An Exploration of the Genetics and Molecular Mechanisms Underlying Conserved Longevity Interventions

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Abstract

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Aging is a degenerative process that causes a time-dependent deterioration of virtually every biological system in the majority of species. Age is the primary risk factor for many human diseases, including the top causes of death modern societies. Developing treatments to slow the aging process has the potential increase human life span and simultaneously prevent or improve outcomes in countless diseases. Studying aging in mammals is challenging due to the relatively high longevity of most mammalian species and the costs associated with maintaining populations of mammals in the laboratory for their entire life span. The invertebrate organisms *Saccharomyces cerevisiae, Caenorhabditis elegans*, and *Drosophila melanogaster* have emerged as central models in aging due to relatively short life spans, ease of maintenance in the laboratory, well characterized genetics, and the availability of a wide range of genetic and biochemical tools. By focusing on genetic pathways and interventions that influence longevity in a similar manner across these evolutionarily divergent species, we can gain insight into the biology of aging in mammals. The application of genome-scale techniques in aging research has started to define the range of genetic and environmental factors involved in longevity determination, and the high degree of intercommunication between these factors. This dissertation reviews current progress toward identifying and understanding conserved longevity interventions and presents several current lines of investigation aimed both at developing tools for analyzing the complex interactions between aging factors and at probing the mechanism of action of specific longevity interventions.

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The first two chapters represent compiled review material from several sources related to the various aspects of the biology of aging. The sections on chronological aging were compiled by Brady Olsen.

A large portion of the early conceptual development of the epistasis system presented in Chapter 3 should be attributed to Kristen Steffen, as should construction of many of the strains and coordination of the replicative life span data collection.

Chapter 4 details two studies related to sirtuins. The first study exploring sirtuin overexpression in worms and flies (Burnett et al., 2011) was a collaborative effort involving multiple research groups at several institutions, and was led by David Gems and Linda Partridge at the University College London. The second study exploring yeast Sir2 (Delaney et al., 2011) represents a combined effort between Joe Delaney, a fellow graduate student in the Kaeberlein lab, and I. Many of the strains and rDNA assays from my portion of this work were performed by Ben Dulken.

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Introduction

Aging is commonly defined as a degenerative process characterized by a progressive decline in fitness resulting in mortality. Age is a primary risk factor for many of the top causes of death in the developed world, including heart disease, stroke, diabetes, and cancer. While biomedical research has traditionally focused on finding treatments for specific diseases, developing interventions to slow the aging process can potentially target a wide spectrum of ailments simultaneously. In fact, extending human longevity to a similar degree to that already achieved in multiple model organisms using dietary restriction is predicted to have a greater impact on life span than completely curing cancer, heart disease, stroke, and diabetes (Martin et al., 2003). The past few decades have seen an explosion of interest in the area of aging biology, and a great deal of effort is currently aimed at understanding the molecular processes and environmental factors that influence the rate of aging. The identification and characterization of genetic pathways that interact with these processes to modulate longevity is paramount to understanding why we age and developing therapeutic strategies for combating aging and age-related disease.

We are ultimately interested in understanding human aging, making mammalian models attractive targets for aging research. However, mammalian models for aging have several challenges. Life span is the primary endpoint to consider when studying aging, and directly measuring life span in mammals is difficult and expensive because of the relatively long life spans of most mammalian species. Aged populations of animals are also necessary for examining the age-related changes in secondary phenotypes, such as tissue degeneration or gene expression profiles. For this reason, research into mammalian aging has often been limited to looking at secondary endpoints that tend to correlate with longevity, such as stress-resistance and metabolic parameters. The cost and labor associated with maintaining relatively large cohorts of long-lived animals in a laboratory environment is particularly prohibitive to large-scale approaches looking for longevity phenotypes in mammals, such as high-throughput genetic or chemical screens. An alternative approach has been to develop model systems with characteristics that lend themselves to the types of studies common in aging research. These characteristics include short life span, rapid reproduction resulting in a large number of offspring, ease of maintenance in the laboratory environment, well-characterized biology including fully sequenced genomes, and availability of powerful tools for genetic manipulation. Three non-mammalian organisms have emerged as particularly prominent models of aging: the budding yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, and the fruit fly *Drosophila melanogaster*.

The use of non-mammalian models in aging-related research raises an important question: are findings in relatively simple eukaryotes applicable to human aging? From a genetic standpoint the relevant issue is whether genetic pathways that play a role in controlling aging and longevity in one species are common among evolutionarily divergent species or unique to that particular lineage. This raises two questions: is there good reason to think that aging is an evolved process, and can we identify genetic factors that play a conserved role in longevity determination across divergent species?

EVOLUTIONARY THEORIES OF AGING

"When one or more individuals have provided a sufficient number of successors they themselves, as consumers of nourishment in a constantly increasing degree, are an injury to those successors. Natural selection therefore weeds them out, and in many cases favors such races as die almost immediately after they have left successors." ~Alfred Russel Wallace, 1865–1870

How does a process that ultimately results in the death of each individual in a species evolve by natural selection? The first attempts by evolutionary biologists to answer this question followed closely on the heels of the theory of natural selection itself. The earliest written argument was made by Alfred Russel Wallace in an informal note, the essence of which is captured in the above quote (Wallace, 1889). August Weismann, a German biologist and evolutionary theorist, expanded Wallace's ideas into a theory describing aging as a programmed process to end an organism's life in the absence of accident or predation in order to make way for succeeding generations (Weismann, 1889). In contrast to these early theories, modern evolutionary biology views aging not as an advantageous trait that is selected for directly, but as a side-effect of a decline in the force of natural selection with increasing age combined with selection for other traits. Although the early "programmed aging" theories have lost favor, they illustrate the magnitude of the problem aging posed to evolutionary theory.

The models proposed by Wallace and Weismann describe aging as a programmed process that evolved through direct selection on senescence as a beneficial trait (Weismann, 1889). These models suffer from a reliance on group theory and do not provide an explanation for why an individual with a mutation that confers increased life span—and thus an increased opportunity to produce offspring—would not be selected over individuals without such a mutation (Kirkwood, 2005). This view of aging was challenged in 1952, when Peter Medawar proposed the "mutation accumulation" theory, revolutionizing the way most biologists think about the evolution of aging (Medawar, 1952). The underlying reasoning is based on the observation that, even in the absence of aging or other intrinsic

decline, most species experience a substantial rate of mortality from external forces such as accident, infectious disease, or predation. For a given species, fewer individuals live to progressively older ages, diminishing the force of natural selection in an age-dependent manner and resulting in stronger selection against genes that are deleterious early in life relative to genes that are deleterious late in life (Fisher, 1930; Haldane, 1941). The mutation accumulation theory states that genes with late-acting deleterious effects will accumulate in the germline, resulting in an increase in mortality with age (Medawar, 1952; Medawar, 1946).

George C. Williams refined Medawar's reasoning by incorporating the concept of pleiotropy. In the "antagonistic pleiotropy" model of aging, age-dependent increase in mortality is caused by an accumulation of genes that function to the benefit of the organism early in life but become deleterious with advanced age, thus providing a means by which senescence can be selected for indirectly (Williams, 1957). A related theory, proposed by Thomas Kirkwood and termed "disposable soma," states that natural selection will favor genes that promote redirection of resources from maintenance of soma to reproduction, resulting in an accumulation of damage that increases with age (Kirkwood, 1977).

The theories of mutation accumulation, antagonistic pleiotropy, and disposable soma all represent aging as a result of negligible natural selection with advanced age rather than a programmed process. An important extension of these arguments is that natural selection is concerned with overall fitness, which does not necessarily correlate with enhanced longevity (Kirkwood and Holliday, 1979). Organisms should therefore possess genes that optimize fitness and not maximize life span.

THE DISCOVERY OF CONSERVED AGING FACTORS

With a coherent evolutionary theory of aging established, the next question is whether conserved genetic determinants of longevity can be identified. The development of multiple, evolutionarily divergent models of aging provided a platform on which this question might be answered. As an extension of this idea, if genetic mechanisms can be identified that modulate life span across divergent simple eukaryotes, it is reasonable to expect that at least a subset of these mechanisms will be conserved in humans (Kaeberlein, 2004).

From the evolutionary insights by Medawar in the 1950s, our understanding of the aging process progressed steadily for roughly the next 30 years through the study of interventions that influence life span, such as dietary restriction and secondary age-associated phenotypes (e.g. stress resistance). The advances in genetics in the 1990s led to rapid identification of several key pathways and processes

involved in the determination of life span and the demonstration that these factors play a conserved role in aging across multiple species.

The best-studied example of a conserved longevity intervention is dietary restriction, which has been defined as a reduction in food consumption in the absence of malnutrition (Kennedy et al., 2007; Masoro, 2005; Spindler, 2010; Weindruch and Walford, 1988). Dietary restriction has long been known to increase life span in many different species, including yeast, worms, flies, and rodents (Chapman and Partridge, 1996; Fabrizio et al., 2004a; Good and Tatar, 2001; Jiang et al., 2000; Lakowski and Hekimi, 1998; Lin et al., 2000; McCay et al., 1935). Dietary restriction has also been reported to increase life span and health span in a nonhuman primate, the rhesus macaque, with the caveat that significance was achieved only when more than two-thirds of the deaths were censored as non-age-related (Colman et al., 2009). Several factors have been proposed to contribute to the health and longevity benefits of dietary restriction. To date, while dietary restriction is effective at extending life span in a variety of species, it remains unknown whether dietary restriction acts via similar mechanisms in different species, let alone whether dietary restriction can significantly improve longevity or health span in humans. Genetic interventions that extend life span in divergent species include reduced insulin/IGF-1-like signaling (IIS) (Bluher et al., 2003; Holzenberger et al., 2003; Kenyon et al., 1993; Tatar et al., 2001), increased sirtuin activity (Kaeberlein et al., 1999; Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001) and reduced target of rapamycin (TOR) signaling (Kaeberlein et al., 2005d; Kapahi et al., 2004; Powers et al., 2006; Vellai et al., 2003). Each of these pathways is discussed in greater detail in Chapter 1.

The genomics revolution of the past decade and the development of methodology for studying aging and life span determinants on the genomic-scale have provided the first glimpse of the large number of factors involved in controlling aging and the degree of conservation of these factors between evolutionarily divergent organisms. Our understanding of the aging process and its interaction with disease has also started to yield its first clinical applications in humans. Pharmacological agents targeting sirtuins and TOR signaling are now in clinical trials for treatment of age-associated pathologies such as cancer and metabolic disease. The discovery of new longevity genes and novel aspects of known aging pathways has accelerated at a break-neck pace and we expect that this knowledge will rapidly be translated to clinical practice as well.

In a broad sense, this dissertation is focused on understanding the molecular mechanisms underlying conserved longevity interventions. The first two chapters review the current state of research related to conserved aging biology, including a description of the three most common invertebrate models of aging and an overview of discoveries related to prominent conserved aging pathways (Chapter 1), and a discussion of current and emerging genome-scale methods and technologies for studying aging and what insights they have provided to date (Chapter 2). Chapter 3 provides a final report for a long-running

genome-wide screen for yeast genes involved in replicative life span determination and presents a formalized system for epistasis analysis of life span phenotypes developed to analyze complex relationships between different aging pathways. The remaining chapters discuss several studies aimed at developing a mechanistic understanding of specific conserved longevity interventions. These include a description of two recent studies examining the role of sirtuins in the aging processes of yeast, worms, and flies (Chapter 4), the characterization of the effects of caffeine on worm life span and health span (Chapter 5), and the investigation of the role of manganese homeostasis in the response of yeast to dietary restriction (Chapter 6).

