion control in Sir2 null: w^{1118}; Sir2$^{4.5}$/Sir2$^{5.26}$;UAS-D-NAAM^{42}/+; *elav*-Gal4 insertion control in Sir2 null: w^{1118};Sir2$^{4.5}$/Sir2$^{5.26}$;*elav*-Gal4/+.

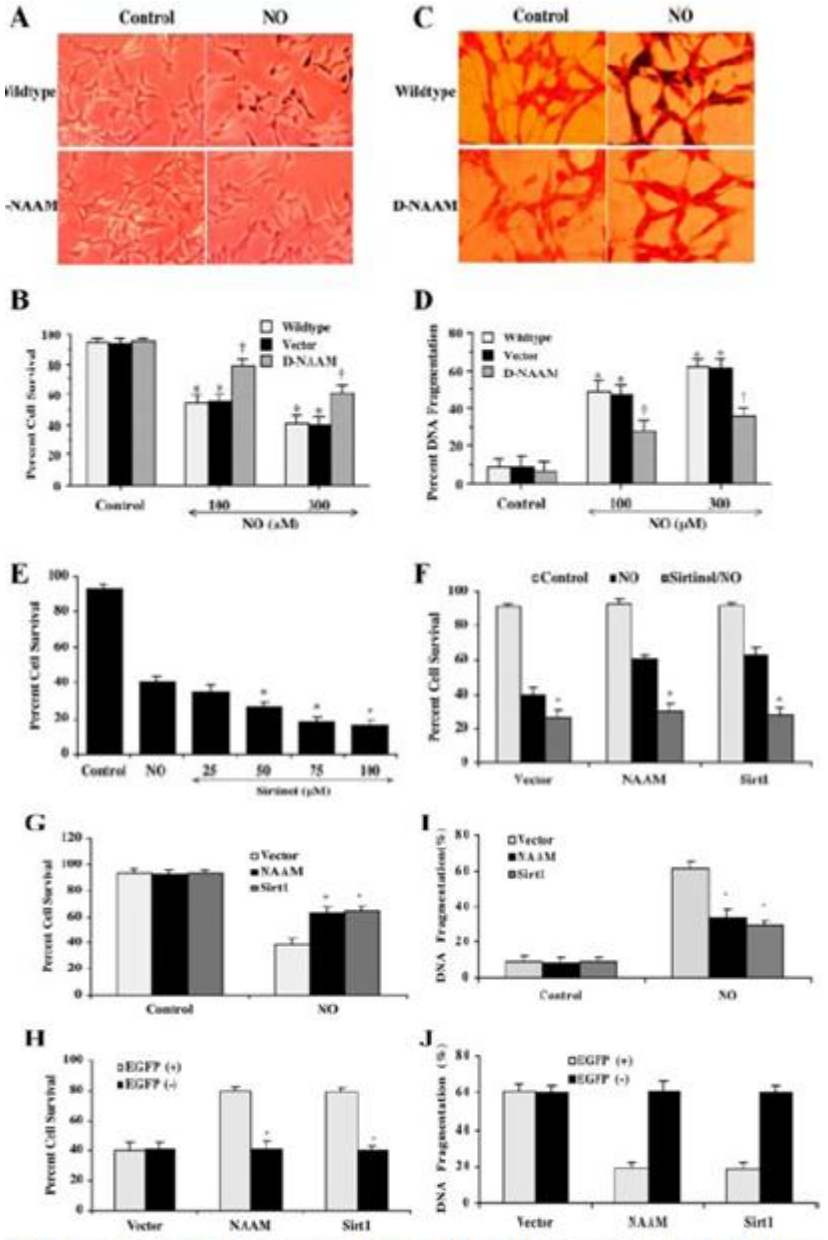


FIGURE 7. **D-NAAM expression protects human neuronal cells from oxidative stress-induced cell death.** *A–D*, SH-SY5Y cells transfected with control vector or D-NAAM or nontransfected cells were exposed for 24 h to 100 or 300 μM NOC-9 and analyzed for cell death using trypan blue exclusion (*A* and *B*) or assayed for apoptotic cell death using deoxynucleotidyltransferase-mediated dUTP nick end labeling assay (*C* and *D*). For quantification of cell death and DNA fragmentation, on average, 200 cells were counted in triplicate samples (*B* and *D*). † denotes *p* value <0.01, and *asterisks* denote statistically nonsignificant difference between nontransfected and vector-transfected cells. *E* and *F*, control SH-SY5Y cells (*E*) or cells expressing EGFP control vector, EGFP-D-NAAM or EGFP-Sirt1 (*F*) were treated with the indicated concentrations of sirtinol (*E*) or 50 μM sirtinol (*F*) 1 h prior to exposure to 300 μM NOC-9 and cell survival was determined 24 h later as in *B*. *G–J*, SH-SY5Y cells were transfected with EGFP control vector, EGFP-D-NAAM, or EGFP-Sirt1; cell survival in response to 300 μM NOC-9 was analyzed as in *B* (*G*), and apoptotic cell death was analyzed as in *D* (*I*). Alternatively, EGFP positive and negative cells were analyzed separately (*H* and *J*). The *asterisks* denote *p* value <0.01.

Appendix C: Figures from Chapter 3

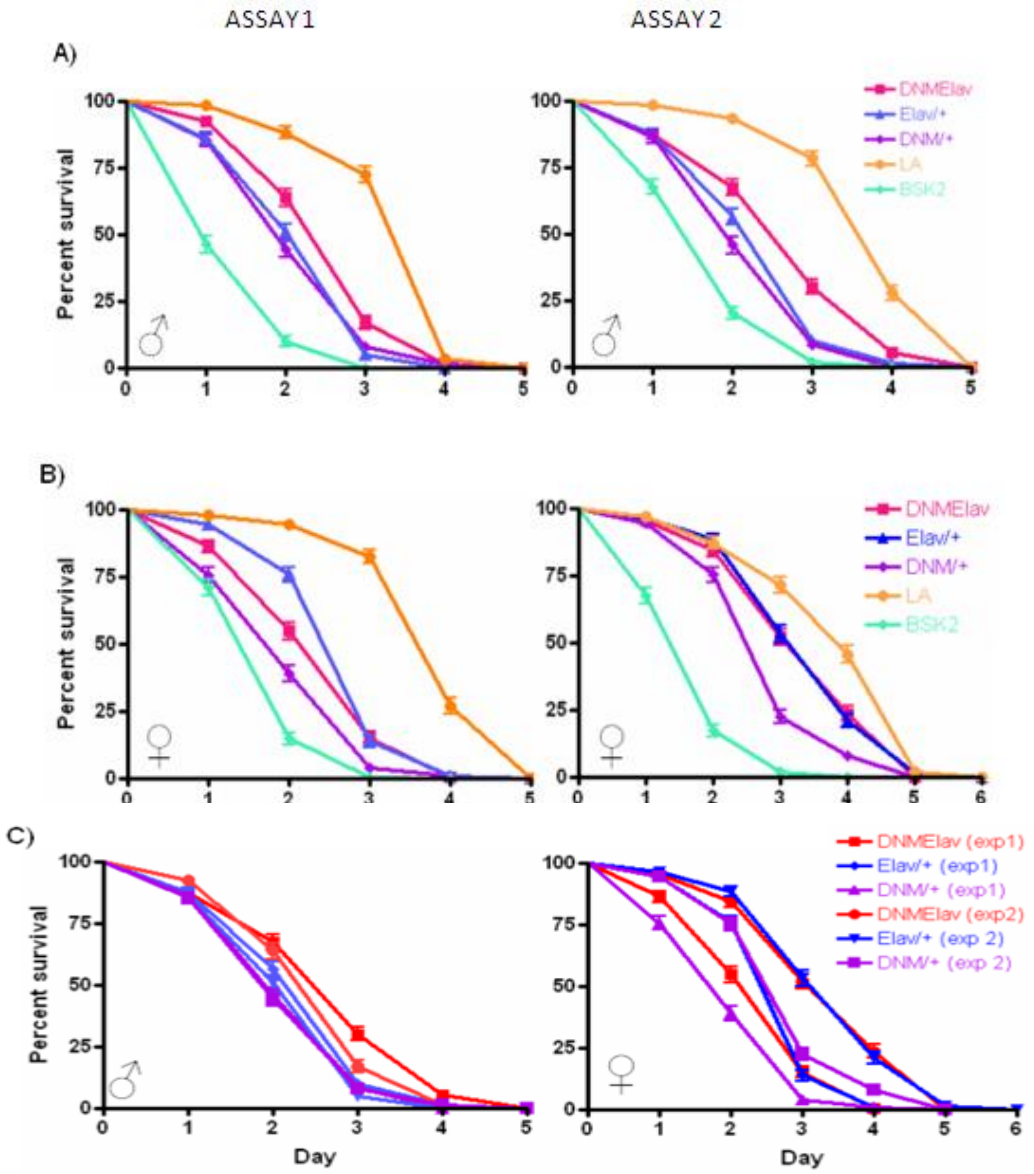


Fig 1. *DNAAM* nervous system expression results in a small but significant increase in paraquat resistance in males (a) but not females (b) in each individual experiment. In figs A and B results of the first assay are shown on the left, and results of the second assay are shown on the right. In all assays, La acts as our positive control (oxidative stress resistant), while bsk2 is our negative control (oxidative stress susceptible). Importantly, lifespan of *DNAAM* expression and heterozygous controls are always in between that of La and *bsk2* flies. When the DNAAM experimental data of both experiments are superimposed, we can see the variation between replicates that may account for small changes in survival (c), After careful analysis of experimental variance we believe these small changes are likely not gene dependent, but instead are the result of normal variance and genetic background.