Chapter 1: Common Non-Mammalian Models in Aging Research and the Identification of Conserved Aging Pathways

CHAPTER SUMMARY

The push to understand the aging process and its underlying molecular causes through genetic studies of longevity encompasses a variety of common model organisms ranging from the invertebrate budding yeast, nematodes, and flies to mammalian models such as mice and primates. The relative simplicity and low cost of conducting aging research in invertebrate models has led to the discovery of numerous interventions and genetic pathways that impact the aging process. By focusing on longevity-associated pathways with a high degree of evolutionary conservation, these findings can be leveraged to gain insight into mammalian aging. The depth of knowledge in this area has reached a point where meaningful comparisons can be made between models to understand which genetic aspects of aging are conserved among evolutionarily divergent organisms and which are unique to a specific lineage. Such comparative analyses have identified dietary restriction, IIS, TOR signaling, and sirtuins as conserved longevity pathways. This chapter reviews the three most commonly used invertebrate models of aging and several of the most studied conserved longevity interventions in these models.

INTRODUCTION

One of the primary goals of aging research is the development of interventions to impede the aging process in an effort to fight age-associated pathologies in humans. From the standpoint of human aging, it is optimal to investigate interventions in mammalian systems; however, directly studying longevity is difficult and expensive in mammals due to their long life spans. For example, life span experiments require approximately 3 years in mice, 25 years in rhesus monkeys, and are ethically and functionally impractical in humans. To circumvent these difficulties, short-lived, genetically tractable organisms have been developed as models of aging biology.

The rationale that conserved longevity interventions are more likely to be relevant for human health than species-specific interventions has spurred the hunt for genes, pharmacological agents, and environmental alterations that function to modulate aging in multiple evolutionarily divergent species (Kaeberlein, 2004). This chapter describes the current state of knowledge in this area. First, the methods used to study aging in each of the three most common invertebrate model systems are discussed. Second, the major classes of conserved aging genes are described. Finally, an overview is presented of the complex relationships between known conserved pathways that influence aging and how they interact with the response to environmental nutrients.

INVERTEBRATE AGING MODELS

Aging has been studied in a wide variety of model organisms, both mammalian and non-mammalian. Invertebrate organisms have emerged as the preeminent model systems in aging research. The most prominent are the budding yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster*, which share a number of characteristics that make them ideal for aging research: relatively short life span, rapid production of large numbers of offspring, ease of maintenance and manipulation in the laboratory environment, well characterized biology, fully sequenced genomes, and the availability of powerful genetic tools. Several other non-mammalian organisms are also actively being used to study aging on a smaller scale, including bacteria (Ackermann et al., 2003; Nystrom, 2007; Stewart et al., 2005), fission yeast (Barker and Walmsley, 1999; Roux et al., 2006), other nematode and fly species (Carey et al., 2002; Davies et al., 2005; Sutphin and Kaeberlein, 2008), and fish (Terzibasi et al., 2007; Valenzano et al., 2006). This chapter focuses primarily on comparative genetics of aging in the three most common models.

THE BUDDING YEAST *SACCHAROMYCES CEREVISIAE*

S. cerevisiae has been used as a model organism for aging research for more than 60 years (Mortimer and Johnston, 1959). Since the life span is a primary endpoint in aging research, using yeast as an aging model immediately raises a vital question: at what point is a yeast cell considered dead? When dealing with macroscopic animals the answer to this question is fairly straight-forward: an animal is considered dead at the point of failure of the majority of its macro-scale systems (e.g. the respiratory system and the circulatory system). Individual cells that are technically still alive will only remain so for a period of time that is short relative to the life span of the animal. To an extent this is also true for smaller animals that are on the verge between the micro- and macroscopic worlds, such as worms and flies. While the failure of major systems is more difficult to judge, by general acceptance these animals are considered dead at the point when they fail to respond to external stimuli.

The path is less clear for single cell systems. In fact, two distinct paradigms have been defined for yeast aging: chronological and replicative (Steinkraus et al., 2008) (Figure 1.1). Replicative life span refers to the number of cell divisions an individual cell completes before undergoing replicative senescence (Kaeberlein, 2006; Mortimer and Johnston, 1959). Chronological life span refers to the length of time that a yeast cell can retain viability in a non-dividing state (Fabrizio and Longo, 2003; Fabrizio et al., 2001; Kaeberlein, 2006). Replicative life span might be considered analogous to aging in mitotic tissue, such as skin and blood, and chronological life span to aging in non-mitotic tissue, such as heart or brain, although it is unclear if this analogy, based on the proliferative potential of mammalian tissues, truly holds. Indeed, accumulating evidence suggests a considerable degree of conservation in factors that influence aging, even between mitotic models, such as yeast replicative aging and primarily non-mitotic models, such as *C. elegans*, in which only the germline is mitotically active during adulthood.

Yeast Replicative Aging

Replicative life span is the older of the two yeast aging models and has been studied in greater detail. Including the study outlined in Chapter 3, more than 200 genes are reported for which reduced expression increases yeast replicative life span to date (Bitterman et al., 2003; Jazwinski, 2000; Steinkraus et al., 2008). Replicative life span is measured by microdissection of daughter cells away from mother cells while tallying the number of daughters produced at each age point (Steffen et al., 2009). Replicative longevity varies widely among different laboratory strains, with the most strains having an average replicative life span between 18 and 26 generations (Kaeberlein, 2006). The most extensively

studied yeast strains are the parental strains of the yeast open reading frame (ORF) deletion collection, which are closely related to the *S. cerevisiae* wild type strain S288C (Kaeberlein et al., 2005b; Mortimer and Johnston, 1986). This collection has been used for genome-wide screens for single-gene deletions that increase either chronological life span or replicative life span (Chapter 3) (Kaeberlein et al., 2005d; Powers et al., 2006). Environmental parameters such as temperature and medium composition are also known to influence replicative life span. One primary molecular cause of replicative aging in yeast is thought to be the mother cell-specific accumulation of extrachromosomal ribosomal DNA circles (ERCs) (Defossez et al., 1999; Sinclair and Guarente, 1997), although additional uncharacterized factors are also known to contribute to replicative aging. Evidence suggests that these factors may include age-associated genomic instability, mitochondrial retrograde signaling, accumulation of oxidatively damaged proteins in the mother cell, and altered histone acetylation near telomeres (Aguilaniu et al., 2003; Dang et al., 2009; Kaeberlein et al., 1999; Kirchman et al., 1999; McMurray and Gottschling, 2003).

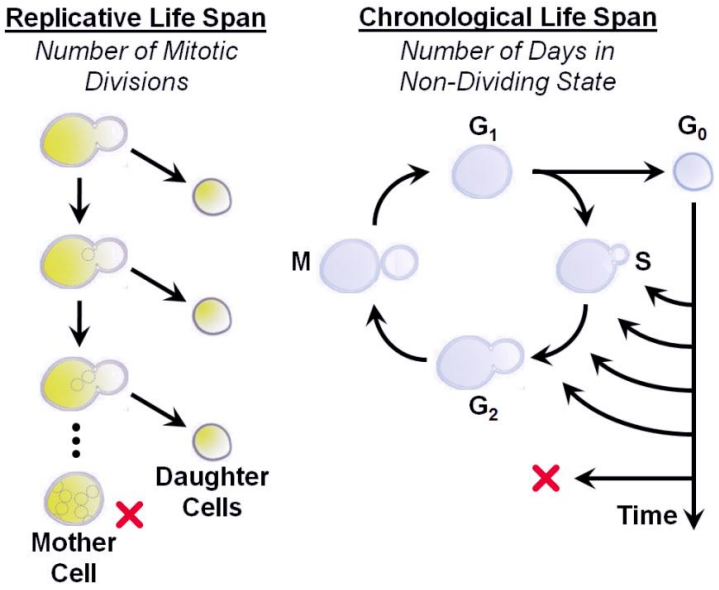


Figure 1.1. Yeast aging models. Two models of aging are used in yeast. Replicative life span (left) is a measure of the number of cell divisions a cell completes before undergoing replicative senescence. Chronological life span (right) is a measure of the number of days a cell remains viable in a post replicative state.

Dietary restriction has been studied in the context of replicative life span, most commonly by reducing the glucose content of the growth medium, although other forms include reducing the amino acid content of the medium or by replacing the media glucose with an alternative carbon source (Delaney et al., 2011; Jiang et al., 2000; Lin et al., 2000). Replicative life span extension has been reported at multiple glucose concentrations ranging from 0.5 to 0.005% glucose (Kaeberlein, 2006; Lin et al., 2002) compared to the standard concentration in yeast medium of 2%. The glucose concentration at which replicative life span is maximally extended is dependent on the genetic background of the strain, and there has been substantial debate regarding whether the mechanism by which dietary restriction extends replicative life span is similar at different glucose concentrations (Kaeberlein and Powers, 2007).

Yeast Chronological Aging

Chronological aging has typically been measured by culturing cells into a post-diauxic quiescent-like state in synthetically defined growth medium and monitoring the viability of cells over time. Viability is defined by the ability of cells to reenter the cell cycle and resume vegetative growth in the presence of a nutrient-rich medium (Fabrizio and Longo, 2003; Murakami and Kaeberlein, 2009). Alternative growth conditions for monitoring chronological aging have been described but not widely used, including maintaining cells in water at a high temperature after 2–3 days of standard culture and aging cells in rich growth medium rather than synthetic defined medium (Harris et al., 2001; Piper et al., 2006). Like replicative life span, chronological life span varies among different laboratory strains and is robustly influenced by the composition of the growth medium (Fabrizio et al., 2005; Murakami et al., 2008; Smith et al., 2007a). Chronological senescence is correlated with an accumulation of oxidatively damaged proteins, mitochondrial dysfunction, and induction of the yeast apoptotic-like response (Aerts et al., 2009; Fabrizio et al., 2003; Herker et al., 2004). Recently, acetic acid toxicity associated with acidification of the growth medium has been identified as a primary molecular cause of chronological senescence under standard conditions (Burtner et al., 2009). Although the molecular causes of chronological senescence appear to be distinct from replicative senescence, chronologically aged cells show a reduced replicative life span, suggesting that underlying similarities may exist (Ashrafi et al., 1999).

Similar to the case for replicative aging, life span extension from dietary restriction in the yeast chronological aging paradigm can be accomplished by reducing the glucose concentration of the growth medium from 2 to 0.5% or lower (Murakami et al., 2008). A form of extreme dietary restriction has also

been described in which cells are transferred from expired growth medium to water after 2–4 days of aging (Fabrizio et al., 2005).

Contributions of Yeast to Our Understanding of Aging

S. cerevisiae has several features that make it useful as a model organism for aging research, including short life span, well-characterized genetic and molecular methods, low relative cost, cell type homogeneity, and a vast organismal information base. These advantages have facilitated unbiased screens for genes that influence life span in yeast, as well as candidate gene approaches. Several dozen genetic determinants of yeast longevity have been identified from these studies, at least some of which appear to play a conserved role in the aging of multicellular eukaryotes. These advantages have made yeast a prominent model for aging genetics and has been instrumental in the discovery and characterization of several of the best studied genetic pathways involved in life span determination. These pathways include dietary restriction, sirtuins, TOR signaling, and mitochondrial metabolism (Tables 1.1 and 1.2).

One tool that has greatly facilitated studies of longevity and other processes in yeast is a collection of isogenic single-gene deletion strains encompassing a majority of non-essential ORFs in the yeast genome. The yeast ORF deletion collection contains more than 20,000 unique strains (6,061 single ORF deletions in one or more strains), with full-genome collections of homozygous and heterozygous diploids as well as haploid deletions in both mating types (Winzeler et al., 1999). Screens have been carried out across this deletion collection for many different phenotypes, including sensitivity to a variety of stresses, metabolism of different carbon sources, and growth rate (Que and Winzeler, 2002; Scherens and Goffeau, 2004). Essentially any yeast-based assay that can be modified for moderate- or high-throughput capacity can, in principle, be used in conjunction with the deletion collection to perform genome-wide queries of the process under study. Chapter 2 describes large-scale studies of both replicative and chronological life span using the yeast ORF deletion collections in detail and touches briefly on alternative strategies for studying aging in yeast.

Table 1.1 Genetic interactions common aging interventions with respect to replicative life span. The data in this table was compiled from numerous sources (Kaeberlein et al., 2004, 2005b; Kaeberlein et al., 1999; Kaeberlein et al., 2005d; Lamming et al., 2005; Lin et al., 2000; Tsuchiya et al., 2006). Table entries indicate the effect of each intervention on replicative life span in each genetic background (N.E. = no effect).

	Genetic background			
Intervention	***sir2Δ***	***fob1Δ***	***sir2Δ fob1Δ***	***tor1Δ***
Dietary restriction (0.05% glucose)	N.E.	↑	↑	N.E.
tor1 Δ	N.E.	↑	↑	N.E.
SIR2 overexpression	N.E.	N.E.	N.E.	↑
fob1 Δ	↑	N.E.	N.E.	↑

Early Investigations of Yeast Replicative Aging

The first conceptual development of replicative aging in yeast centered on the observation that mother cells retained a chitinous ring of scar tissue, termed a "bud scar", that was visible under a microscope following each cell division that marked the site of cytokinesis. Following the characterization of bud scars and the finite replicative capacity of yeast cells (Barton, 1950), a first study designed to investigate a cause for aging in yeast was reported by Mortimer and Johnston (1959). Although the term "replicative life span" was not applied to the method until later, the study used a microdissection assay essentially identical to what is commonly used in replicative life span assays today to test the hypothesis that the number of cell division a yeast cell undergoes is limited by the cell surface area, based on the observation that permanent, non-overlapping bud scars remained on the cell surface following each division (Bacon et al., 1966; Barton, 1950; Seichertova et al., 1973). They found instead that, as the cell divides, the surface area increases at a rate that more than compensates for the bud scar area, leading to the speculation that reduced surface-to-volume ratio may limit metabolic processes. While early ideas for the cause of yeast replicative aging are now largely dismissed in favor of recent models, the concept of yeast replicative aging itself has become mainstream. After this initial foray, replicative aging was virtually neglected for more than two decades, when Muller et al. (1980) provided an important characterization of yeast replicative aging by demonstrating that the number of mitotic divisions, and not the time elapsed since budding, was the limiting factor in replicative life span, a finding supported by the observation that cultured cells do not lyse immediately after senescing (Mortimer and Johnston, 1959).

Table 1.2 Effects of common yeast aging interventions on life span and secondary, age-associated phenotypes. The data in this table was compiled from numerous sources (Bonawitz et al., 2007; Bryk et al., 1997; Defossez et al., 1999; Fabrizio et al., 2005; Fritze et al., 1997; Gottlieb and Esposito, 1989; Huang and Moazed, 2003; Kaeberlein et al., 2005a; Kaeberlein et al., 2004, 2005b; Kaeberlein et al., 1999; Kaeberlein and Powers, 2007; Lin et al., 2000; Lin et al., 2002; Longo et al., 1997; Riesen and Morgan, 2009; Rusche et al., 2003; Smith et al., 2009; Smith and Boeke, 1997; Wang et al., 2008; Wei et al., 2008; Wei et al., 2009).

	Phenotype						
Intervention	**Replicative Life Span**	**Chronological Life Span**	**Oxidative Stress Resistance**	**Thermo-tolerance**	**Telomere Silencing**	**rDNA Recombination**	**rDNA Silencing**
Dietary restriction (0.05% glucose)	↑	↑	↑	↑	No effect	No effect	No effect
tor1 Δ	↑	↑	↑	↑	No effect	↓	No effect
sir2 Δ	↓	No effect	↓	?	↓	↑	↓
fob1Δ	↑	?	?	?	?	↓	↓
sir2 Δ fob1Δ	No effect	?	?	?	?	↓	?

Sirtuins and the ribosomal DNA

The role of sirtuins in life span determination was first discovered using the yeast replicative model of aging and is closely linked to the influence of ERCs on yeast aging. The silent information regulator (SIR) complex was first identified in a screen for stress resistance and maintenance of viability at 4°C, two phenotypes that correlate with longevity in the strain background used for this screen (Kennedy et al., 1995). The SIR complex includes Sir2, Sir3, and Sir4 and acts to repress transcription at telomeres, ribosomal DNA (rDNA), and the silent mating-type locus (Rusche et al., 2003). The screen specifically identified a semi-dominant mutation in *SIR4*, *sir4-42*, that resulted in a redirection of the Sir2 complex from the telomeres and silent mating-type locus to rDNA (Kennedy et al., 1995; Kennedy et al., 1997). Sir2, a conserved NAD-dependent histone deacetylase, has since emerged as the vital component of the SIR complex with respect to aging. In addition to its role in the SIR complex, Sir2 has a Sir3- and Sir4-independent role in preventing rDNA recombination and has been shown to silence a Pol II gene artificially inserted into the rDNA (Table 1.2) (Bryk et al., 1997; Defossez et al., 1999; Gottlieb and Esposito, 1989; Smith and Boeke, 1997). Mutants lacking *SIR2* have a life span that is roughly 50%

shorter than wild type, while overexpressing *SIR2* extends replicative life span by 30–40% (Kaeberlein et al., 2004; Kaeberlein et al., 1999; Kennedy et al., 1995).

Egilmez and Jazwinski (1989) first suggested that deleterious factors may accumulate with age in yeast cells and contribute to replicative senescence based on changes in generation time with replicative age in yeast. Yeast cell division is asymmetric, with the mother retaining a larger portion of the cell contents than the daughter. This phenomenon gives the mother cell the potential to preferentially retain the majority of deleterious factors that accumulate with age resulting in daughter cells with renewed replicative capacity (Egilmez and Jazwinski, 1989; Kennedy et al., 1994). ERCs represent the first such aging factor identified (Sinclair and Guarente, 1997). Yeast rDNA consists of a tandem repeat of a 9.1 kb sequence coding for the ribosomal RNA (Petes and Botstein, 1977; Philippsen et al., 1978; Rustchenko and Sherman, 1994). ERCs form through homologous recombination between rDNA repeats and accumulate with age in mother cells as a consequence of two factors: (1) a replication of origin within the rDNA that allows ERCs to self-replicate, and (2) the lack of a CEN element, causing biased segregation toward the mother cell during asymmetric division (Murray and Szostak, 1983). Cell senescence is thought to occur when ERCs accumulate past an unknown threshold level.

Several lines of evidence support a model in which Sir2 promotes longevity by preventing rDNA recombination and thus inhibiting ERC formation. First, deletion of *SIR2* increases rDNA recombination by 6–10 fold, increases ERC accumulation, and reduces replicative life span (Table 1.2), while overexpression of *SIR2* extends life span (Kaeberlein et al., 1999). Second, deletion of *FOB1*, a replication fork barrier protein with rDNA-specific activity that increases rDNA recombination, extends replicative life span, dramatically reduces ERC levels, and prevents the short replicative life span caused by deletion of *SIR2* (Table 1.2) (Defossez et al., 1999; Kaeberlein et al., 1999).

The life span characteristics of *sir2Δ* and *fob1Δ* strains suggest that promotion of ERC formation is not the only mechanism by which Sir2 influences longevity. Similar ERC levels are observed in both *sir2Δ fob1Δ* and *fob1Δ* strains (Kaeberlein et al., 1999); however, *FOB1* deletion alone results in extension of replicative life span relative to wild type, while deletion of both *SIR2* and *FOB1* together results in a replicative life span similar to wild type (Table 1.2). This suggests that Sir2 has a pro-longevity function independent of both Fob1 and ERC accumulation. Further support for this idea comes from the recent finding that life span extension by *SIR2* overexpression is largely dependent on *SIR3*, while inhibition of rDNA recombination is *SIR3*-independent (Dang et al., 2009). This study also shows that Sir2 protein levels decline with increasing age, resulting in enhanced histone H4K16 acetylation at a variety of sub-telomeric sites, and potentially others. Together these data suggest a model whereby increased Sir2 activity leads to altered transcription at key non-nucleolar loci resulting in activation of a second pathway influencing life span in yeast. A second possibility for an ERC-independent role for Sir2

in aging is increased oxidative stress resistance, which stems from the finding that *SIR2* overexpression suppresses the short life span of yeast exposed to hydrogen peroxide (Oberdoerffer et al., 2008). This model is supported by the finding that yeast lacking Sir2 are unable to maintain asymmetric segregation of hydrogen peroxide and carbonylated proteins to the mother cells during division (Aguilaniu et al., 2003; Erjavec and Nystrom, 2007). Erjavec and Nystrom (2007) found that the reduction in hydrogen peroxide results from a Sir2-dependent segregation of the cytosolic catalase Ctt1 toward the daughter cell during division. Sirtuin-associated life span extension has also been linked to oxidative damage in nematodes (Hekimi and Guarente, 2003). Another possible mechanism is highlighted by several prior studies implicating Sir2 in mediating repair of DNA damage (Lee et al., 1999; Martin et al., 1999; McAinsh et al., 1999; Mills et al., 1999; Tamburini and Tyler, 2005).

Asymmetric Segregation, Oxidative Damage, and Mitochondria

An aspect of yeast replicative aging that has generated much interest and has already been touched on several times in this chapter is the ability of mother cells to generate daughters with renewed replicative potential. The disparate replicative potential between mother and daughter suggests that the yeast cell divides asymmetrically, with the mother retaining and accumulating one or more "aging factors", thus sacrificing its own replicative potential to promote that of the daughter (Egilmez and Jazwinski, 1989; Kennedy et al., 1994). ERCs, discussed above with respect to sirtuins, were the first example of such an aging factor (Sinclair and Guarente, 1997). Two additional cellular components, dysfunctional mitochondria and oxidatively damaged proteins, have more recently been implicated as potential candidates.

Near the end of a mother cell's replicative life span the division asymmetry between mother and daughter breaks down, resulting in daughters with reduced replicative potential (Jazwinski et al., 1989; Johnston, 1966; Kennedy et al., 1994). Lai et al. (2002) performed a screen for temperature sensitive mutants lacking division asymmetry and identified mutants that exhibited clonal senescence at the restrictive temperature. One of these mutations was identified as a point mutation in *ATP2,* encoding the β-subunit of the mitochondrial ATP synthase. The *ATP2* mutants showed a time-dependent loss in mitochondrial membrane potential followed by a loss of mitochondrial mass, particularly in younger cells. They also found that older mother cells tended to segregate dysfunctional mitochondria to their daughters and propose dysfunctional mitochondria as an asymmetrically segregated aging factor in normal replicative aging. A later study found that the abnormal segregation of mitochondria in *ATP2*

mutants can be rescued by overexpression of Pex6, a peroxin protein, and suggested that Pex6 may promote mitochondrial biogenesis (Seo et al., 2007).

Another potential category of aging factor are reactive oxygen species (ROS), which have long been at the center of the debate on causes of aging and are a central player in the free-radical theory of aging. One form of oxidative damage that is considered irreversible and has been correlated with age in various organisms, including replicative age in yeast, is protein carbonylation (Nystrom, 2005). Protein carbonyls have been proposed as a yeast aging factor based on the observations that both protein carbonyls (Aguilaniu et al., 2003; Erjavec and Nystrom, 2007) and aggregates containing heavily carbonylated proteins (Erjavec et al., 2007) are asymmetrically retained in mother cells during division. The proper asymmetric segregation of oxidatively damaged proteins appears to be dependent on a functioning actin cytoskeleton (Aguilaniu et al., 2003; Erjavec et al., 2007), which has independently been linked to ROS and life span through the actin bundling protein, Scp1 (Gourlay et al., 2004).