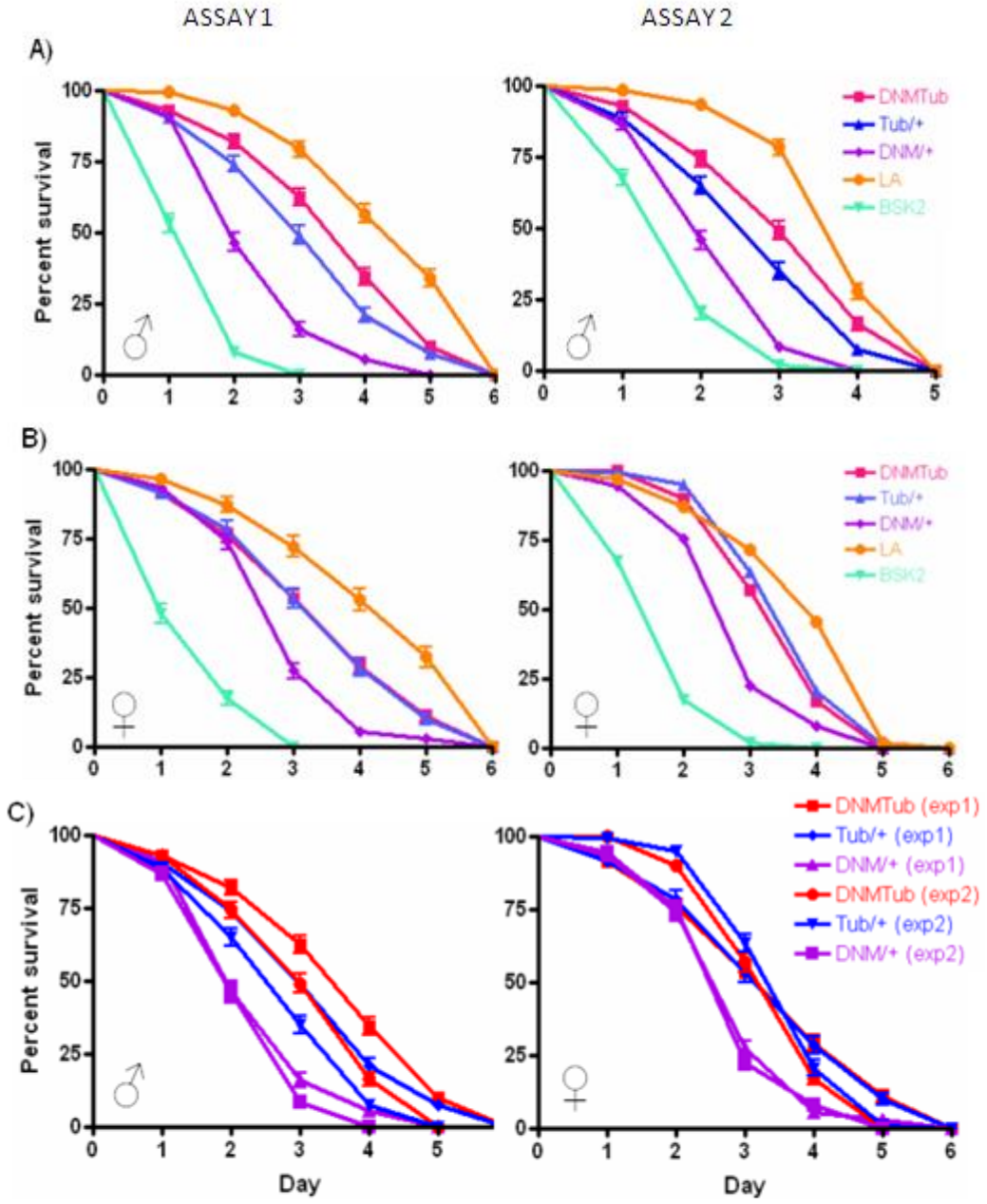


Fig 2. Ubiquitous expression of *DNAAM* results in a similar increase in male lifespan on dietary paraquat in individual experiments as was observed in nervous system expression (a), yet has no effect on female lifespan (b). As with *DNAAM* nervous system expression, lifespan of ubiquitous expressed *DNAAM* and its controls is in between the positive and negative controls La and *bsk2*. Superimposition of the two experimental data sets shows that in male flies (left panel) the driver control (*tub/+)* varies and is not always significantly different from *DNAAM* over-expression. In females, the variation in controls is smaller, and *DNAAM* over-expression is not significantly different from the *tub/+* controls (c, right panel).

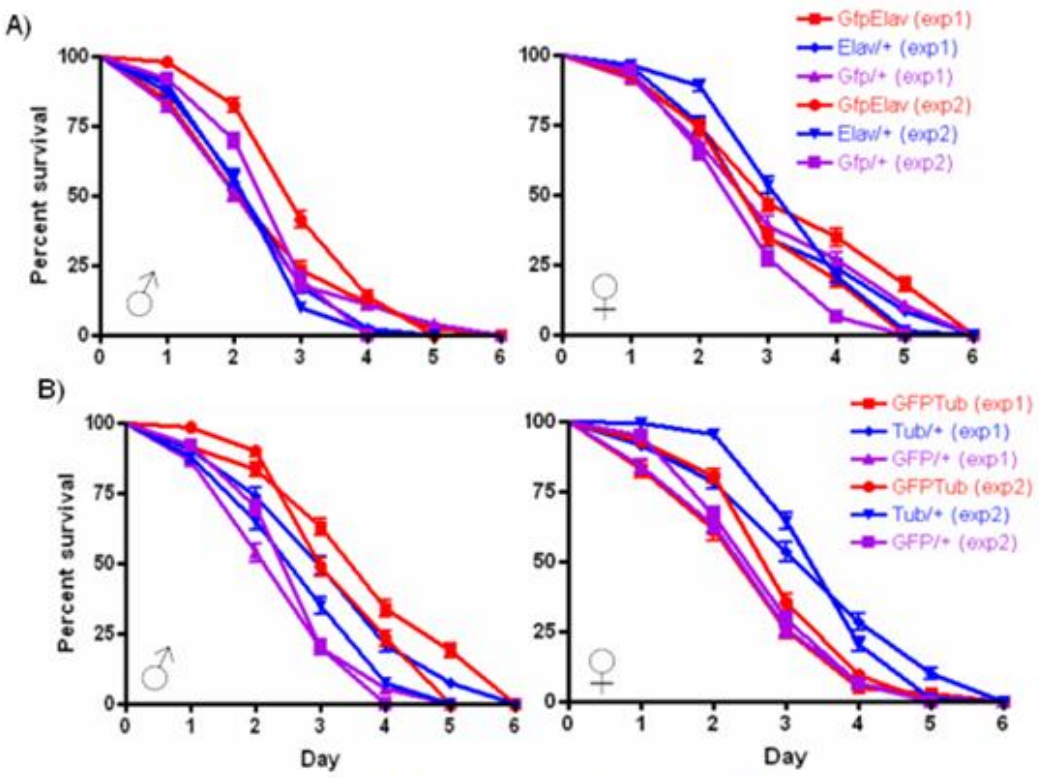


Fig 3. Nervous system expression of *GFP* (a) does not result in a consistent increase in paraquat resistance in either males (left panel) or females (right panel). Ubiquitous *GFP* expression did result in a significant increase in paraquat resistance in males in individual experiments (see supplemental data), however as shown by superimposition of the two experiments the *tub/+* control is not always significantly different from *GFP* expression (b, left panel). Ubiquitous *GFP* expression in females does not increase paraquat resistance (b, right panel). These results help us interpret the data observed for *DNAAM* over-expression (fig 1,2).

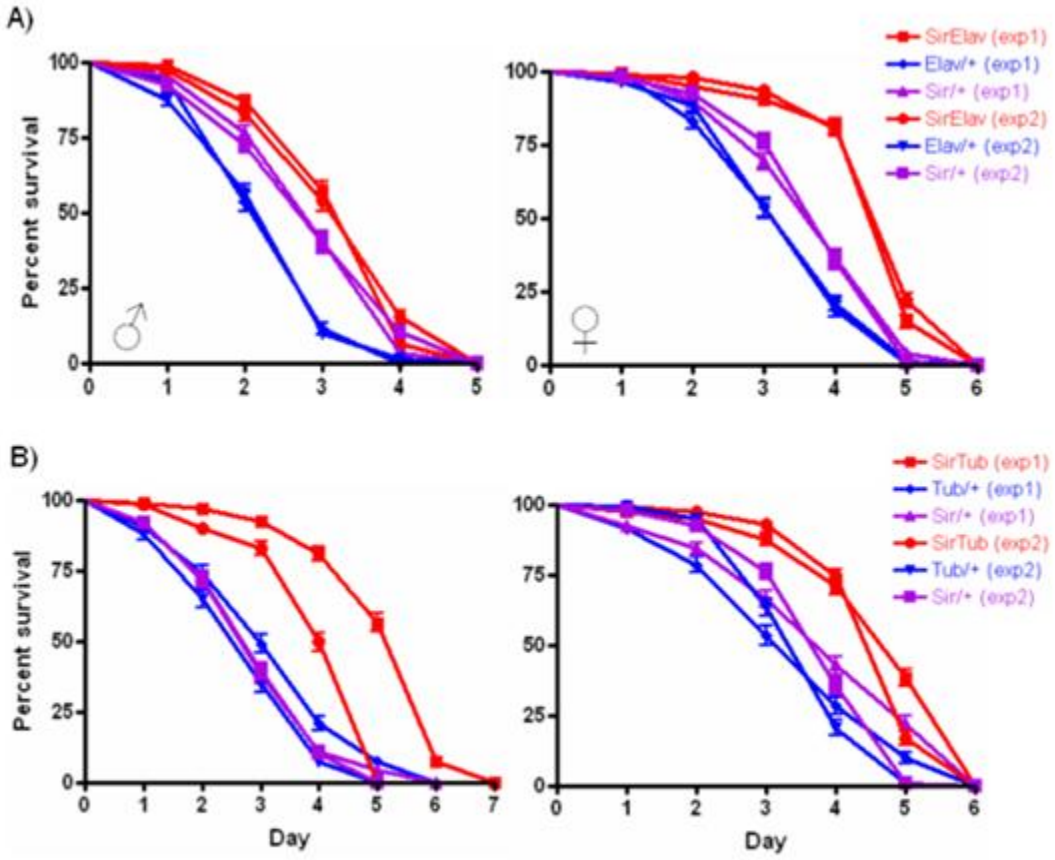


Fig 4. When *Sir2* is expressed in the nervous system, male flies exhibit a consistent increase in paraquat resistance that is always greater than the two controls (a, left panel) but may not be gene dependant. In females, *Sir2* nervous system expression results in a reproducible increase in paraquat resistance. Ubiquitous *Sir2* expression results in a large and reproducible increase in paraquat resistance in both males and females (b).

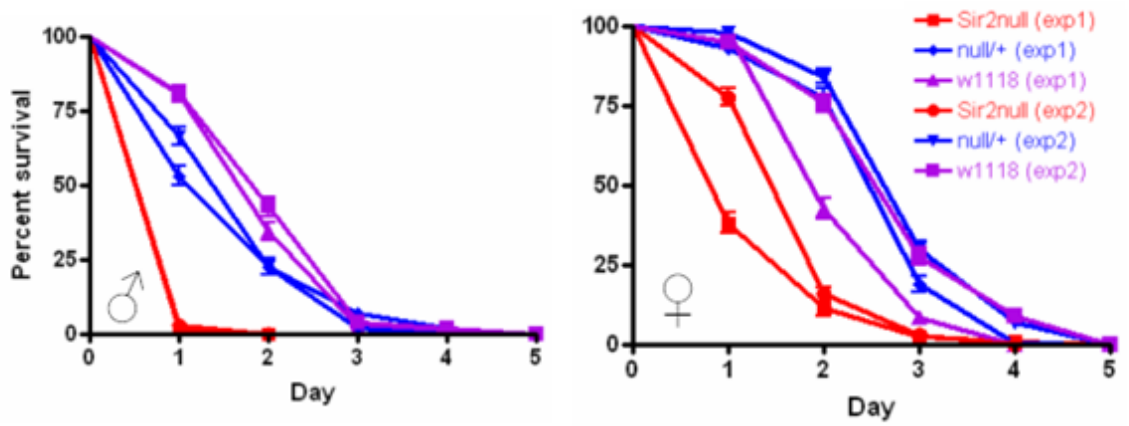


Fig5. ***Sir2*** **null** ***Drosophila*** **exhibit an increased sensitivity to paraquat induced oxidative stress. A. When** ***Sir2*** **is mutated, male flies perish at an extremely high rate compared with control animals. Male sensitivity to paraquat is more severe than our negative control** ***bsk*²** **flies (see supp data). B. Female flies exhibit a similar profound decrease in paraquat resistance.**