The Retrograde Response and Mitochondrial Back-Signaling

Another process related to the mitochondria that has been linked to regulation of yeast replicative life span is the retrograde response, a signaling pathway that alters the expression of metabolic and stress response genes in response to mitochondrial dysfunction (Epstein et al., 2001). Changes in metabolic gene expression induced by the retrograde response cause a shift in cellular metabolism to the preferential use of lipid/acetate as a carbon source. Acetate is processed through the glyoxylate cycle, an efficient alternative to the TCA cycle. This shift is thought to be a compensatory mechanism for dealing with a progressive age-dependent decline in mitochondrial function (and therefore TCA cycle activity) (Jazwinski, 2004). Genetic and environmental interventions that induce the retrograde response lead to an extension of replicative life span in a manner that is dependent on *RTG2*, a gene coding for key signaling enzyme in the retrograde response pathway (Kirchman et al., 1999). Retrograde signaling is regulated upstream by both TOR (Komeili et al., 2000; Tate and Cooper, 2003) and RAS (Kirchman et al., 1999) through the Mks1 transcription factor (Matsuura and Anraku, 1993; Pierce et al., 2001).

Interestingly, the induction of the retrograde response is also associated with an increase in ERC production (Conrad-Webb and Butow, 1995). In addition to its role in retrograde response signaling, Rtg2 is a suppressor of ERCs (Borghouts et al., 2004). The two roles apparently cannot be performed simultaneously, as Rtg2 ERC suppression is reduced while the retrograde response is active (Borghouts et al., 2004). An aging cell may therefore have to balance the benefits of activating the retrograde response against the deleterious effects of ERC accumulation.

A second pathway related to mitochondria function was recently discovered when a study identified *MRPL25*, which encodes a component of the large subunit of the mitochondrial ribosome, as a mediator of replicative life span (Heeren et al., 2009). Deletion of *MRPL25* caused respiratory deficiency, increased oxidative stress resistance, and extended median replicative life span by 60% in a manner that was non-additive with deletion of *TOR1*. Mutants lacking *MRPL25* were also resistant to growth inhibition by rapamycin and blocked cytoplasmic translocation of the Sfp1 transcription factor from the nucleus in response to treatment with rapamycin. The mechanism for replicative life span extension by deletion of *MRPL25* appears to involve signaling from the mitochondria to the nucleus through Sfp1, suggesting a possible link to the retrograde response; however, Heeren et al. (2009) observed increased replicative life span in the absence of detectable retrograde response. To distinguish the two signaling pathways, they coined the term "mitochondrial back-signaling". Mitochondrial back-signaling thus represents a pathway linking the mitochondria to TOR signaling with respect to replicative life span.

Loss of Heterozygosity

One age-related pathology not intuitively associated with aging in a single-celled organism is cancer. Even though yeast cannot get cancer in the same sense as multicellular eukaryotes, working with yeast has many practical advantages over working in multicellular systems or cell culture and yeast models have been developed to study the events that give rise to cancer. As humans age, we experience an exponential increase in the incidence rate of many cancers (DePinho, 2000) which is thought to arise from genetic mutation (Knudson, 2001). The observation that normal mutation rates in human tissue culture cannot account for the diversity of genetic mutation in most cancers had led to the hypothesis that cells undergo genetic changes that result in an increased mutation rate early in the development of cancer (Loeb, 1991; Loeb et al., 2003; Nowak et al., 2002). Related to this hypothesis is the question of whether mutation rates inherently increases with age.

To address this question, McMurray and Gottschling (2003) developed a system in yeast to quantify one type of mutation. In diploid yeast, heterozygous cells with one normally functioning allele and one non-functioning allele of a particular gene usually show a wild type phenotype, with the normal allele compensating for the mutant allele and allowing normal function of the gene (excepting genes for which haploinsufficiency is relevant). Such individuals are particularly susceptible to loss of function mutations that inactivate the normal allele of the gene, an event termed "loss of heterozygosity". McMurray and Gottschling (2003) inserted a normal copy of a gene affecting colony color into one copy of a chromosome, creating an artificial heterozygous locus. By allowing single mother cells to divide and

monitoring the color of colonies produced by individual daughter cells, they were able to measure loss of heterozygosity as a function of the mother cell's replicative age. Indeed, the authors observed a marked increase in loss of heterozygosity with age (McMurray and Gottschling, 2003).

Importantly, while loss of heterozygosity increases with replicative age in yeast (Carr and Gottschling, 2008), the rate of increase does not appear to correlate with the replicative life span of the strain (McMurray and Gottschling, 2003). Loss of heterozygosity is therefore interesting as a model to study mutation rates with respect to cancer, but probably not relevant to the intrinsic aging process in yeast.

Apoptosis

Cell suicide, or apoptosis, is a well-studied biological phenomenon in multicellular organisms that allows specific cells to be removed during the development of complex tissues or potentially dangerous damaged cells to be destroyed for the benefit of the whole organism. The lack of an apparent evolutionary benefit for such a process in a single-celled organism initially caused controversy about the presence of an apoptotic pathway in yeast. Today, however, a number of yeast orthologs to mammalian apoptosis genes have been discovered and apoptotic-like cell death has been linked to mating, colony formation, and aging (Buttner et al., 2006; Eisenberg et al., 2007; Frohlich et al., 2007). With respect to aging, both replicatively and chronologically aged cells that die have increased ROS and display apoptotic phenotypes (Fabrizio et al., 2004a; Herker et al., 2004; Laun et al., 2001).

The known causative role for oxidative damage in apoptosis combined with the increased ROS in aged yeast cells, the role of mitochondria in producing ROS, and the asymmetric distribution of dysfunctional mitochondria to mother cells during division suggest that apoptosis may play a role in yeast aging through changes in mitochondrial function. Mitochondria and oxidative stress have been connected with both forms of yeast aging. In the replicative paradigm, overexpression of *NDE1* or *NDE2*, which encodes components of the yeast electron transport chain, extends life span (Lin et al., 2004), consistent with a model where increasing electron transport chain efficiency inhibits aging via decreased ROS production (Korshunov et al., 1997; Starkov, 1997). In support of this model, enhancing respiration through mitochondrial uncoupling leads to a decrease in ROS production and an increase in both replicative and chronological life span (Barros et al., 2004; Starkov, 1997). In the chronological paradigm, overexpression of superoxide dismutase Sod1 or Sod2 extends life span and deletion of *SOD2* prevents the life span extension resulting from deletion of *CYR1*, which encodes an adenylate cyclase required for production of cyclic AMP that controls a variety of downstream processes including

metabolism and stress resistance, or *SCH9* (Fabrizio et al., 2003). Acetic acid, which is known to induce apoptosis (Ludovico et al., 2001), has also recently been identified as a primary mechanism of chronological aging in yeast (Burtner et al., 2009).

The importance of apoptosis in yeast aging has yet to be fully characterized. At the very least, yeast apoptosis provides a useful pathway for studying genetic interactions for age-related diseases that affect humans, such as cancer. Readers interested in further information related to yeast apoptosis are referred to several in-depth reviews (Buttner et al., 2006; Eisenberg et al., 2007; Frohlich et al., 2007).

A great deal of progress has been made in advancing our understanding of yeast aging through genetic and, of late, genomic studies. Through these studies a large number of genes involved in the aging process have been identified. Collectively, the field has been quite successful at extending both chronological and replicative life span. Most of the aging genes identified thus far are regulatory components and include genes involved in signal transduction, transcription, or translation. Homologs of a subset these genes are likely to have similar effects in mammals. Regulatory factors are often pleiotropic in function and it remains unclear which downstream targets drive aging in yeast. The next challenge facing aging researchers is to use the available knowledge of these regulatory factors to work downstream and uncover the spectrum of molecular events that lead to age-associated deterioration in yeast and other organisms. ERCs and acetic acid represent the first steps down this path in the replicative and chronological aging paradigms, respectively, but these factors are only part of the story. What else is involved? Reactive oxygen species and DNA damage? Mitochondrial degeneration? Loss of protein homeostasis? Epigenetic drift? Some as yet unidentified molecular mechanism? The answer will likely involve some or all of these possibilities. We anticipate that research in the coming years, driven in combination by unbiased genome-scale longevity studies and focused hypothesis-driven experiments, will provide the answers.

THE NEMATODE *CAENORHABDITIS ELEGANS*

C. elegans has arguably become the most informative model organism for genetic studies of basic mechanisms of aging. When measured under standard conditions (20°C on solid nematode growth medium), the life span of the common lab strain (N2) is about 3 weeks. Life span in wild type worms is strongly influenced by temperature, and can range from a little over two weeks at 25°C to more than four weeks at 15°C for wild type worms at commonly studied temperatures. *C. elegans* are typically fed a diet of *Escherichia coli* OP50 bacteria grown as a lawn on the surface of an agar plate and viability is determined by the ability of adult animals to move in response to touch (Sutphin and Kaeberlein, 2009).

The *C. elegans* life cycle takes around 3 days and consists of externally laid eggs, four larval stages, and a reproductively active adult stage. The majority of adult animals are hermaphrodites that self-fertilize to produce several hundred offspring per adult. Rare male worms arise spontaneously and mate with hermaphrodites to produce broods that are half male and half hermaphrodite. Cells in adult animals are post-mitotic with the exception of the germline.

Studies in *C. elegans* have identified more than 300 genes that are associated with increased life span when their function is diminished (Braeckman and Vanfleteren, 2007; Smith et al., 2008b). Most of these genes were identified from large-scale RNA interference (RNAi) screens carried out using libraries that cover roughly 90% of the known ORFs in the nematode genome (Arum and Johnson, 2007; Chen et al., 2007a; Curran and Ruvkun, 2007; Dillin et al., 2002; Hamilton et al., 2005; Hansen et al., 2005; Lee et al., 2003). RNAi is particularly powerful in *C. elegans*, as efficient gene knockdown can be achieved by simply feeding animals bacteria expressing double-stranded RNA with sequence corresponding to the gene of interest. Many of the currently known *C. elegans* aging genes can be broadly classified, based on epistasis grouping and known or predicted function, into one or more of the following classes: (1) IIS, (2) mitochondrial function, (3) protein synthesis/mRNA translation, (4) chemosensory function, (5) dietary restriction, or (6) hypoxic response.

The molecular mechanisms that cause *C. elegans* to age are not known, but analysis of tissue-specific aging has led to the conclusion that neuronal cells largely retain function in old animals, while muscle cells in many animals show a gradual decline in function beginning near the transition to the post-reproductive stage of adulthood (Herndon et al., 2002). Associated with this general decline in muscle function is a decrease in pharyngeal pumping, resulting in reduced food consumption (Huang et al., 2004; Kenyon et al., 1993; Smith et al., 2008a), and an accumulation of autofluorescent age pigment throughout the body (Gerstbrein et al., 2005; Klass, 1977). If a live food source is used, bacterial colonization of the gut can also contribute to senescence; however, the relevance of this to normal aging is unclear, as animals fed a killed bacterial food source show a similar progression of age-associated phenotypes with life span extended by only a few days (Garigan et al., 2002; Garsin et al., 2003).

Dietary restriction in *C. elegans* has been studied using a variety of methods and there is currently little consensus regarding which methods are most appropriate (Greer and Brunet, 2009; Mair et al., 2009). Most methods of dietary restriction in *C. elegans* involve reducing the amount of bacterial food provided to the worms, but differ in whether the food is alive or killed, whether the growth environment is solid agar-based or liquid, and whether the amount of food is constant or varied (akin to feeding/fasting cycles in rodents) over the course of the experiment (Greer and Brunet, 2009). Under at least some conditions on agar-based medium, complete removal of the bacterial food during adulthood has been observed to increase life span maximally, a dietary restriction regimen referred to as bacterial deprivation

(Kaeberlein et al., 2006b; Lee et al., 2006). Age at onset of dietary restriction also varies from study to study and may influence the resulting life span; however, at least in the case of bacterial deprivation, similar median and maximal life span extension has been demonstrated for dietary restriction initiated between day 4 and day 14, with similar maximal life span extension achieved for dietary restriction initiated as late as day 24 (Smith et al., 2008a).

THE FRUIT FLY *DROSOPHILA MELANOGASTER*

D. melanogaster is the earliest invertebrate player in aging research, with studies of life span dating back to 1916 (Loeb and Northrop, 1916). The fly life cycle lasts 1 to 2 weeks and consists of three easily distinguishable growth stages (embryo, larva, and pupae) followed by the reproductively active adult stage. Similar to *C. elegans*, the majority of the cells in the adult fruit fly are post-mitotic, with exceptions in the germline and a subset of gut cells. Flies are typically maintained in vials with a cornmeal–sugar–yeast or sugar–yeast agar-based food source. Unlike yeast and worms, flies cannot be frozen and must be actively maintained. Wild type *D. melanogaster* has a median life span between 1 and 2 months when maintained at 25°C.

The fruit fly has been used extensively to explore non-genetic environmental manipulations that extend life span. Dietary restriction can be accomplished by diluting yeast or other components in the food source (Bass et al., 2007a; Chapman and Partridge, 1996; Good and Tatar, 2001). Fruit flies also experience a strong inverse relationship between environmental temperature and life span (Helfand and Rogina, 2003; Miquel et al., 1976), and brief exposure to mild stressors such as high temperature or low-level radiation can result in increased life span (Hercus et al., 2003; Le Bourg et al., 2004; Vaiserman et al., 2003). Flies generally have a strong inverse correlation between reproduction and longevity. Strains bred for longevity by selecting offspring from late life reproduction show reduced egg laying early in life relative to ancestral strains (Luckinbill et al., 1984; Rose, 1984). In *Drosophila subobscura*, life span extension observed in response to dietary restriction is accompanied by a reduction in egg production (Marden et al., 2003). Preventing mating can also double female life span (Smith, 1958), though, in *D. melanogaster*, seminal factors have been implicated in shortening female life span as opposed to some intrinsic cost associated with reproduction (Ueyama and Fuyama, 2003).

As a model system, *Drosophila* offers a variety of powerful genetic techniques for studying aging at a genetic level. While high-throughput methodology for studying life span has yet to be developed, gene and pathway-specific approaches, as well as smaller scale candidate gene and random mutation studies, have been useful in testing a variety of aging theories and in identifying new players in fly aging.

Drosophila genes that play a role in modifying aging have been identified in a variety of pathways, including IIS, mitochondrial function, oxidative stress resistance, sirtuins, and TOR signaling.

CONSERVED LONGEVITY DETERMINANTS

Invertebrate models offer many powerful advantages in the context of aging research and a substantial portion of the knowledge we possess about how and why organisms age has been generated using these models. An important consideration when interpreting evidence from invertebrate systems is relevance to human aging. The growing body of aging research using divergent mammalian and invertebrate species has led to the discovery and characterization of several aspects of longevity control that have been evolutionarily conserved, of which dietary restriction is the most studied. Work from several groups has led to the identification of more than two dozen conserved aging genes, and comparative genetic analyses are beginning to place these genes into known aging pathways (Table 1.3). Three (at least partially) distinct genetic pathways have been found to modulate aging in evolutionarily divergent organisms: IIS, sirtuins, and TOR signaling (Table 1.4).

In addition to genetic factors, several environmental interventions, such as dietary restriction and transient heat shock, are known to influence longevity in multiple evolutionarily divergent organisms (Table 1.4). Dietary restriction in particular has been shown to extend life span in yeast, worms, flies, mice, spiders, rats, dogs and hamsters (Kennedy et al., 2007; Masoro, 2005; Weindruch and Walford, 1988). There is likely a set of key environmental conditions that induce a similar set of responses—increased longevity, enhanced resistance to stress, reduced fecundity—in a wide range of organisms. However, this does not necessarily guarantee that the molecular mechanisms that mediate these responses are the same in different organisms and there are cases where it appears that certain age-associated responses, including increased life span, are mechanistically implemented in different ways in different organisms.

Table 1.3. Conserved aging genes. Orthologs are only show if altered expression has been reported to increases life span in each included organism. The data in this table was compiled from numerous sources (Barber et al., 2006; Bluher et al., 2003; Chiocchetti et al., 2007; Clancy et al., 2001; Curran and Ruvkun, 2007; D'Mello N et al., 1994; Dell'agnello et al., 2007; Dorman et al., 1995; Fabrizio et al., 2003; Fabrizio et al., 2004b; Fabrizio et al., 2001; Giannakou et al., 2004; Hamilton et al., 2005; Hansen et al., 2005; Hansen et al., 2007; Harrison et al., 2009; Henderson et al., 2006; Henderson and Johnson, 2001; Hertweck et al., 2004; Holzenberger et al., 2003; Hwangbo et al., 2004; Kaeberlein et al., 1999; Kaeberlein et al., 2005d; Kapahi et al., 2004; Kenyon et al., 1993; Kim et al., 1999; Kim and Sun, 2007; Klass, 1983; Koc et al., 2004; Lakowski and Hekimi, 1998; Lans and Jansen, 2007; Lin et al., 2000; Liu et al., 2005; Menuz et al., 2009; Mitsui et al., 2002; Orr and Sohal, 1994; Pan et al., 2007; Paradis et al., 1999; Powers et al., 2006; Rogina and Helfand, 2004; Rogina et al., 2002; Ruan et al., 2002; Schriner et al., 2005; Selman et al., 2008; Selman et al., 2009; Smith et al., 2008b; Taguchi et al., 2007; Tatar et al., 1997; Tatar et al., 2001; Tedesco et al., 2008; Tissenbaum and Guarente, 2001; Umeda-Kameyama et al., 2007; Urban et al., 2007; Vellai et al., 2003; Wolkow et al., 2002; Yokoyama et al., 2002; Zordan et al., 2006).

Longevity Pathway	Known or Predicted Protein Function	Encoding Gene				References
		Yeast	Worms	Flies	Mice	
Insulin/IGF-1-Like Signaling	AKT/Protein Kinase B	*SCH9**	*akt-1, akt-2***			Fabrizio et al. (2001) ; Hamilton et al. (2005), Hertweck et al. (2004)
	FOXO Family Transcription Factor	n/a	*daf-16*	*dFOXO*		Henderson and Johnson (2001); Giannakou et al. (2004), Hwangbo et al. (2004)
	Insulin Receptor Substrate (IRS)	n/a		*Chico*	*Irs1, Irs2*	Clancy et al. (2001); Selman et al. (2008), Taguchi et al. (2007)
	Insulin/IGF-1-like Receptor	n/a	*daf-2*	*InR*	*Insr, Igf1r*	Kenyon et al. (1993); Tatar et al. (2001); Blüher et al. (2003), Holzenberger et al. (2003)
	Phosphoinositide 3-Kinase (PI3K)	n/a	*age-1, aap-1*		*PI3K γ*	Klass (1983), Dorman et al. (1995), Wolkow et al. (2002); Barber et al. (2006)
Sirtuins	Histone Deacytelase	*SIR2*	*sir-2.1*	*dSir2*		Kaeberlein et al. (1999); Tissenbaum and Guarente (2001); Rogina and Helfand (2004)
	Histone Deacytelase	*RPD3*		*Rpd3*		Kim et al. (1999); Rogina et al. (2002)
mRNA Translation/ TOR Signaling	Large Subunit Ribosomal Protein	*RPL19A*	*rpl-19*			Smith et al. (2008); Hansen et al. (2007)
	Large Subunit Ribosomal Protein	*RPL6B*	*rpl-6*			Smith et al. (2008); Hansen et al. (2007)
	Large Subunit Ribosomal Protein	*RPL9A*	*rpl-9*			Smith et al. (2008); Hansen et al. (2007)
	S6 Kinase	*SCH9**	*rsks-1*	*dS6K*	*S6K1*	Fabrizio et al. (2001), Fabrizio et al. (2004), Urban et al. (2007); Hansen et al. (2007), Pan et al. (2007); Kapahi et al. (2004); Selman et al. (2009)
	Small Subunit Ribosomal Protein	*RPS6B*	*rps-6*			Chiocchetti et al. (2007); Hansen et al. (2007)
	Target of Rapamycin Kinase	*TOR1*	*let-363*	*dTOR*	*mTOR*†	Kaeberlein et al. (2005), Powers et al. (2006); Vellai et al. (2003); Kapahi et al. (2004); Harrison et al. (2009)
	Translation Initiation Factor	*TIF1, TIF2*	*inf-1*			Smith et al. (2008); Curran and Ruvkun (2007)
	Translation Initiation Factor	*TIF4631*	*ifg-1*			Smith et al. (2008); Henderson et al. (2006), Curran and Ruvkun (2007), Pan et al. (2007)

Table 1.3 (continued).

Longevity Pathway	Known or Predicted Protein Function	Encoding Gene				References
		Yeast	Worms	Flies	Mice	
Stress Resistance	Catalase			*Cat*	*Cat*‡	Orr and Sohal (1994); Schriner et al. (2005)
	Heat Shock Protein		*hsp-6*	*Hsp70*		Yokoyama et al. (2002); Tatar et al. (1997)
	Superoxide Dismutase	*SOD1*		*Sod1*		Fabrizio et al. (2003); Orr and Sohal (1994)
Unknown	3-Phosphoinositide-Dependent Kinase	*PKH2*	*pdk-1*			Smith et al. (2008); Paradis et al. (1999)
	Alpha-Mannosyltransferase	*ALG12*	*T27F7.3*			Smith et al. (2008); Curran and Ruvkun (2007)
	Ammonium Transporter	*MEP1, MEP2*	*amt-2*			Powers et al. (2006); Kim and Sun (2007)
	CCCH-Type Zn-Finger Protein	*TIS11*	*pos-1*			Smith et al. (2008); Curran and Ruvkun (2007)
	Ceramide Synthase Component	*LAG1*	*hyl-1*			D'mello et al. (1994); Tedesco et al. (2008), Menuz et al. (2009)
	Coenzyme Q7 Homolog		*clk-1*		*Coq7*	Lakowski and Hekimi (1998); Liu et al. (2005)
	Cytoskeletal Linker Protein	*YGR130C*	*erm-1*			Smith et al. (2008); Curran and Ruvkun (2007)
	DEAD-Box Helicase	*DBP3*	*B0511.6*			Smith et al. (2008); Curran and Ruvkun (2007)
	Endosomal Complex Adaptor Protein	*HSE1*	*sem-5*			Smith et al. (2008); Curran and Ruvkun (2007)
	G Protein, Alpha Subunit	*GPA2*	*gpa-1, gpa-5, odr-3*			Lin et al. (2000); Lans and Jansen (2007)
	Ion Transporter					(1998)
	Isocitrate Dehydrogenase	*IDH1, IDH2*	*F43G9.1*			Smith et al. (2008); Hamilton et al. (2005)
	Metalloprotease	*AFG3*	*spg-7*			Smith et al. (2008); Curran and Ruvkun (2007)
	Methionine Sulfoxide Reductase A	*MXR1*		*Eip71CD*		Koc et al. (2004); Ruan et al. (2002)
	Polyphosphoinositide Phosphatase	*INP51, INP53*	*unc-26*			Smith et al. (2008); Lakowski and Hekimi (1998)
	Protein Phosphatase Regulatory Subunit	*SIS2*	*Y46H3C.6*			Smith et al. (2008); Hamilton et al. (2005)
	RAB-Family GTPase	*YPT6*	*rab-10*			Smith et al. (2008); Hansen et al. (2005)
	S-Adenosylmethionine Synthetase	*SAM1*	*sams-3*			Smith et al. (2008); Curran and Ruvkun (2007)
	Surfeit Gene 1			*Surf1*	*Surf1*	Zordan et al. (2006); Dell'Agnello et al. (2007)
	Thioredoxin	*TrxT*			*Txn1*‡	Umeda-Kameyama et al. (2007); Mitsui et al. (2002)
	Transcription Elongation Factor	*SPT4*	*spt-4*			Smith et al. (2008); Hamilton et al. (2005)

* *SCH9* has been suggested as a yeast homolog to both mammalian Akt/PKB and mammalian S6K and shows S6K activity

** *akt-1(RNAi) akt-2(ok393)* is longer-lived than wild type, akt-1(ok525), and akt-2(ok393)

† Predicted based on life span extension from treatment with TOR inhibitor rapamycin

‡ Mouse life span extension shown by overexpressing the human version of the gene

Table 1.4. Conserved environmental and genetic interventions known to influence life span. Arrows indicate whether the intervention increases (↑), decreases (↓), is not applicable to (n/a) or has an unknown effect on (?) life span in each aging model.