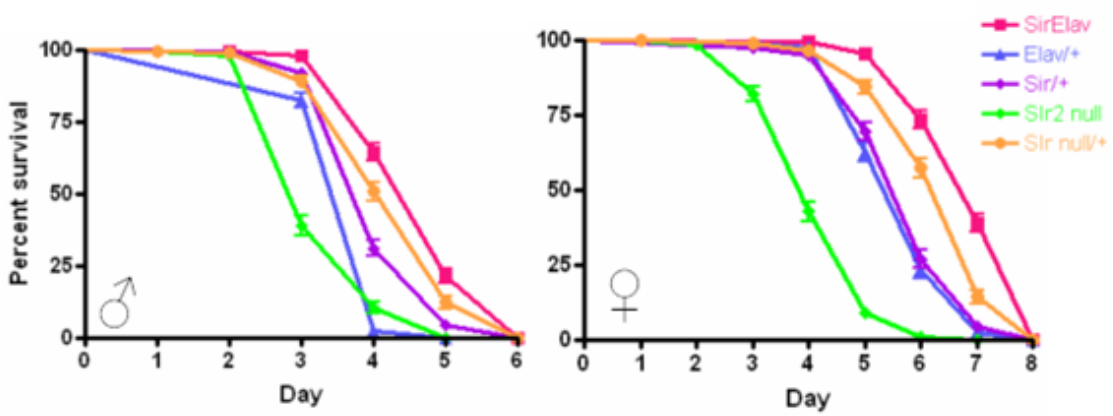
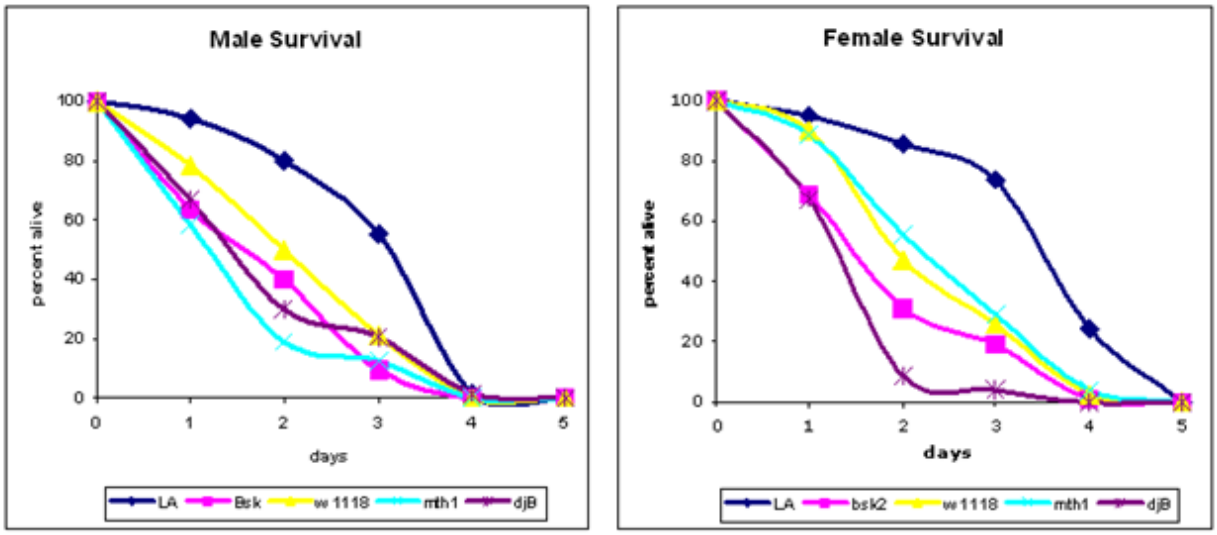
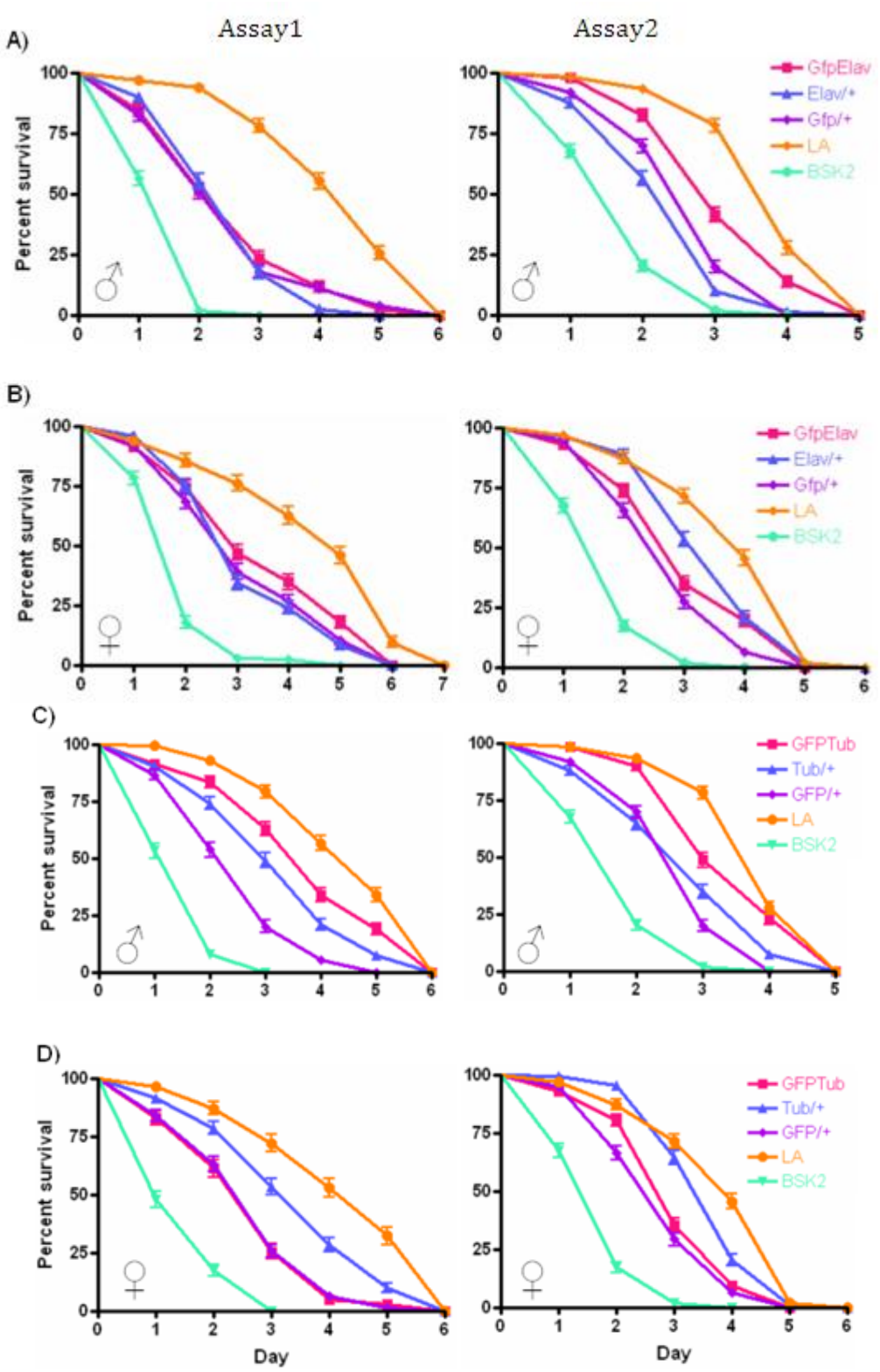


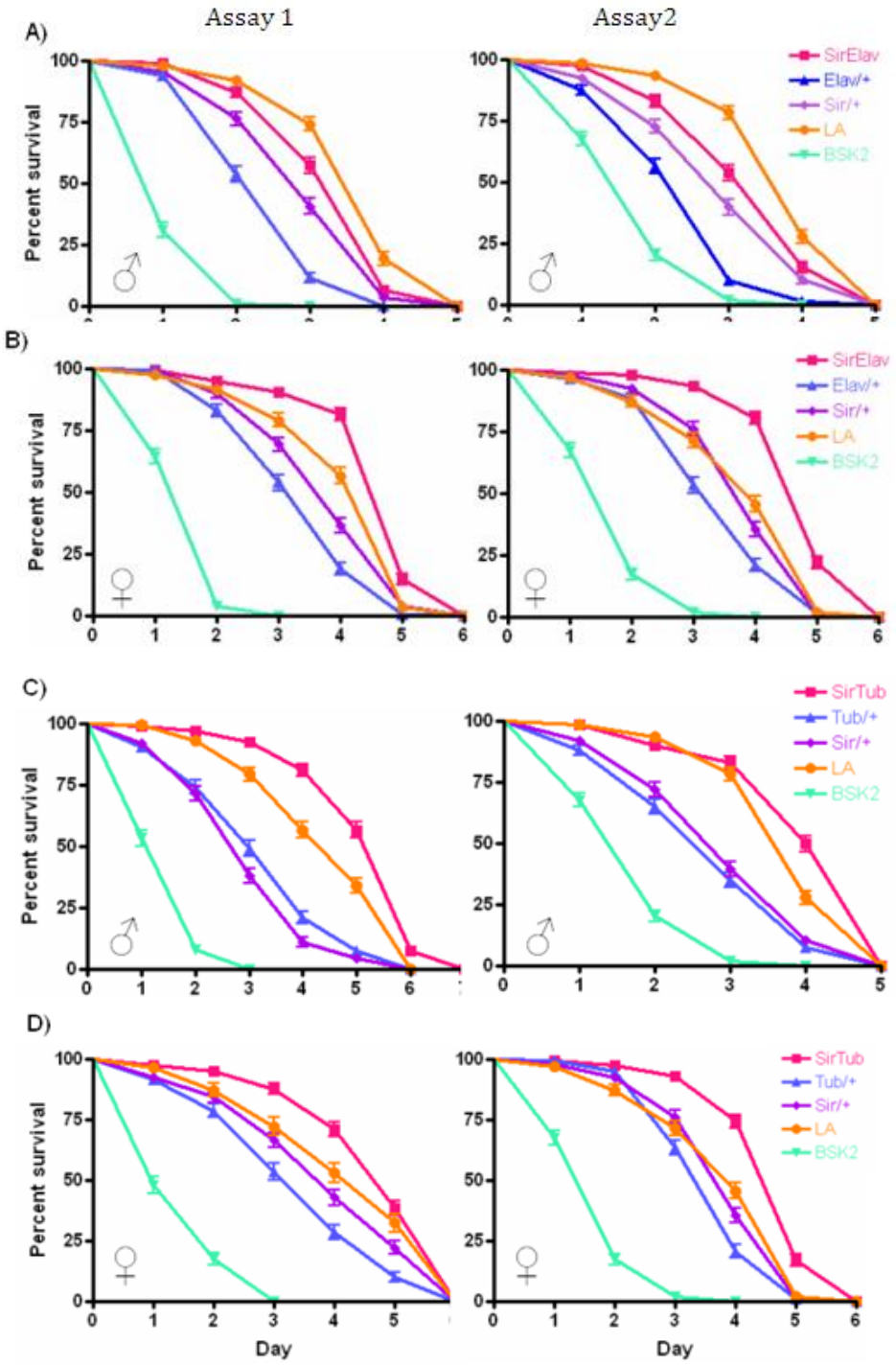
Fig 6. ***Sir2*** **is involved in hydrogen peroxide tolerance. Male (right) and female (left) flies that over-express Sir2 have an increased tolerance to hydrogen peroxide versus their respective controls. Mutation of** ***Sir2*** **results in a reduced tolerance in both sexes (green line).**



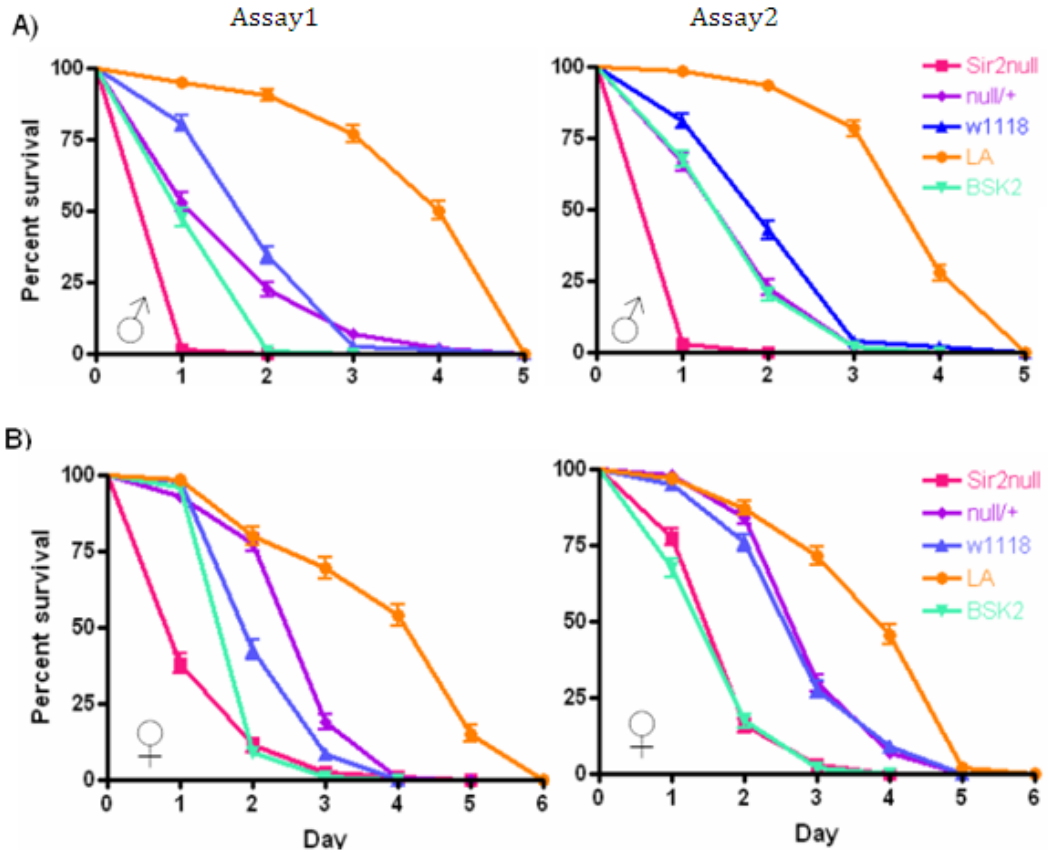
Supplemental Figure 1: The La strain was chosen as the positive control (blue line) because in both sexes it exhibited a much greater resistance to 15mM paraquat than w^{1118} (yellow line). Both *bsk2* (pink line) and *djß* (purple line) were more susceptible to paraquat than w^{1118}, however, due to a higher fecundity *bsk2* was chosen as the negative control.