		Yeast		Worms	Flies	Mice
		Replicative	Chronological			
Environmental Interventions	Dietary Restriction	↑	↑	↑	↑	↑
	Transient Exposure to Stress	↑	?	↑	↑	↑
	Antioxidants	?	↑	?	↑	↑
Genetic Interventions	Reduced IIS	n/a	n/a	↑	↑	↑
	Increased Sirtuin Activity	↑	↓	↑	↑	?
	Reduced TOR Signaling	↑	↑	↑	↑	?

As our knowledge of the genetic pathways that influence aging becomes more complete, developing models of how the pathways interact with respect to both the upstream environmental and pharmacological interventions and the downstream molecular targets will be important in developing treatments targeting human aging and age-related disease. While each pathway is at least partially distinct, there is already evidence for interaction between pathways through both influence from environmental conditions and action on downstream targets. For example, each pathway has been independently proposed as a potential mediator of the beneficial effects of dietary restriction. The following sections describe the current state of knowledge surrounding dietary restriction and each of these conserved longevity pathways with respect to aging, as well as the relationship between each pathway and dietary restriction.

IIS PROMOTES AGING

Among multicellular eukaryotes, IIS pathways mediate growth, stress resistance, and longevity in response to environmental conditions. The longevity related IIS pathways share a core set of similar features in divergent organisms, including insulin-like molecules, one or more insulin/IGF-1-like receptors, a phosphatidylinositol 3-kinase (PI3K), an Akt kinase, and a FoxO-family transcription factor (Figure 1.2). Downstream genetic targets of IIS are regulated by controlling nuclear localization of the FoxO-family transcription factor, with increased IIS resulting in decreased transcription factor activity.

Worms and flies each possess a single IIS receptor that mediates signals from multiple insulin-like ligands (at least 30 in worms and 8 in flies) (Bartke, 2008; Toivonen and Partridge, 2009). The *C. elegans* insulin/IGF-1-like receptor, PI3K, Akt kinase, and FoxO-family transcription factor are encoded by *daf-2*, *age-1*, *akt-1/2*, and *daf-16*, respectively (Kimura et al., 1997; Lin et al., 1997; Morris et al., 1996; Ogg et al., 1997). Worms with reduced IIS caused by mutations that decrease activity of either *daf-2* or *age-1* have a life span increased in a non-additive, *daf-16*-dependent manner (Dorman et al., 1995; Kenyon et al., 1993). Life span extension by reduced IIS therefore requires DAF-16, which regulates a diverse set of processes including fat storage, metabolism, development, fertility, and resistance to heat and oxidative stress (Finch and Ruvkun, 2001; Gems et al., 1998; Larsen, 1993). Extension of life span via mutation of *daf-2* also requires AAK-2, the catalytic subunit of the adenosine monophosphate-activated protein (AMP) kinase. Overexpression of *aak-2* is sufficient to increase life span (Apfeld et al., 2004).

Reduced IIS is thought to increase life span in worms, at least in part, by upregulating stress-response proteins. Long-lived worms with reduced IIS are resistant to multiple forms of environmental stress including reactive oxygen species, exposure to UV, increased temperature, viral infection, and bacterial pathogenesis (Martin et al., 1996; Murakami and Johnson, 1996). Transient heat shock is sufficient to extend life span (Butov et al., 2001; Lithgow et al., 1995; Michalski et al., 2001; Yashin et al., 2002) and causes nuclear localization of DAF-16 (Henderson and Johnson, 2001; Lin et al., 2001a). Overexpression of the *C. elegans* heat-shock factor-1 (HSF-1) is sufficient to increase life span, and deletion of the *hsf-1* blocks life span extension from *daf-2* knockdown (Hsu et al., 2003; Morley and Morimoto, 2004). HSF-1 also activates multiple longevity genes including several that encode small heat-shock proteins (Hsu et al., 2003). Reducing IIS probably does not optimally activate the heat-shock response for life span extension however, as heat shock produces a further increase in life span and upregulation of small heat-shock protein genes in long-lived *age-1* mutants (Walker et al., 2001).

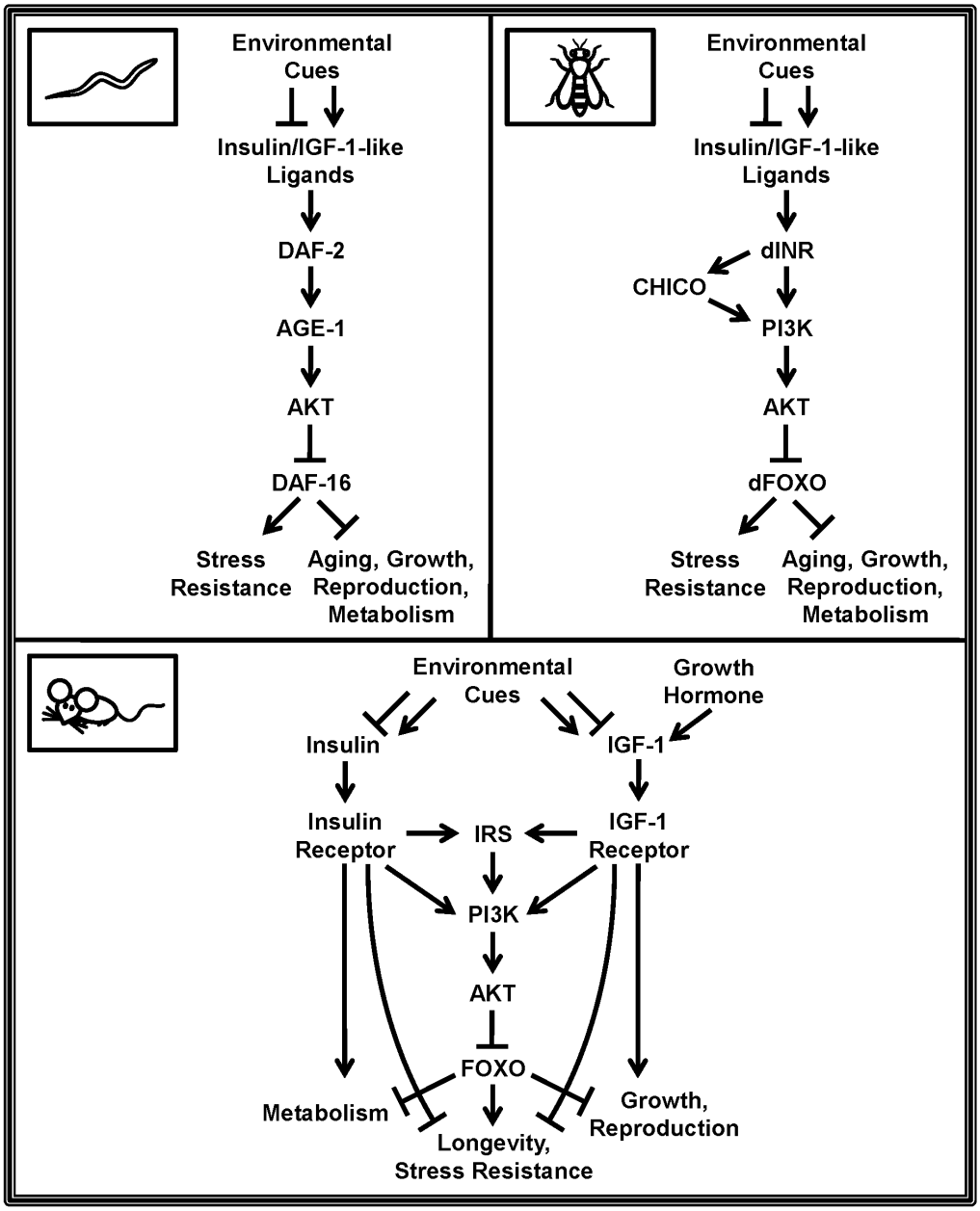


Figure 1.2. Insulin and IGF-1-like signaling pathways play a conserved role in aging in nematodes, flies, and mice.

In addition to their role in aging, *daf-2* and *daf-16* regulate entry into the dauer larval stage, a long-lived alternate development pathway. Dauer larvae are sexually immature and characterized by a thick cuticle, constricted pharynx, and sealed buccal and anal cavities resulting in an inability to eat or defecate and an increased resistance to environmental stresses such as harsh chemical treatment and desiccation (Cassada and Russell, 1975; Riddle, 1988). At least three environmental factors contribute to

the decision to enter the dauer larval stage: population density, temperature, and food availability (Golden and Riddle, 1982, 1984). Dauer larvae exposed to favorable environmental conditions (i.e. low population density, temperature within an optimal range, and abundant food) resume development and proceed to become reproductively active adults. Complete inhibition of *daf-2* results in constitutive entry into the dauer larva stage regardless of environmental signals, and worms with mutations in *daf-16* fail to enter the dauer larva stage or do so inefficiently (Gottlieb and Ruvkun, 1994). As might be expected, dauer larvae share many similarities with worms that have reduced (but not abolished) IIS, including enhanced longevity and stress resistance, suggesting that the benefits of reduced IIS may represent an adult dauer-like state and may potentially be subject to the same trade-offs, such as reduced reproduction. Notably, the dauer response can be decoupled from the pro-longevity effects of reduced IIS, as RNAi knockdown of *daf-2* starting well into adulthood—even post-reproductively—dramatically increases life span without altering development or influencing reproductive potential (Dillin et al., 2002; Smith et al., 2008a).

Similar to worms, reduction of IIS signaling in flies via mutations in InR, the gene encoding the insulin/IGF-1-like receptor, or Chico, the gene encoding the insulin receptor substrate (IRS), increases stress resistance and longevity (Clancy et al., 2001; Tatar et al., 2001; Tu et al., 2002). The influence of IIS on life span appears to be partially gender specific in flies, as mutation of InR extends only the female life span (Tatar et al., 2001). In the Chico mutants, both heterozygous and homozygous female flies displayed increased life span, whereas only heterozygous males displayed increased life span, relative to wild type (Clancy et al., 2001; Tu et al., 2002). This is in contrast to worms, in which mutation of *daf-2* increases hermaphrodite and male life spans to a similar degree (Gems and Riddle, 2000). Transient heat shock also extends fly life span (Hercus et al., 2003). Furthermore, partial genetic ablation of the median neurosecretory cells (MNCs) both reduces expression of MNC-specific *Drosophila* insulin-like peptide (dilp) genes and increases life span (Broughton et al., 2005). This suggests that the role of IIS in aging is cell non-autonomous. While it is not conclusively known whether these phenotypes are dependent on dFOXO, the *D. melanogaster* FoxO-family transcription factor (Junger et al., 2003; Kramer et al., 2003; Puig et al., 2003), there is evidence consistent with that conclusion. Reduced cell division caused by mutations that decrease IIS require dFOXO (Junger et al., 2003), and adult fat-body-specific overexpression of dFOXO is sufficient to extend life span (Giannakou et al., 2007; Giannakou et al., 2004; Hwangbo et al., 2004).