Supplemental figure 2. A, B) *GFP elav* males and females do not show a consistent increase in paraquat resistance, perhaps due to replicate control variance. C, D) Male *GFP tub* flies do show resistance to paraquat in independent experiments, however when replicates are overlapped, this increase is not always significantly different from controls. D) Female *GFP tub* flies show no increase in paraquat resistance.



Supplemental figure 3. **A) *Sir2 elav* males show a small increase in paraquat resistance that is maintained in both replicates and when replicates are overlapped, however due to results with *DNAAM* and *GFP* this may not be a gne dependent effect (see text). B) Female *Sir2* flies exhibit a strong reproducible resistance to paraquat, because this is not observed in other experiments this is probably a gene dependant effect. C, D) Male and Female *Sir2 tub* flies exhibit a strong reproducible resistance to paraquat.**



Supplemental figure 4. A, B) When *Sir2* is mutated, both sexes show a severe reduction in their ability to tolerate dietary paraquat. This is highlighted by the fact that male flies (A) are more susceptible than the *bsk2* paraquat sensitive control, and female flies (B) either more susceptible or not significantly different from the *bsk2* paraquat sensitive control.

REFERENCES

Abdulwahid Arif, I., and Ahmad Khan, H. (2010). Environmental toxins and Parkinson's disease: putative roles of impaired electron transport chain and oxidative stress. Toxicol Ind Health *26*, 121-128.

Anderson, R.M., Bitterman, K.J., Wood, J.G., Medvedik, O., Cohen, H., Lin, S.S., Manchester, J.K. Gordon, J.I., and Sinclair, D.A. (2002). Manipulation of a nuclear NAD+ salvage pathway delays aging without altering steady-state NAD+ levels. J Biol Chem *277*, 18881-18890.

Anderson, R.M., Bitterman, K.J., Wood, J.G., Medvedik, O., and Sinclair, D.A. (2003). Nicotinamide and PNC1 govern lifespan extension by calorie restriction in Saccharomyces cerevisiae. Nature *423*, 181-185.

Anekonda, T.S., and Reddy, P.H. (2006). Neuronal protection by sirtuins in Alzheimer's disease. J Neurochem *96*, 305-313.

Araki, T., Sasaki, Y., and Milbrandt, J. (2004). Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. Science *305*, 1010-1013.

Arden, K.C. (2004). FoxO: linking new signaling pathways. Mol Cell *14*, 416-418.

Arking, R., Buck, S., Novoseltev, V.N., Hwangbo, D.S., and Lane, M. (2002). Genomic plasticity, energy allocations, and the extended longevity phenotypes of Drosophila. Ageing Res Rev *1*, 209-228.

Arking, R., Buck, S., Wells, R.A., and Pretzlaff, R. (1988). Metabolic rates in genetically based long lived strains of Drosophila. Exp Gerontol *23*, 59-76.

Arking, R., and Wells, R.A. (1990). Genetic alteration of normal aging processes is responsible for extended longevity in Drosophila. Dev Genet *11*, 141-148.

Astrom, S.U., Cline, T.W., and Rine, J. (2003). The Drosophila melanogaster sir2+ gene is nonessential and has only minor effects on position-effect variegation. Genetics *163*, 931-937.

Balan, V., Miller, G.S., Kaplun, L., Balan, K., Chong, Z.Z., Li, F., Kaplun, A., VanBerkum, M.F., Arking, R., Freeman, D.C.*, et al.* (2008). Life span extension and neuronal cell protection by Drosophila nicotinamidase. J Biol Chem *283*, 27810-27819.

Banks, A.S., Kon, N., Knight, C., Matsumoto, M., Gutierrez-Juarez, R., Rossetti, L., Gu, W., and Accili, D. (2008). SirT1 gain of function increases energy efficiency and prevents diabetes in mice. Cell Metab *8*, 333-341.

Barger, J.L., Kayo, T., Vann, J.M., Arias, E.B., Wang, J., Hacker, T.A., Wang, Y., Raederstorff, D., Morrow, J.D., Leeuwenburgh, C.*, et al.* (2008). A low dose of dietary resveratrol partially mimics caloric restriction and retards aging parameters in mice. PLoS One *3*, e2264.

Barja, G. (2002). Endogenous oxidative stress: relationship to aging, longevity and caloric restriction. Ageing Res Rev *1*, 397-411.

Barthel, A., Schmoll, D., and Unterman, T.G. (2005). FoxO proteins in insulin action and metabolism. Trends Endocrinol Metab *16*, 183-189.

Bass, T.M., Grandison, R.C., Wong, R., Martinez, P., Partridge, L., and Piper, M.D. (2007). Optimization of dietary restriction protocols in Drosophila. J Gerontol A Biol Sci Med Sci *62*, 1071-1081.

Bass, T.M., Weinkove, D., Houthoofd, K., Gems, D., and Partridge, L. (2007). Effects of resveratrol on lifespan in Drosophila melanogaster and Caenorhabditis elegans. Mech Ageing Dev *128*, 546-552.

Bauer, J.H., and Helfand, S.L. (2009). Sir2 and longevity: the p53 connection. Cell Cycle *8*, 1821.

Bauer, J.H., Morris, S.N., Chang, C., Flatt, T., Wood, J.G., and Helfand, S.L. (2009). dSir2 and Dmp53 interact to mediate aspects of CR-dependent lifespan extension in D. melanogaster. Aging (Albany NY) *1*, 38-48.

Bauer, J.H., Poon, P.C., Glatt-Deeley, H., Abrams, J.M., and Helfand, S.L. (2005). Neuronal expression of p53 dominant-negative proteins in adult Drosophila melanogaster extends life span. Curr Biol *15*, 2063-2068.

Baur, J.A., Chen, D., Chini, E.N., Chua, K., Cohen, H.Y., de Cabo, R., Deng, C., Dimmeler, S., Gius, D., Guarente, L.P.*, et al.* (2010). Dietary restriction: standing up for sirtuins. Science *329*, 1012-1013; author reply 1013-1014.

Baur, J.A., Pearson, K.J., Price, N.L., Jamieson, H.A., Lerin, C., Kalra, A., Prabhu, V.V., Allard, J.S., Lopez-Lluch, G., Lewis, K.*, et al.* (2006). Resveratrol improves health and survival of mice on a high-calorie diet. Nature *444*, 337-342.

Bayne, A.C., Mockett, R.J., Orr, W.C., and Sohal, R.S. (2005). Enhanced catabolism of mitochondrial superoxide/hydrogen peroxide and aging in transgenic Drosophila. Biochem J *391*, 277-284.

Belenky, P., Racette, F.G., Bogan, K.L., McClure, J.M., Smith, J.S., and Brenner, C. (2007). Nicotinamide riboside promotes Sir2 silencing and extends lifespan via Nrk and Urh1/Pnp1/Meu1 pathways to NAD+. Cell *129*, 473-484.

Bennett, B., Carosone-Link, P., Zahniser, N.R., and Johnson, T.E. (2006). Confirmation and fine mapping of ethanol sensitivity quantitative trait loci, and candidate gene testing in the LXS recombinant inbred mice. J Pharmacol Exp Ther *319*, 299-307.

Berdichevsky, A., Viswanathan, M., Horvitz, H.R., and Guarente, L. (2006). C. elegans SIR-2.1 interacts with 14-3-3 proteins to activate DAF-16 and extend life span. Cell *125*, 1165-1177.

Bhadra, U., Pal-Bhadra, M., and Birchler, J.A. (1997). A trans-acting modifier causing extensive overexpression of genes in Drosophila melanogaster. Mol Gen Genet *254*, 621-634.

Bishop, N.A., and Guarente, L. (2007). Two neurons mediate diet-restriction-induced longevity in C. elegans. Nature *447*, 545-549.

Bitterman, K.J., Anderson, R.M., Cohen, H.Y., Latorre-Esteves, M., and Sinclair, D.A. (2002). Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. J Biol Chem *277*, 45099-45107.

Blander, G., and Guarente, L. (2004). The Sir2 family of protein deacetylases. Annu Rev Biochem *73*, 417-435.

Bogan, K.L., and Brenner, C. (2008). Nicotinic acid, nicotinamide, and nicotinamide riboside: a molecular evaluation of NAD+ precursor vitamins in human nutrition. Annu Rev Nutr *28*, 115-130.

Boily, G., He, X.H., Pearce, B., Jardine, K., and McBurney, M.W. (2009). SirT1-null mice develop tumors at normal rates but are poorly protected by resveratrol. Oncogene *28*, 2882-2893.

Boily, G., Seifert, E.L., Bevilacqua, L., He, X.H., Sabourin, G., Estey, C., Moffat, C., Crawford, S., Saliba, S., Jardine, K.*, et al.* (2008). SirT1 regulates energy metabolism and response to caloric restriction in mice. PLoS One *3*, e1759.

Bokov, A., Chaudhuri, A., and Richardson, A. (2004). The role of oxidative damage and stress in aging. Mech Ageing Dev *125*, 811-826.

Bonilla, E., Medina-Leendertz, S., Villalobos, V., Molero, L., and Bohorquez, A. (2006). Paraquat-induced oxidative stress in Drosophila melanogaster: effects of melatonin, glutathione, serotonin, minocycline, lipoic acid and ascorbic acid. Neurochem Res *31*, 1425-1432.

Bordone, L., Cohen, D., Robinson, A., Motta, M.C., van Veen, E., Czopik, A., Steele, A.D., Crowe, H., Marmor, S., Luo, J.*, et al.* (2007). SIRT1 transgenic mice show phenotypes resembling calorie restriction. Aging Cell *6*, 759-767.

Bordone, L., and Guarente, L. (2005). Calorie restriction, SIRT1 and metabolism: understanding longevity. Nat Rev Mol Cell Biol *6*, 298-305.

Bordone, L., Motta, M.C., Picard, F., Robinson, A., Jhala, U.S., Apfeld, J., McDonagh, T., Lemieux, M., McBurney, M., Szilvasi, A.*, et al.* (2006). Sirt1 regulates insulin secretion by repressing UCP2 in pancreatic beta cells. PLoS Biol *4*, e31.

Borra, M.T., Smith, B.C., and Denu, J.M. (2005). Mechanism of human SIRT1 activation by resveratrol. J Biol Chem *280*, 17187-17195.

Brand, A.H., Manoukian, A.S., and Perrimon, N. (1994). Ectopic expression in Drosophila. Methods Cell Biol *44*, 635-654.

Breese, C.R., Ingram, R.L., and Sonntag, W.E. (1991). Influence of age and long-term dietary restriction on plasma insulin-like growth factor-1 (IGF-1), IGF-1 gene expression, and IGF-1 binding proteins. J Gerontol *46*, B180-187.

Brewer, G.J. (2010). Epigenetic oxidative redox shift (EORS) theory of aging unifies the free radical and insulin signaling theories. Exp Gerontol *45*, 173-179.