Unlike invertebrates, mammalian IIS involves only three insulin-like peptides (insulin, IGF-1, and IGF-2), and three receptor monomers (one insulin receptor and two IGF-1 receptors). The receptor monomers combine to form five types of dimeric receptors. These include separate receptors for insulin and IGF-1 ligands (Taguchi and White, 2008), both of which appear to play a role in determining life span. Female mice with a heterozygous IGF-1 receptor knockout live ~30% longer than wild type mice

(Holzenberger et al., 2003), while both male and female fat-specific insulin receptor knockout mice with an adipose-specific insulin receptor knockout live ~18% longer than wild type (Bluher et al., 2003). Growth hormone, which is not present in invertebrates, also appears to interact with IIS in modulating life span in mice. IGF-1 production is promoted by increased growth hormone activity and mice with mutations in the growth hormone receptor or defects in the pituitary gland (Ames and Snell dwarf mice) show reduced growth hormone and IGF-1 levels and increased life span relative to controls (Brown-Borg et al., 1996; Coschigano et al., 2003; Flurkey et al., 2002). As with flies, the role of FoxO proteins in mouse life span extension from reduced IIS is unknown. However, FoxO proteins are known to function in IIS pathways that affect metabolism (Burgering and Kops, 2002) and have been implicated in the increased stress resistance of certain long-lived mouse strains (Nemoto and Finkel, 2002).

Interestingly, while insulin sensitivity is typically associated with longevity in mice, there are several examples of mutations that both increase insulin resistance and extend life span. These include KLOTHO overexpression (Kurosu et al., 2005), IRS1 knockout (Selman et al., 2008), and brain-specific IRS2 knockout (Taguchi et al., 2007). These findings are difficult to interpret in light of the potential for pleiotropic effects, as resistance to both insulin and IGF-1 was observed in all cases.

The evolution of multiple IIS pathways in mammals has several implications for the role of IIS in aging. Functions performed by the single IIS pathway in invertebrates that are related to life span extension may be divided between the insulin and the IGF-1 branches of IIS in mammals. Indeed, while there is evidence for overlapping function, insulin signaling is primarily involved in regulating metabolism, while IGF-1 modulates growth and development (Kim and Accili, 2002; Rincon et al., 2005). Multiple pathways would also have eased pleiotropic evolutionary restrictions and allowed the insulin and IGF-1 branches to specialize further and/or acquire new functions.

SIRTUINS: PLAYING BOTH SIDES?

Sir2 orthologs (sirtuins) are present in organisms from yeast to humans and function as NAD-dependent protein deacetylases (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000; Tanner et al., 2000). Sir2 is a histone deacetylase that promotes transcriptional silencing at three specific loci in the yeast genome: the rDNA, the silent mating (HM) loci, and regions near the telomeres (Aparicio et al., 1991; Bryk et al., 1997; Gottschling et al., 1990; Ivy et al., 1986; Rine and Herskowitz, 1987; Smith and Boeke, 1997). Unlike yeast, the reported substrates of Sir2 orthologs in multicellular eukaryotes appear to be primarily non-histone and include endoplasmic reticulum-stress response factors (Viswanathan et al.,

2005), FoxO-family transcription factors (Brunet et al., 2004; Motta et al., 2004; van der Horst et al., 2004), peroxisome proliferator-activated receptor γ coactivator 1α (Gerhart-Hines et al., 2007; Rodgers et al., 2005), p53 (Luo et al., 2001; Vaziri et al., 2001), and several others (Dali-Youcef et al., 2007; Finkel et al., 2009).

A role for Sir2 orthologs in aging was first demonstrated by the observation that overexpression of Sir2 is sufficient to increase yeast replicative life span (Kaeberlein et al., 1999). Subsequent studies demonstrated a similar longevity-enhancing effect associated with overexpression of *sir-2.1* in worms and dSir2 in flies (Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001), though a recent report detailing an investigation of the strain backgrounds and overexpression systems calls both reports into question and is discussed in detail in Chapter 4. To date, there has been no report showing that increased expression of SIRT1 in mammals is sufficient to increase life span, although SIRT1 transgenic mice are reported to have improved metabolic profiles (Banks et al., 2008; Bordone et al., 2007) and show resistance to colon cancer (Firestein et al., 2008).

One surprising feature of the longevity-promoting functions of sirtuins is the apparently distinct mechanisms by which they act in different organisms (Figure 1.3). In yeast, Sir2 is thought to slow replicative aging by promoting genomic stability in the rDNA and repressing the formation of ERCs (Kaeberlein et al., 1999), one cause of replicative senescence in yeast cells (Sinclair and Guarente, 1997). Unlike yeast, there is no evidence that sirtuins modulate the formation of ERCs in multicellular eukaryotes, nor are there data suggesting that ERCs cause aging in higher organisms. Instead, sirtuins appear to have evolved different pro-longevity functions in these organisms. For example, in *C. elegans*, evidence suggests that *sir-2.1* modulates the downstream targets of IIS by interacting with *daf-16* in a 14-3-3-dependent manner (Berdichevsky et al., 2006; Wang et al., 2006). In flies, the relevant downstream targets of dSir2 have yet to be described, but it has been proposed that dSir2 acts in a longevity-promoting pathway with the Rpd3 histone deacetylase (Rogina and Helfand, 2004). Whether Sir2 orthologs really function to slow aging by different mechanisms in different organisms, or whether there exist as yet uncharacterized conserved sirtuin functions, is a question of continuing interest.

In contrast to the pro-longevity effects associated with sirtuins, recent studies have suggested that sirtuin proteins may also promote aging in some systems or tissues. For example, yeast chronological life span is limited by Sir2 activity (Fabrizio et al., 2005; Kennedy et al., 2005). SIRT1-deficient mouse embryonic fibroblasts are highly resistant to replicative senescence and have increased replicative potential under chronic oxidative stress (Chua et al., 2005), in stark contrast to the observed decrease in replicative life span of yeast lacking Sir2 (Kennedy et al., 1995). A recent study found reduced IIS and Ras/ERK signaling in mice lacking SIRT1 (Li et al., 2008). Li et al. (2008) also found that SIRT1 knockdown enhanced oxidative stress resistance in mouse neuronal cell culture and that SIRT1 knockout

mice had reduced oxidation of proteins and lipids in the brain, in contrast to the finding in flies that neuron-specific overexpression of dSir2 is sufficient for life span extension (Rogina and Helfand, 2004), suggesting that both increasing and decreasing sirtuin activity may have neuroprotective consequences. These studies reinforce the idea that sirtuins perform different functions in different organisms and imply that the biology of sirtuins is more complex than initially suspected. Further effort will be required to unravel the intricacies of the action of sirtuins on longevity and to determine what similarities and differences exist between evolutionarily divergent species.

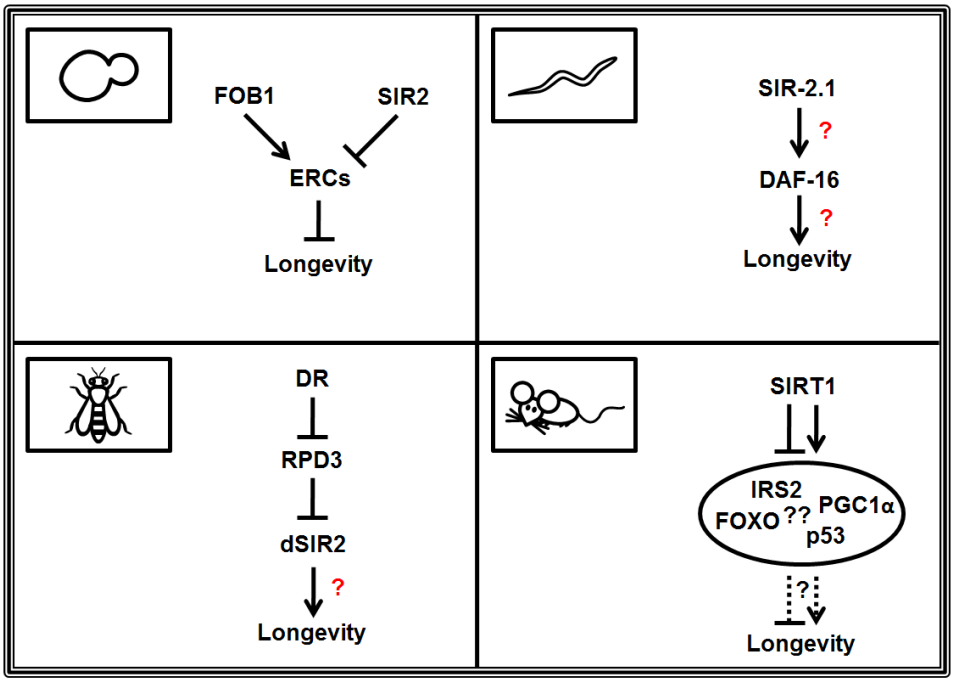


Figure 1.3. Sir2 orthologs promote longevity in yeast, nematodes, and flies by distinct mechanisms. The ability of SIRT1 overexpression to increase mouse life span has yet to be established, but SIRT1 influences a variety of age-associated phenotypes in mice, possibly via multiple substrate targets. Red question marks indicate relationships established using strains that have been called into question by a recent study detailed in Chapter 4.

REDUCED TOR SIGNALING CONSISTENTLY EXTENDS LIFE SPAN

The TOR kinase is a highly conserved nutrient- and growth factor-responsive protein that is essential for viability in eukaryotic species (Stanfel et al., 2009). TOR was first identified as the molecular target of an antifungal compound (rapamycin) produced by the bacterium *Streptomyces hygroscopicus* (Vezina et al., 1975). Rapamycin was subsequently shown to inhibit the activity of protein products of two partially redundant yeast genes: *TOR1* and *TOR2* (Heitman et al., 1991). TOR proteins have since been identified in a variety of species, including humans, and have been shown to act in two distinct complexes: TOR complex 1 (TORC1) and TOR complex 2 (TORC2) (De Virgilio and Loewith, 2006; Martin and Hall, 2005). Although both TOR complexes are essential for viability (Guertin et al., 2006; Helliwell et al., 1998), only TORC1 is sensitive to rapamycin. While most aging studies to date have focused on TORC1, one report reveals a role for TORC2 in *C. elegans* aging (Soukas et al., 2009). TORC1 serves as a key regulatory nexus for mounting an appropriate response to nutrients, growth cues, and cellular energy status (Wullschleger et al., 2006). TORC1 is activated by environmental nutrient availability in the form of both amino acids and glucose and is also responsive to IIS (through Akt) as well as the energy-sensing AMPK (Arsham and Neufeld, 2006; Bhaskar and Hay, 2007). The link between TOR and aging has been definitively demonstrated in four different organisms (Stanfel et al., 2009). Reduced TOR signaling is sufficient to increase life span in mice (Harrison et al., 2009), worms (Jia et al., 2004; Vellai et al., 2003), flies (Kapahi et al., 2004), and both yeast aging paradigms (Kaeberlein et al., 2005d; Powers et al., 2006). Aside from dietary restriction, TOR inhibition is the only intervention known to slow aging in each of these model systems (Kaeberlein and Kennedy, 2009). The importance of TOR signaling in yeast replicative and chronological life span determination was uncovered from independent, unbiased longevity screens of the yeast ORF deletion collection. Deletion of *TOR1* was found to increase both replicative and chronological life span, as did pharmacological inhibition of TOR using rapamycin (Kaeberlein et al., 2005d; Powers et al., 2006). RNAi knockdown of the gene coding for TOR (*let-363*) or the TORC1 component raptor (*daf-15*) is sufficient to increase life span in worms (Jia et al., 2004; Vellai et al., 2003) and transgenic expression of a dominant-negative allele of TOR increases life span in flies (Kapahi et al., 2004). The effect of reduced TOR signaling on life span in a mammalian system was recently demonstrated by a study in which mice were fed a diet supplemented with rapamycin. Supplementation with rapamycin beginning at 600 days of age resulted in a significant increase in life span (Harrison et al., 2009).