Brunet, A., Sweeney, L.B., Sturgill, J.F., Chua, K.F., Greer, P.L., Lin, Y., Tran, H., Ross, S.E., Mostoslavsky, R., Cohen, H.Y., *et al.* (2004). Stress-dependent regulation of FOXO transcription factors by the SIRT1 deacetylase. Science *303*, 2011-2015.

Buck, S., Vettraino, J., Force, A.G., and Arking, R. (2000). Extended longevity in Drosophila is consistently associated with a decrease in developmental viability. J Gerontol A Biol Sci Med Sci *55*, B292-301.

Busso, N., Karababa, M., Nobile, M., Rolaz, A., Van Gool, F., Galli, M., Leo, O., So, A., and De Smedt, T. (2008). Pharmacological inhibition of nicotinamide phosphoribosyltransferase/visfatin enzymatic activity identifies a new inflammatory pathway linked to NAD. PLoS One *3*, e2267.

Cadenas, S., Buckingham, J.A., Samec, S., Seydoux, J., Din, N., Dulloo, A.G., and Brand, M.D. (1999). UCP2 and UCP3 rise in starved rat skeletal muscle but mitochondrial proton conductance is unchanged. FEBS Lett *462*, 257-260.

Carter, C.S., Hofer, T., Seo, A.Y., and Leeuwenburgh, C. (2007). Molecular mechanisms of life- and health-span extension: role of calorie restriction and exercise intervention. Appl Physiol Nutr Metab *32*, 954-966.

Chen, D., Steele, A.D., Lindquist, S., and Guarente, L. (2005). Increase in activity during calorie restriction requires Sirt1. Science *310*, 1641.

Chen, D., Steele, A.D., Lindquist, S., and Guarente, L. (2005). Increase in activity during calorie restriction requires Sirt1. Science *310*, 1641.

Chen, J., Zhou, Y., Mueller-Steiner, S., Chen, L.F., Kwon, H., Yi, S., Mucke, L., and Gan, L. (2005). SIRT1 protects against microglia-dependent amyloid-beta toxicity through inhibiting NF-kappaB signaling. J Biol Chem *280*, 40364-40374.

Cheng, H.L., Mostoslavsky, R., Saito, S., Manis, J.P., Gu, Y., Patel, P., Bronson, R., Appella, E., Alt, F.W., and Chua, K.F. (2003). Developmental defects and p53 hyperacetylation in Sir2 homolog (SIRT1)-deficient mice. Proc Natl Acad Sci U S A *100*, 10794-10799.

Cho, Y., Griswold, A., Campbell, C., and Min, K.T. (2005). Individual histone deacetylases in Drosophila modulate transcription of distinct genes. Genomics *86*, 606-617.

Chong, Z.Z., Kang, J.Q., and Maiese, K. (2004). AKT1 drives endothelial cell membrane asymmetry and microglial activation through Bcl-xL and caspase 1, 3, and 9. Exp Cell Res *296*, 196-207.

Clancy, D.J., Gems, D., Hafen, E., Leevers, S.J., and Partridge, L. (2002). Dietary restriction in long-lived dwarf flies. Science *296*, 319.

Cohen, D.E., Supinski, A.M., Bonkowski, M.S., Donmez, G., and Guarente, L.P. (2009). Neuronal SIRT1 regulates endocrine and behavioral responses to calorie restriction. Genes Dev *23*, 2812-2817.

Cohen, H.Y., Miller, C., Bitterman, K.J., Wall, N.R., Hekking, B., Kessler, B., Howitz, K.T., Gorospe, M., de Cabo, R., and Sinclair, D.A. (2004). Calorie restriction promotes mammalian cell survival by inducing the SIRT1 deacetylase. Science *305*, 390-392.

Colman, R.J., Anderson, R.M., Johnson, S.C., Kastman, E.K., Kosmatka, K.J., Beasley, T.M., Allison, D.B., Cruzen, C., Simmons, H.A., Kemnitz, J.W., *et al.* (2009). Caloric restriction delays disease onset and mortality in rhesus monkeys. Science *325*, 201-204.

Colman, R.J., Roecker, E.B., Ramsey, J.J., and Kemnitz, J.W. (1998). The effect of dietary restriction on body composition in adult male and female rhesus macaques. Aging (Milano) *10*, 83-92.

Daitoku, H., Yamagata, K., Matsuzaki, H., Hatta, M., and Fukamizu, A. (2003). Regulation of PGC-1 promoter activity by protein kinase B and the forkhead transcription factor FKHR. Diabetes *52*, 642-649.

D'Amati, G., Casali, C., and Giordano, C. (1999). [Mitochondrial diseases]. Pathologica *91*, 49-50.

Denu, J.M. (2005). The Sir 2 family of protein deacetylases. Curr Opin Chem Biol *9*, 431-440.

Du, X., Wang, W., Kim, R., Yakota, H., Nguyen, H., and Kim, S.H. (2001). Crystal structure and mechanism of catalysis of a pyrazinamidase from Pyrococcus horikoshii. Biochemistry *40*, 14166-14172.

Dudas, S.P., and Arking, R. (1995). A coordinate upregulation of antioxidant gene activities is associated with the delayed onset of senescence in a long-lived strain of Drosophila. J Gerontol A Biol Sci Med Sci *50*, B117-127.

Edwards, I.J., Rudel, L.L., Terry, J.G., Kemnitz, J.W., Weindruch, R., Zaccaro, D.J., and Cefalu, W.T. (2001). Caloric restriction lowers plasma lipoprotein (a) in male but not female rhesus monkeys. Exp Gerontol *36*, 1413-1418.

Fabrizio, P., Pozza, F., Pletcher, S.D., Gendron, C.M., and Longo, V.D. (2001). Regulation of longevity and stress resistance by Sch9 in yeast. Science *292*, 288-290.

Finkel, T., and Holbrook, N.J. (2000). Oxidants, oxidative stress and the biology of ageing. Nature *408*, 239-247.

Fisher, A.L., and Lithgow, G.J. (2006). The nuclear hormone receptor DAF-12 has opposing effects on Caenorhabditis elegans lifespan and regulates genes repressed in multiple long-lived worms. Aging Cell *5*, 127-138.

Fontana, L., Meyer, T.E., Klein, S., and Holloszy, J.O. (2004). Long-term calorie restriction is highly effective in reducing the risk for atherosclerosis in humans. Proc Natl Acad Sci U S A *101*, 6659-6663.

Frankel, S., and Rogina, B. (2006). Sir2, caloric restriction and aging. Pathol Biol (Paris) *54*, 55-57.

Frankel, S., Ziafazeli, T., and Rogina, B. (2010). dSir2 and longevity in Drosophila. Exp Gerontol.

Furukawa, A., Tada-Oikawa, S., Kawanishi, S., and Oikawa, S. (2007). H2O2 accelerates cellular senescence by accumulation of acetylated p53 via decrease in the function of SIRT1 by NAD+ depletion. Cell Physiol Biochem *20*, 45-54.

Furuyama, T., Banerjee, R., Breen, T.R., and Harte, P.J. (2004). SIR2 is required for polycomb silencing and is associated with an E(Z) histone methyltransferase complex. Curr Biol *14*, 1812-1821.

Furuyama, T., Kitayama, K., Yamashita, H., and Mori, N. (2003). Forkhead transcription factor FOXO1 (FKHR)-dependent induction of PDK4 gene expression in skeletal muscle during energy deprivation. Biochem J *375*, 365-371.

Gallo, C.M., Smith, D.L., Jr., and Smith, J.S. (2004). Nicotinamide clearance by Pnc1 directly regulates Sir2-mediated silencing and longevity. Mol Cell Biol *24*, 1301-1312.

Garsin, D.A., Villanueva, J.M., Begun, J., Kim, D.H., Sifri, C.D., Calderwood, S.B., Ruvkun, G., and Ausubel, F.M. (2003). Long-lived C. elegans daf-2 mutants are resistant to bacterial pathogens. Science *300*, 1921.

Gems, D., Sutton, A.J., Sundermeyer, M.L., Albert, P.S., King, K.V., Edgley, M.L., Larsen, P.L., and Riddle, D.L. (1998). Two pleiotropic classes of daf-2 mutation affect larval arrest, adult behavior, reproduction and longevity in Caenorhabditis elegans. Genetics *150*, 129-155.

Ghislain, M., Talla, E., and Francois, J.M. (2002). Identification and functional analysis of the Saccharomyces cerevisiae nicotinamidase gene, PNC1. Yeast *19*, 215-224.

Giannakou, M.E., Goss, M., Junger, M.A., Hafen, E., Leevers, S.J., and Partridge, L. (2004). Long-lived Drosophila with overexpressed dFOXO in adult fat body. Science *305*, 361.

Goodrick, C.L. (1980). Effects of long-term voluntary wheel exercise on male and female Wistar rats. I. Longevity, body weight, and metabolic rate. Gerontology *26*, 22-33.

Grandison, R.C., Piper, M.D., and Partridge, L. (2009). Amino-acid imbalance explains extension of lifespan by dietary restriction in Drosophila. Nature *462*, 1061-1064.

Gredilla, R., and Barja, G. (2005). Minireview: the role of oxidative stress in relation to caloric restriction and longevity. Endocrinology *146*, 3713-3717.

Gredilla, R., Sanz, A., Lopez-Torres, M., and Barja, G. (2001). Caloric restriction decreases mitochondrial free radical generation at complex I and lowers oxidative damage to mitochondrial DNA in the rat heart. FASEB J *15*, 1589-1591.

Griswold, A.J., Chang, K.T., Runko, A.P., Knight, M.A., and Min, K.T. (2008). Sir2 mediates apoptosis through JNK-dependent pathways in Drosophila. Proc Natl Acad Sci U S A *105*, 8673-8678.

Grover, D., Ford, D., Brown, C., Hoe, N., Erdem, A., Tavare, S., and Tower, J. (2009). Hydrogen peroxide stimulates activity and alters behavior in Drosophila melanogaster. PLoS One *4*, e7580.

Guarente, L. (2005). NO link between calorie restriction and mitochondria. Nat Chem Biol *1*, 355-356.

Haigis, M.C., and Sinclair, D.A. (2010). Mammalian sirtuins: biological insights and disease relevance. Annu Rev Pathol *5*, 253-295.

Halliwell, B. (2007). Oxidative stress and cancer: have we moved forward? Biochem J *401*, 1-11.

Harman, D. (1956). Aging: a theory based on free radical and radiation chemistry. J Gerontol *11*, 298-300.

Harman, D. (1972). The biologic clock: the mitochondria? J Am Geriatr Soc *20*, 145-147.

Harper, J.M., Salmon, A.B., Chang, Y., Bonkowski, M., Bartke, A., and Miller, R.A. (2006). Stress resistance and aging: influence of genes and nutrition. Mech Ageing Dev *127*, 687-694.

Harper, J.M., Salmon, A.B., Leiser, S.F., Galecki, A.T., and Miller, R.A. (2007). Skin-derived fibroblasts from long-lived species are resistant to some, but not all, lethal stresses and to the mitochondrial inhibitor rotenone. Aging Cell *6*, 1-13.

Hashimoto, T., Horikawa, M., Nomura, T., and Sakamoto, K. (2010). Nicotinamide adenine dinucleotide extends the lifespan of Caenorhabditis elegans mediated by sir-2.1 and daf-16. Biogerontology *11*, 31-43.

Helfand, S.L., and Rogina, B. (2003). Genetics of aging in the fruit fly, Drosophila melanogaster. Annu Rev Genet *37*, 329-348.

Helfand, S.L., and Rogina, B. (2003). Molecular genetics of aging in the fly: is this the end of the beginning? Bioessays *25*, 134-141.

Holloszy, J.O. (1993). Exercise increases average longevity of female rats despite increased food intake and no growth retardation. J Gerontol *48*, B97-100.

Holzenberger, M., Dupont, J., Ducos, B., Leneuve, P., Geloen, A., Even, P.C., Cervera, P., and Le Bouc, Y. (2003). IGF-1 receptor regulates lifespan and resistance to oxidative stress in mice. Nature *421*, 182-187.

Honda, Y., and Honda, S. (1999). The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. FASEB J *13*, 1385-1393.

Howitz, K.T., Bitterman, K.J., Cohen, H.Y., Lamming, D.W., Lavu, S., Wood, J.G., Zipkin, R.E., Chung, P., Kisielewski, A., Zhang, L.L.*, et al.* (2003). Small molecule activators of sirtuins extend Saccharomyces cerevisiae lifespan. Nature *425*, 191-196.

Hunt, L., Holdsworth, M.J., and Gray, J.E. (2007). Nicotinamidase activity is important for germination. Plant J *51*, 341-351.

Imai, S., Armstrong, C.M., Kaeberlein, M., and Guarente, L. (2000). Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. Nature *403*, 795-800.

Imai, S., Johnson, F.B., Marciniak, R.A., McVey, M., Park, P.U., and Guarente, L. (2000). Sir2: an NAD-dependent histone deacetylase that connects chromatin silencing, metabolism, and aging. Cold Spring Harb Symp Quant Biol *65*, 297-302.

Jackson, M.D., Schmidt, M.T., Oppenheimer, N.J., and Denu, J.M. (2003). Mechanism of nicotinamide inhibition and transglycosidation by Sir2 histone/protein deacetylases. J Biol Chem *278*, 50985-50998.

Jacobson, J., Lambert, A.J., Portero-Otin, M., Pamplona, R., Magwere, T., Miwa, S., Driege, Y., Brand, M.D., and Partridge, L. (2010). Biomarkers of aging in Drosophila. Aging Cell *9*, 466-477.

Jia, K., and Levine, B. (2007). Autophagy is required for dietary restriction-mediated life span extension in C. elegans. Autophagy *3*, 597-599.

Jiang, H.Y., Koike, T., Li, P., Wang, Z.H., Kawata, Y., and Oshida, Y. (2010). Combined Effects of Short-term Calorie Restriction and Exercise on Insulin Action in Normal Rats. Horm Metab Res.

Kaeberlein, M., Kirkland, K.T., Fields, S., and Kennedy, B.K. (2004). Sir2-independent life span extension by calorie restriction in yeast. PLoS Biol *2*, E296.

Kaeberlein, M., McVey, M., and Guarente, L. (1999). The SIR2/3/4 complex and SIR2 alone promote longevity in Saccharomyces cerevisiae by two different mechanisms. Genes Dev *13*, 2570-2580.

Kaeberlein, M., Powers, R.W., 3rd, Steffen, K.K., Westman, E.A., Hu, D., Dang, N., Kerr, E.O., Kirkland, K.T., Fields, S., and Kennedy, B.K. (2005). Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. Science *310*, 1193-1196.

Kaeberlein, M., and Rabinovitch, P.S. (2006). Medicine: grapes versus gluttony. Nature *444*, 280-281.

Kaeberlein, T.L., Smith, E.D., Tsuchiya, M., Welton, K.L., Thomas, J.H., Fields, S., Kennedy, B.K., and Kaeberlein, M. (2006). Lifespan extension in Caenorhabditis elegans by complete removal of food. Aging Cell *5*, 487-494.

Kaneto, H., Katakami, N., Matsuhisa, M., and Matsuoka, T.A. (2010). Role of reactive oxygen species in the progression of type 2 diabetes and atherosclerosis. Mediators Inflamm *2010*, 453892.

Kang, H.T., Lee, H.I., and Hwang, E.S. (2006). Nicotinamide extends replicative lifespan of human cells. Aging Cell *5*, 423-436.

Kang, J.Q., Chong, Z.Z., and Maiese, K. (2003). Critical role for Akt1 in the modulation of apoptotic phosphatidylserine exposure and microglial activation. Mol Pharmacol *64*, 557-569.

Kang, M.K., and Park, N.H. (2007). Extension of cell life span using exogenous telomerase. Methods Mol Biol *371*, 151-165.

Kang, S.C., Kim, M.J., and Choi, U.K. (2007). Shelf-life extension of fresh-cut iceberg lettuce (Lactuca sativa L) by different antimicrobial films. J Microbiol Biotechnol *17*, 1284-1290.

Kapahi, P., Zid, B.M., Harper, T., Koslover, D., Sapin, V., and Benzer, S. (2004). Regulation of lifespan in Drosophila by modulation of genes in the TOR signaling pathway. Curr Biol *14*, 885-890.

Kaplow, M.E., Mannava, L.J., Pimentel, A.C., Fermin, H.A., Hyatt, V.J., Lee, J.J., and Venkatesh, T.R. (2007). A genetic modifier screen identifies multiple genes that interact with Drosophila Rap/Fzr and suggests novel cellular roles. J Neurogenet *21*, 105-151.

Kealy, R.D., Lawler, D.F., Ballam, J.M., Mantz, S.L., Biery, D.N., Greeley, E.H., Lust, G., Segre, M., Smith, G.K., and Stowe, H.D. (2002). Effects of diet restriction on life span and age-related changes in dogs. J Am Vet Med Assoc *220*, 1315-1320.

Kelly, D.P., and Scarpulla, R.C. (2004). Transcriptional regulatory circuits controlling mitochondrial biogenesis and function. Genes Dev *18*, 357-368.

Kenyon, C. (2005). The plasticity of aging: insights from long-lived mutants. Cell *120*, 449-460.

Kenyon, C., Chang, J., Gensch, E., Rudner, A., and Tabtiang, R. (1993). A C. elegans mutant that lives twice as long as wild type. Nature *366*, 461-464.

Kim, H., Lee, D.K., Choi, J.W., Kim, J.S., Park, S.C., and Youn, H.D. (2003). Analysis of the effect of aging on the response to hypoxia by cDNA microarray. Mech Ageing Dev *124*, 941-949.

Kim, J.H., Chu, S.C., Gramlich, J.L., Pride, Y.B., Babendreier, E., Chauhan, D., Salgia, R., Podar, K., Griffin, J.D., and Sattler, M. (2005). Activation of the PI3K/mTOR pathway by BCR-ABL contributes to increased production of reactive oxygen species. Blood *105*, 1717-1723.

Kirby, K., Hu, J., Hilliker, A.J., and Phillips, J.P. (2002). RNA interference-mediated silencing of Sod2 in Drosophila leads to early adult-onset mortality and elevated endogenous oxidative stress. Proc Natl Acad Sci U S A *99*, 16162-16167.

Klass, M.R. (1977). Aging in the nematode Caenorhabditis elegans: major biological and environmental factors influencing life span. Mech Ageing Dev *6*, 413-429.

Kobayashi, Y., Furukawa-Hibi, Y., Chen, C., Horio, Y., Isobe, K., Ikeda, K., and Motoyama, N. (2005). SIRT1 is critical regulator of FOXO-mediated transcription in response to oxidative stress. Int J Mol Med *16*, 237-243.

Ku, H.H., Brunk, U.T., and Sohal, R.S. (1993). Relationship between mitochondrial superoxide and hydrogen peroxide production and longevity of mammalian species. Free Radic Biol Med *15*, 621-627.

Kume, S., Uzu, T., Horiike, K., Chin-Kanasaki, M., Isshiki, K., Araki, S., Sugimoto, T., Haneda, M., Kashiwagi, A., and Koya, D. (2010). Calorie restriction enhances cell adaptation to hypoxia through Sirt1-dependent mitochondrial autophagy in mouse aged kidney. J Clin Invest *120*, 1043-1055.

Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., Messadeq, N., Milne, J., Lambert, P., Elliott, P.*, et al.* (2006). Resveratrol improves mitochondrial function and protects against metabolic disease by activating SIRT1 and PGC-1alpha. Cell *127*, 1109-1122.

Lakowski, B., and Hekimi, S. (1996). Determination of life-span in Caenorhabditis elegans by four clock genes. Science *272*, 1010-1013.

Landry, J., Slama, J.T., and Sternglanz, R. (2000). Role of NAD(+) in the deacetylase activity of the SIR2-like proteins. Biochem Biophys Res Commun *278*, 685-690.

Landry, J., Sutton, A., Tafrov, S.T., Heller, R.C., Stebbins, J., Pillus, L., and Sternglanz, R. (2000). The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. Proc Natl Acad Sci U S A *97*, 5807-5811.

Larrosa, M., Yanez-Gascon, M.J., Selma, M.V., Gonzalez-Sarrias, A., Toti, S., Ceron, J.J., Tomas-Barberan, F., Dolara, P., and Espin, J.C. (2009). Effect of a low dose of dietary resveratrol on colon microbiota, inflammation and tissue damage in a DSS-induced colitis rat model. J Agric Food Chem *57*, 2211-2220.

Lavara-Culebras, E., and Paricio, N. (2007). Drosophila DJ-1 mutants are sensitive to oxidative stress and show reduced lifespan and motor deficits. Gene *400*, 158-165.

Lebovitz, R.M., Zhang, H., Vogel, H., Cartwright, J., Jr., Dionne, L., Lu, N., Huang, S., and Matzuk, M.M. (1996). Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. Proc Natl Acad Sci U S A *93*, 9782-9787.

Lee, C.K., Allison, D.B., Brand, J., Weindruch, R., and Prolla, T.A. (2002). Transcriptional profiles associated with aging and middle age-onset caloric restriction in mouse hearts. Proc Natl Acad Sci U S A *99*, 14988-14993.

Lee, C.K., Klopp, R.G., Weindruch, R., and Prolla, T.A. (1999). Gene expression profile of aging and its retardation by caloric restriction. Science *285*, 1390-1393.

Lee, G.D., Wilson, M.A., Zhu, M., Wolkow, C.A., de Cabo, R., Ingram, D.K., and Zou, S. (2006). Dietary deprivation extends lifespan in Caenorhabditis elegans. Aging Cell *5*, 515-524.

Liao, C.Y., Rikke, B.A., Johnson, T.E., Diaz, V., and Nelson, J.F. (2010). Genetic variation in the murine lifespan response to dietary restriction: from life extension to life shortening. Aging Cell *9*, 92-95.

Lim, C.S., Potts, M., and Helm, R.F. (2006). Nicotinamide extends the replicative life span of primary human cells. Mech Ageing Dev *127*, 511-514.

Lin, S.J., Defossez, P.A., and Guarente, L. (2000). Requirement of NAD and SIR2 for life-span extension by calorie restriction in Saccharomyces cerevisiae. Science *289*, 2126-2128.

Lin, S.J., Ford, E., Haigis, M., Liszt, G., and Guarente, L. (2004). Calorie restriction extends yeast life span by lowering the level of NADH. Genes Dev *18*, 12-16.

Lin, S.J., Kaeberlein, M., Andalis, A.A., Sturtz, L.A., Defossez, P.A., Culotta, V.C., Fink, G.R., and Guarente, L. (2002). Calorie restriction extends Saccharomyces cerevisiae lifespan by increasing respiration. Nature *418*, 344-348.

Lin, Y.J., Seroude, L., and Benzer, S. (1998). Extended life-span and stress resistance in the Drosophila mutant methuselah. Science *282*, 943-946.

Loeb, J., and Northrop, J.H. (1917). What Determines the Duration of Life in Metazoa? Proc Natl Acad Sci U S A *3*, 382-386.

Longo, V.D., and Kennedy, B.K. (2006). Sirtuins in aging and age-related disease. Cell *126*, 257-268.

Maier, B., Gluba, W., Bernier, B., Turner, T., Mohammad, K., Guise, T., Sutherland, A., Thorner, M., and Scrable, H. (2004). Modulation of mammalian life span by the short isoform of p53. Genes Dev *18*, 306-319.

Masoro, E.J. (2005). Overview of caloric restriction and ageing. Mech Ageing Dev *126*, 913-922.

Masoro, E.J. (2007). The role of hormesis in life extension by dietary restriction. Interdiscip Top Gerontol *35*, 1-17.

Masoro, E.J., McCarter, R.J., Katz, M.S., and McMahan, C.A. (1992). Dietary restriction alters characteristics of glucose fuel use. J Gerontol *47*, B202-208.

Medvedik, O., and Sinclair, D.A. (2007). Caloric restriction and life span determination of yeast cells. Methods Mol Biol *371*, 97-109.

Merry, B.J. (2004). Oxidative stress and mitochondrial function with aging--the effects of calorie restriction. Aging Cell *3*, 7-12.

Michishita, E., Park, J.Y., Burneskis, J.M., Barrett, J.C., and Horikawa, I. (2005). Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. Mol Biol Cell *16*, 4623-4635.

Migliaccio, E., Giorgio, M., Mele, S., Pelicci, G., Reboldi, P., Pandolfi, P.P., Lanfrancone, L., and Pelicci, P.G. (1999). The p66shc adaptor protein controls oxidative stress response and life span in mammals. Nature *402*, 309-313.

Miller, R.A. (2009). Cell stress and aging: new emphasis on multiplex resistance mechanisms. J Gerontol A Biol Sci Med Sci *64*, 179-182.

Morrow, G., Samson, M., Michaud, S., and Tanguay, R.M. (2004). Overexpression of the small mitochondrial Hsp22 extends Drosophila life span and increases resistance to oxidative stress. FASEB J *18*, 598-599.

Mortimer, R.K., and Johnston, J.R. (1959). Life span of individual yeast cells. Nature *183*, 1751-1752.

Moskovitz, J., Bar-Noy, S., Williams, W.M., Requena, J., Berlett, B.S., and Stadtman, E.R. (2001). Methionine sulfoxide reductase (MsrA) is a regulator of antioxidant defense and lifespan in mammals. Proc Natl Acad Sci U S A *98*, 12920-12925.

Motta, M.C., Divecha, N., Lemieux, M., Kamel, C., Chen, D., Gu, W., Bultsma, Y., McBurney, M., and Guarente, L. (2004). Mammalian SIRT1 represses forkhead transcription factors. Cell *116*, 551-563.

Murakami, S., and Johnson, T.E. (1996). A genetic pathway conferring life extension and resistance to UV stress in Caenorhabditis elegans. Genetics *143*, 1207-1218.

Murakami, S., Salmon, A., and Miller, R.A. (2003). Multiplex stress resistance in cells from long-lived dwarf mice. FASEB J *17*, 1565-1566.

Nasrin, N., Kaushik, V.K., Fortier, E., Wall, D., Pearson, K.J., de Cabo, R., and Bordone, L. (2009). JNK1 phosphorylates SIRT1 and promotes its enzymatic activity. PLoS One *4*, e8414.

Nemoto, S., Fergusson, M.M., and Finkel, T. (2004). Nutrient availability regulates SIRT1 through a forkhead-dependent pathway. Science *306*, 2105-2108.

Newman, B.L., Lundblad, J.R., Chen, Y., and Smolik, S.M. (2002). A Drosophila homologue of Sir2 modifies position-effect variegation but does not affect life span. Genetics *162*, 1675-1685.

Nisoli, E., Tonello, C., Cardile, A., Cozzi, V., Bracale, R., Tedesco, L., Falcone, S., Valerio, A., Cantoni, O., Clementi, E.*, et al.* (2005). Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. Science *310*, 314-317.

North, B.J., Marshall, B.L., Borra, M.T., Denu, J.M., and Verdin, E. (2003). The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase. Mol Cell *11*, 437-444.

Nuzhdin, S.V., Pasyukova, E.G., Dilda, C.L., Zeng, Z.B., and Mackay, T.F. (1997). Sex-specific quantitative trait loci affecting longevity in Drosophila melanogaster. Proc Natl Acad Sci U S A *94*, 9734-9739.

Oberdoerffer, P., Michan, S., McVay, M., Mostoslavsky, R., Vann, J., Park, S.K., Hartlerode, A., Stegmuller, J., Hafner, A., Loerch, P.*, et al.* (2008). SIRT1 redistribution on chromatin promotes genomic stability but alters gene expression during aging. Cell *135*, 907-918.

Olsen, A., Vantipalli, M.C., and Lithgow, G.J. (2006). Lifespan extension of Caenorhabditis elegans following repeated mild hormetic heat treatments. Biogerontology *7*, 221-230.

Pallos, J., Bodai, L., Lukacsovich, T., Purcell, J.M., Steffan, J.S., Thompson, L.M., and Marsh, J.L. (2008). Inhibition of specific HDACs and sirtuins suppresses pathogenesis in a Drosophila model of Huntington's disease. Hum Mol Genet *17*, 3767-3775.

Parashar, V., Frankel, S., Lurie, A.G., and Rogina, B. (2008). The effects of age on radiation resistance and oxidative stress in adult Drosophila melanogaster. Radiat Res *169*, 707-711.

Parashar, V., and Rogina, B. (2009). dSir2 mediates the increased spontaneous physical activity in flies on calorie restriction. Aging (Albany NY) *1*, 529-541.

Perez, V.I., Bokov, A., Van Remmen, H., Mele, J., Ran, Q., Ikeno, Y., and Richardson, A. (2009). Is the oxidative stress theory of aging dead? Biochim Biophys Acta *1790*, 1005-1014.

Pfluger, P.T., Herranz, D., Velasco-Miguel, S., Serrano, M., and Tschop, M.H. (2008). Sirt1 protects against high-fat diet-induced metabolic damage. Proc Natl Acad Sci U S A *105*, 9793-9798.

Piazza, N., Gosangi, B., Devilla, S., Arking, R., and Wessells, R. (2009). Exercise-training in young Drosophila melanogaster reduces age-related decline in mobility and cardiac performance. PLoS One *4*, e5886.

Picard, F., and Guarente, L. (2005). Molecular links between aging and adipose tissue. Int J Obes (Lond) *29 Suppl 1*, S36-39.

Picard, F., Kurtev, M., Chung, N., Topark-Ngarm, A., Senawong, T., Machado De Oliveira, R., Leid, M., McBurney, M.W., and Guarente, L. (2004). Sirt1 promotes fat mobilization in white adipocytes by repressing PPAR-gamma. Nature *429*, 771-776.

Pletcher, S.D., Libert, S., and Skorupa, D. (2005). Flies and their golden apples: the effect of dietary restriction on Drosophila aging and age-dependent gene expression. Ageing Res Rev *4*, 451-480.

Porcu, M., and Chiarugi, A. (2005). The emerging therapeutic potential of sirtuin-interacting drugs: from cell death to lifespan extension. Trends Pharmacol Sci *26*, 94-103.

Reiling, J.H., and Sabatini, D.M. (2006). Stress and mTORture signaling. Oncogene *25*, 6373-6383.

Revollo, J.R., Grimm, A.A., and Imai, S. (2004). The NAD biosynthesis pathway mediated by nicotinamide phosphoribosyltransferase regulates Sir2 activity in mammalian cells. J Biol Chem *279*, 50754-50763.

Revollo, J.R., Grimm, A.A., and Imai, S. (2007). The regulation of nicotinamide adenine dinucleotide biosynthesis by Nampt/PBEF/visfatin in mammals. Curr Opin Gastroenterol *23*, 164-170.

Rogina, B., Benzer, S., and Helfand, S.L. (1997). Drosophila drop-dead mutations accelerate the time course of age-related markers. Proc Natl Acad Sci U S A *94*, 6303-6306.

Rogina, B., and Helfand, S.L. (2004). Sir2 mediates longevity in the fly through a pathway related to calorie restriction. Proc Natl Acad Sci U S A *101*, 15998-16003.

Rogina, B., Helfand, S.L., and Frankel, S. (2002). Longevity regulation by Drosophila Rpd3 deacetylase and caloric restriction. Science *298*, 1745.

Rongvaux, A., Galli, M., Denanglaire, S., Van Gool, F., Dreze, P.L., Szpirer, C., Bureau, F., Andris, F., and Leo, O. (2008). Nicotinamide phosphoribosyl transferase/pre-B cell colony-enhancing factor/visfatin is required for lymphocyte development and cellular resistance to genotoxic stress. J Immunol *181*, 4685-4695.

Salmon, A.B., Murakami, S., Bartke, A., Kopchick, J., Yasumura, K., and Miller, R.A. (2005). Fibroblast cell lines from young adult mice of long-lived mutant strains are resistant to multiple forms of stress. Am J Physiol Endocrinol Metab *289*, E23-29.

Salmon, A.B., Richardson, A., and Perez, V.I. (2010). Update on the oxidative stress theory of aging: does oxidative stress play a role in aging or healthy aging? Free Radic Biol Med *48*, 642-655.

Sandmeier, J.J., French, S., Osheim, Y., Cheung, W.L., Gallo, C.M., Beyer, A.L., and Smith, J.S. (2002). RPD3 is required for the inactivation of yeast ribosomal DNA genes in stationary phase. EMBO J *21*, 4959-4968.

Sanz, A., Caro, P., Ayala, V., Portero-Otin, M., Pamplona, R., and Barja, G. (2006). Methionine restriction decreases mitochondrial oxygen radical generation and leak as well as oxidative damage to mitochondrial DNA and proteins. FASEB J *20*, 1064-1073.

Sanz, A., Fernandez-Ayala, D.J., Stefanatos, R.K., and Jacobs, H.T. (2010). Mitochondrial ROS production correlates with, but does not directly regulate lifespan in Drosophila. Aging (Albany NY) *2*, 220-223.

Sarbassov, D.D., Ali, S.M., and Sabatini, D.M. (2005). Growing roles for the mTOR pathway. Curr Opin Cell Biol *17*, 596-603.

Sasaki, Y., Vohra, B.P., Lund, F.E., and Milbrandt, J. (2009). Nicotinamide mononucleotide adenylyl transferase-mediated axonal protection requires enzymatic activity but not increased levels of neuronal nicotinamide adenine dinucleotide. J Neurosci *29*, 5525-5535.

Sauve, A.A. (2008). NAD+ and vitamin B3: from metabolism to therapies. J Pharmacol Exp Ther *324*, 883-893.

Sauve, A.A. (2008). A SIR-tain acetyl complex is caught by a sulfur trap. Structure *16*, 1289-1292.

Sauve, A.A., and Schramm, V.L. (2003). Sir2 regulation by nicotinamide results from switching between base exchange and deacetylation chemistry. Biochemistry *42*, 9249-9256.

Sharp, Z.D., and Bartke, A. (2005). Evidence for down-regulation of phosphoinositide 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR)-dependent translation regulatory signaling pathways in Ames dwarf mice. J Gerontol A Biol Sci Med Sci *60*, 293-300.

Shen, J., and Tower, J. (2010). Drosophila foxo acts in males to cause sexual-dimorphism in tissue-specific p53 life span effects. Exp Gerontol *45*, 97-105.

Shigenaga, M.K., Hagen, T.M., and Ames, B.N. (1994). Oxidative damage and mitochondrial decay in aging. Proc Natl Acad Sci U S A *91*, 10771-10778.

Shimada, H., Hirai, K., Simamura, E., and Pan, J. (1998). Mitochondrial NADH-quinone oxidoreductase of the outer membrane is responsible for paraquat cytotoxicity in rat livers. Arch Biochem Biophys *351*, 75-81.

Silva, R.M., Duarte, I.C., Paredes, J.A., Lima-Costa, T., Perrot, M., Boucherie, H., Goodfellow, B.J., Gomes, A.C., Mateus, D.D., Moura, G.R.*, et al.* (2009). The yeast PNC1 longevity gene is up-regulated by mRNA mistranslation. PLoS One *4*, e5212.

Simonsen, A., Cumming, R.C., Lindmo, K., Galaviz, V., Cheng, S., Rusten, T.E., and Finley, K.D. (2007). Genetic modifiers of the Drosophila blue cheese gene link defects in lysosomal transport with decreased life span and altered ubiquitinated-protein profiles. Genetics *176*, 1283-1297.

Sinclair, D.A., and Guarente, L. (1997). Extrachromosomal rDNA circles--a cause of aging in yeast. Cell *91*, 1033-1042.

Smith, J.S., Brachmann, C.B., Celic, I., Kenna, M.A., Muhammad, S., Starai, V.J., Avalos, J.L., Escalante-Semerena, J.C., Grubmeyer, C., Wolberger, C.*, et al.* (2000). A phylogenetically conserved NAD+-dependent protein deacetylase activity in the Sir2 protein family. Proc Natl Acad Sci U S A *97*, 6658-6663.

Soh, J.W., Hotic, S., and Arking, R. (2007). Dietary restriction in Drosophila is dependent on mitochondrial efficiency and constrained by pre-existing extended longevity. Mech Ageing Dev *128*, 581-593.

Sohal, R.S., Ku, H.H., Agarwal, S., Forster, M.J., and Lal, H. (1994). Oxidative damage, mitochondrial oxidant generation and antioxidant defenses during aging and in response to food restriction in the mouse. Mech Ageing Dev *74*, 121-133.

Sohal, R.S., and Weindruch, R. (1996). Oxidative stress, caloric restriction, and aging. Science *273*, 59-63.

Sonntag, W.E., Lynch, C.D., Cefalu, W.T., Ingram, R.L., Bennett, S.A., Thornton, P.L., and Khan, A.S. (1999). Pleiotropic effects of growth hormone and insulin-like growth factor (IGF)-1 on biological aging: inferences from moderate caloric-restricted animals. J Gerontol A Biol Sci Med Sci *54*, B521-538.

Sun, J., Folk, D., Bradley, T.J., and Tower, J. (2002). Induced overexpression of mitochondrial Mn-superoxide dismutase extends the life span of adult Drosophila melanogaster. Genetics *161*, 661-672.

Sun, J., Molitor, J., and Tower, J. (2004). Effects of simultaneous over-expression of Cu/ZnSOD and MnSOD on Drosophila melanogaster life span. Mech Ageing Dev *125*, 341-349.

Sun, J., and Tower, J. (1999). FLP recombinase-mediated induction of Cu/Zn-superoxide dismutase transgene expression can extend the life span of adult Drosophila melanogaster flies. Mol Cell Biol *19*, 216-228.

Tang, B.L. (2006). SIRT1, neuronal cell survival and the insulin/IGF-1 aging paradox. Neurobiol Aging *27*, 501-505.

Tanny, J.C., Dowd, G.J., Huang, J., Hilz, H., and Moazed, D. (1999). An enzymatic activity in the yeast Sir2 protein that is essential for gene silencing. Cell *99*, 735-745.

Tatar, M., Bartke, A., and Antebi, A. (2003). The endocrine regulation of aging by insulin-like signals. Science *299*, 1346-1351.

Tissenbaum, H.A., and Guarente, L. (2001). Increased dosage of a sir-2 gene extends lifespan in Caenorhabditis elegans. Nature *410*, 227-230.

Tong, J.J., Schriner, S.E., McCleary, D., Day, B.J., and Wallace, D.C. (2007). Life extension through neurofibromin mitochondrial regulation and antioxidant therapy for neurofibromatosis-1 in Drosophila melanogaster. Nat Genet *39*, 476-485.

Tower, J. (2006). Sex-specific regulation of aging and apoptosis. Mech Ageing Dev *127*, 705-718.

Tsuchiya, M., Dang, N., Kerr, E.O., Hu, D., Steffen, K.K., Oakes, J.A., Kennedy, B.K., and Kaeberlein, M. (2006). Sirtuin-independent effects of nicotinamide on lifespan extension from calorie restriction in yeast. Aging Cell *5*, 505-514.

Tyner, S.D., Venkatachalam, S., Choi, J., Jones, S., Ghebranious, N., Igelmann, H., Lu, X., Soron, G., Cooper, B., Brayton, C*., et al.* (2002). p53 mutant mice that display early ageing-associated phenotypes. Nature *415*, 45-53.

van der Horst, A., Schavemaker, J.M., Pellis-van Berkel, W., and Burgering, B.M. (2007). The Caenorhabditis elegans nicotinamidase PNC-1 enhances survival. Mech Ageing Dev *128*, 346-349.

Van Remmen, H., Ikeno, Y., Hamilton, M., Pahlavani, M., Wolf, N., Thorpe, S.R., Alderson, N.L., Baynes, J.W., Epstein, C.J., Huang, T.T.*, et al.* (2003). Life-long reduction in MnSOD activity results in increased DNA damage and higher incidence of cancer but does not accelerate aging. Physiol Genomics *16*, 29-37.

Vanfleteren, J.R. (1993). Oxidative stress and ageing in Caenorhabditis elegans. Biochem J *292 (Pt 2)*, 605-608.

Vellai, T., Takacs-Vellai, K., Zhang, Y., Kovacs, A.L., Orosz, L., and Muller, F. (2003). Genetics: influence of TOR kinase on lifespan in C. elegans. Nature *426*, 620.

Vettraino, J., Buck, S., and Arking, R. (2001). Direct selection for paraquat resistance in Drosophila results in a different extended longevity phenotype. J Gerontol A Biol Sci Med Sci *56*, B415-425.

Viswanathan, M., Kim, S.K., Berdichevsky, A., and Guarente, L. (2005). A role for SIR-2.1 regulation of ER stress response genes in determining C. elegans life span. Dev Cell *9*, 605-615.

Wallace, D.C. (1999). Mitochondrial diseases in man and mouse. Science *283*, 1482-1488.

Wallace, D.C. (2005). A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu Rev Genet *39*, 359-407.

Wang, C., Wang, M.W., Tashiro, S., Onodera, S., and Ikejima, T. (2005). Roles of SIRT1 and phosphoinositide 3-OH kinase/protein kinase C pathways in evodiamine-induced human melanoma A375-S2 cell death. J Pharmacol Sci *97*, 494-500.

Wang, F., Nguyen, M., Qin, F.X., and Tong, Q. (2007). SIRT2 deacetylates FOXO3a in response to oxidative stress and caloric restriction. Aging Cell *6*, 505-514.

Wang, G., and Pichersky, E. (2007). Nicotinamidase participates in the salvage pathway of NAD biosynthesis in Arabidopsis. Plant J *49*, 1020-1029.

Wang, J., Zhai, Q., Chen, Y., Lin, E., Gu, W., McBurney, M.W., and He, Z. (2005). A local mechanism mediates NAD-dependent protection of axon degeneration. J Cell Biol *170*, 349-355.

Wang, M.C., Bohmann, D., and Jasper, H. (2003). JNK signaling confers tolerance to oxidative stress and extends lifespan in Drosophila. Dev Cell *5*, 811-816.

Wang, Y., Oh, S.W., Deplancke, B., Luo, J., Walhout, A.J., and Tissenbaum, H.A. (2006). C. elegans 14-3-3 proteins regulate life span and interact with SIR-2.1 and DAF-16/FOXO. Mech Ageing Dev *127*, 741-747.

Wang, Y., and Tissenbaum, H.A. (2006). Overlapping and distinct functions for a Caenorhabditis elegans SIR2 and DAF-16/FOXO. Mech Ageing Dev *127*, 48-56.

Wei, M., Fabrizio, P., Hu, J., Ge, H., Cheng, C., Li, L., and Longo, V.D. (2008). Life span extension by calorie restriction depends on Rim15 and transcription factors downstream of Ras/PKA, Tor, and Sch9. PLoS Genet *4*, e13.

Wetter, T.J., Gazdag, A.C., Dean, D.J., and Cartee, G.D. (1999). Effect of calorie restriction on in vivo glucose metabolism by individual tissues in rats. Am J Physiol *276*, E728-738.

Wood, J.G., Rogina, B., Lavu, S., Howitz, K., Helfand, S.L., Tatar, M., and Sinclair, D. (2004). Sirtuin activators mimic caloric restriction and delay ageing in metazoans. Nature *430*, 686-689.

Wu, Z., Lauer, T.W., Sick, A., Hackett, S.F., and Campochiaro, P.A. (2007). Oxidative stress modulates complement factor H expression in retinal pigmented epithelial cells by acetylation of FOXO3. J Biol Chem *282*, 22414-22425.

Yang, H., Baur, J.A., Chen, A., Miller, C., Adams, J.K., Kisielewski, A., Howitz, K.T., Zipkin, R.E., and Sinclair, D.A. (2007). Design and synthesis of compounds that extend yeast replicative lifespan. Aging Cell *6*, 35-43.

Yang, H., Lavu, S., and Sinclair, D.A. (2006). Nampt/PBEF/Visfatin: a regulator of mammalian health and longevity? Exp Gerontol *41*, 718-726.

Yeung, F., Hoberg, J.E., Ramsey, C.S., Keller, M.D., Jones, D.R., Frye, R.A., and Mayo, M.W. (2004). Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J *23*, 2369-2380.

Yu, B.P. (1996). Aging and oxidative stress: modulation by dietary restriction. Free Radic Biol Med *21*, 651-668.

Yu, B.P., and Yang, R. (1996). Critical evaluation of the free radical theory of aging. A proposal for the oxidative stress hypothesis. Ann N Y Acad Sci *786*, 1-11.

Zamocky, M., Furtmuller, P.G., and Obinger, C. (2008). Evolution of catalases from bacteria to humans. Antioxid Redox Signal *10*, 1527-1548.

Zhang, T., Berrocal, J.G., Frizzell, K.M., Gamble, M.J., DuMond, M.E., Krishnakumar, R., Yang, T., Sauve, A.A., and Kraus, W.L. (2009). Enzymes in the NAD+ salvage pathway regulate SIRT1 activity at target gene promoters. J Biol Chem *284*, 20408-20417.

Zhao, T., Zhang, Q., Qi, H., Liu, X., and Li, Z. (2008). Extension of life span and improvement of vitality of Drosophila melanogaster by long-term supplementation with different molecular weight polysaccharides from Porphyra haitanensis. Pharmacol Res *57*, 67-72.

Zhao, X., Sternsdorf, T., Bolger, T.A., Evans, R.M., and Yao, T.P. (2005). Regulation of MEF2 by histone deacetylase 4- and SIRT1 deacetylase-mediated lysine modifications. Mol Cell Biol *25*, 8456-8464.

ABSTRACT

DROSOPHILA NICOTINAMIDASE AND SIR2 IN LONGEVITY REGULATION AND OXIDATIVE STRESS RESPONSE, AND THE IMPLICATIONS FOR AGING THEORY

by

GREGORY MILLER

MAY 2011

Advisor: Dr. Robert Arking

Major: Biological Sciences

Degree: Doctor of Philosophy

The protein deacetylase Sir2 has been shown to increase lifespan in a variety of organisms, possibly through its ability to engender the effects of dietary restriction. The *Drosophila* nicotinamidase DNAAM increases lifespan in a Sir2 dependant manner, and may play a role in oxidative stress resistance. Oxidative damage that accumulates in cells during normal aging contributes to a variety of diseases and pathologies, and can result in an increase in age specific mortality. Accordingly, the oxidative stress theory of aging predicts that organisms with an increased tolerance to oxidative damage will have an increased longevity, while organisms with decreased tolerance will exhibit early mortality. Evidence is mounting, however, that many genetic manipulations that promote resistance to oxidative stress do not correlate with an increase in lifespan. Furthermore, many biochemical and genetic pathways involved in lifespan regulation do not have any known role in oxidative stress response. Here

we report the oxidative stress responses of adult *Drosophila* that over-express Sir2 or the nicotinamidase DNAAM. Both of these gene manipulations are known to increase adult lifespan; however only Sir2 endows strong, reproducible resistance to two different forms of oxidative stress. Sir2 is required for normal oxidative stress resistance, as mutant animals have virtually no tolerance to dietary paraquat and a reduced tolerance to hydrogen peroxide. In order to determine the efficacy of our experimental system, we tested two inert genes, GFP and LacZ, for the effects of over-expression on oxidative stress response. Interestingly, in male flies, small but significant increases in paraquat resistance could be observed when inert genes were expressed. This data highlights an important contribution of background genetics and/or expression system artifacts in eliciting stress response phenotypes. Thus, more comprehensive methodologies for controlling gene expression experiments should be required to confirm small effects on stress response and lifespan. Taken together our data suggests that a correlation between oxidative stress and increased longevity may be gene or gene-pathway dependant, and that pathways independent of oxidative stress response can contribute to increased lifespan.

AUTOBIOGRAPHICAL STATEMENT

Gregory Miller is a life-long native of Michigan who grew up in Grosse Pointe Park and received his undergraduate training at the University of Michigan. He currently lives with his fiancée, Emily Wood, in the city of Hamtramck MI.