While the precise molecular mechanisms by which TOR signaling modulates aging in evolutionarily divergent organisms have yet to be completely characterized, known components of TOR signaling are highly conserved both upstream and downstream of TORC1, including several TOR-

regulated processes that have been suggested to play a role in longevity determination such as regulation of mRNA translation, autophagy, stress response, and mitochondrial metabolism (Figure 1.4). For example, autophagy is induced in a TOR-dependent manner by both dietary restriction and reduced IIS in *C. elegans* and is required for life span extension in both cases (Hansen et al., 2008; Jia and Levine, 2007; Melendez et al., 2003). Altered TOR signaling is thought to be partially responsible for the beneficial effects of dietary restriction, which is discussed further below.

Autophagy, which literally means "self eating", is a degradative process through which cellular components are engulfed by cytoplasmic vesicles and transported to the lysosome/vacuole for degradation (Klionsky, 2007). Autophagy is repressed by TOR signaling and is induced in response to starvation or treatment with TOR inhibitors, such as rapamycin (Noda and Ohsumi, 1998). A decline in the autophagic response has been reported in aging mammals (Cuervo and Dice, 2000), and increased autophagy is required for life span extension in long-lived *C. elegans* mutants with reduced IIS (Melendez et al., 2003). Several recent studies have also uncovered an important role for autophagy in the response to dietary restriction. Dietary restriction induces autophagy in yeast, worms, and flies (Juhasz et al., 2007; Morck and Pilon, 2006; Takeshige et al., 1992) and is reported to be required for life span extension from dietary restriction or TOR-inhibition in both worms and flies (Hansen et al., 2008; Jia and Levine, 2007; Juhasz et al., 2007). Recently, up-regulation of autophagy by spermidine has also been shown to be associated with increased life span in yeast, nematodes, and flies (Eisenberg et al., 2009).

The regulation of mitochondrial metabolism by TOR is a relatively new area of study. In yeast, *tor1Δ* mutants are reported to have increased respiratory activity in the presence of glucose, which is normally fermented to ethanol (Bonawitz et al., 2007). This altered metabolic activity has been implicated in chronological aging, but has not been shown to be important for regulation of replicative life span by TOR signaling. Interestingly, overexpression of the Hap4 transcription factor, which induces expression of many genes involved in respiratory metabolism, has been shown to increase both replicative and chronological life span (Lin et al., 2002; Piper et al., 2006), suggesting that enhanced respiration is associated with longevity in yeast. This mechanism has been attributed to activation of Sir2 however (Lin et al., 2002), which is inconsistent with the observation that deletion of *TOR1* increases life span in a Sir2-independent manner (Table 12.1) (Kaeberlein et al., 2005d). Thus, like autophagy, the importance of mitochondrial metabolism in TOR-mediated control of replicative life span remains unclear.

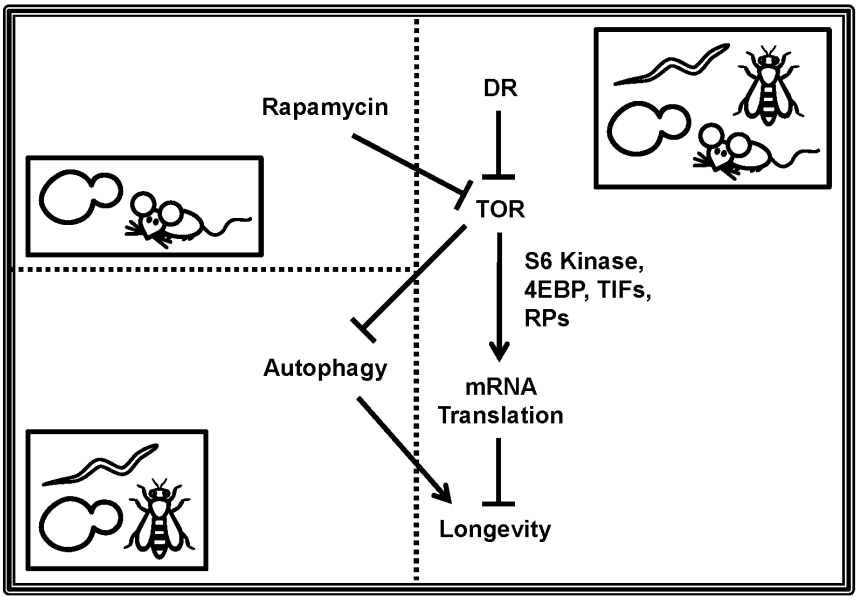


Figure 1.4. A reduction in TOR signaling extends life span in evolutionarily divergent organisms. Mutation of S6 kinase increases life span in yeast, nematodes, flies, and mice. The TOR inhibitor rapamycin increases life span in yeast and mice. Autophagy has been implicated in life-span extension from reduced TOR signaling in yeast, nematodes, and flies.

DIETARY RESTRICTION AND THE SEARCH FOR A MECHANISM

Dietary restriction is commonly defined as a decrease in dietary intake without malnutrition and is the most widely effective and intensely studied intervention known to extend life span. Life extension in response to dietary restriction was first observed in rats in 1934 (McCay and Crowell, 1934) and has since been demonstrated in a wide range of model systems. The effects of dietary restriction on longevity are clearly shared among diverse organisms, but it remains an open question as to whether the underlying molecular mechanisms are also shared. Several hypotheses for how dietary restriction might mediate a reduced rate of aging have been proposed, including reduced inflammation, reduced damage from reactive oxygen species, improved glucose homeostasis, and enhanced resistance to a variety of stresses (Spindler, 2010). To date none of these hypotheses has been definitively shown to play a primary role in mediating the effects of dietary restriction. In addition to enhanced longevity and reduced age-associated disease, two dietary restriction associated phenotypes that seem to be shared between different organisms are a reduction in reproductive rate and an increase in broad-spectrum stress resistance. This observation has led to the hypothesis that life span extension in response to dietary restriction is an evolutionarily

conserved mechanism for maintaining reproductive potential in response to transient environmental fluctuations in food availability (Harrison and Archer, 1988; Holliday, 1989).

One unresolved question regarding dietary restriction is whether the longevity and health benefits are solely due to reduced caloric consumption, as was initially assumed, or whether other dietary factors may also be involved. In support of a more general view of dietary restriction, simply restricting the dietary abundance of methionine in both mice and rats is sufficient to increase life span (Miller et al., 2005; Orentreich et al., 1993). Similar observations have been made with respect to tryptophan in rats (Ooka et al., 1988; Segall and Timiras, 1976; Timiras et al., 1984). An alternative way to interpret these results is that the standard laboratory mouse diet does not contain an ideal balance of amino acids and that a subset, including methionine and tryptophan, are overly abundant. Indeed, Masoro et al. (1989) found that methionine restriction did not contribute to the life span extension resulting from a 40% decrease in food intake in rats. The most convincing evidence for a model of dietary restriction that is not limited to restriction of caloric intake is the finding that food sensing can modulate longevity independent of food consumption in both nematodes and flies (Libert and Pletcher, 2007; Smith et al., 2008a). Whether food sensing modulates longevity in mammals is unknown.

Yeast replicative aging has been used extensively to study the molecular and genetic factors involved in the life span extension resulting from dietary restriction. In yeast, dietary restriction is typically performed by limiting the availability of glucose to cells by reducing the glucose concentration in the media from 2% to either 0.5% or 0.05% (Lin et al., 2000), with optimal life span extension achieved at 0.05% glucose in the strain background of the yeast ORF deletion collection (Kaeberlein et al., 2004; Lin et al., 2000). Other less commonly used forms of dietary restriction include restricting amino acids (Jiang et al., 2000) or replacing media glucose with a nonfermentable carbon source such as glycerol, ethanol, or raffinose (Delaney et al., 2011; Kirchman and Botta, 2007). Genetic models of dietary restriction are also available, including deletion of *HXK2*, which encodes a hexokinase responsible for converting glucose into glucose-6-phosphate for entry into the glycolytic pathway (Walsh et al., 1983). Deletion of *HXK2* extends replicative life span (Lin et al., 2000), although it remains unclear whether this is attributable to reduced cellular hexokinase activity (Rodriguez et al., 2001; Walsh et al., 1991).

The precise molecular mechanisms through which dietary restriction acts to extend life span in yeast are not yet known; however, it is commonly thought that dietary restriction manipulates these mechanisms, at least in part, by influencing several partially redundant nutrient-responsive signaling kinases, including TOR, cyclic AMP-dependent protein kinase (PKA), and Sch9. Mutants with reduced activity for any of these kinases have long replicative life spans that cannot be further extended by dietary restriction (Fabrizio et al., 2004b; Kaeberlein et al., 2005d; Lin et al., 2000). Yeast PKA is an essential

complex consisting of three catalytic subunits and regulated by two upstream sensing pathways, one involving RAS and the other a G protein-coupled receptor system. Two genes, *GPA2* and *GPR1*, encode subunits of the G protein-coupled receptor. Mutants lacking either *GPA2* or *GPR1* are replicatively long-lived relative to wild type and are commonly used as models of reduced PKA activity (Lin et al., 2000). The third kinase, Sch9, shows sequence homology to Akt kinase, a component of IIS (Burgering and Coffer, 1995; Paradis and Ruvkun, 1998), but also functions as a ribosomal S6 kinase, a substrate of TOR and regulator of translation in multicellular eukaryotes (Powers, 2007; Urban et al., 2007). While yeast does not possess a formal IIS pathway, Sch9 may fulfill an equivalent role in both Akt and S6 kinases in multicellular eukaryotes.

INTERACTION BETWEEN DIETARY RESTRICTION AND CONSERVED LONGEVITY PATHWAYS

Dietary restriction alters a multitude of physiological processes and each of the genetic pathways discussed above has independently been proposed as a key mediator of life span extension via dietary restriction. The best and most consistent evidence is for TOR signaling. Life span extension from reduced TOR signaling and dietary restriction is non-additive in yeast replicative aging, worms and flies (Hansen et al., 2007; Juhasz et al., 2007; Kaeberlein et al., 2005d). Clear evidence for the dependence of dietary restriction on other pathways is less straight-forward. In all cases there are observations indicating that life span extension for dietary restriction is at least partially independent, and observations that suggest some level of interaction. The following sections discuss the interplay between dietary restriction and each of the aging pathways described above.

IIS: a partial interaction with dietary restriction with respect to secondary aging phenotypes

Dietary restriction and mutations that reduce IIS have many phenotypic similarities including enhanced longevity, stress resistance, reduced TOR signaling, and increased autophagic protein degradation. This is not surprising, since one of the major environmental factors to modulate IIS is nutrient availability. Reduced IIS is therefore a natural candidate for mediating the beneficial effects of dietary restriction. Interestingly, while dietary restriction and IIS clearly overlap, genetic studies have indicated that they also act through at least partially distinct mechanisms to control longevity.

The relationship between IIS and dietary restriction has been studied most extensively in *C. elegans*. Life span extension from multiple approaches to dietary restriction, including bacterial dilution

in liquid culture, axenic growth in liquid culture, bacterial deprivation, and mutation of *eat-2* (a genetic model resulting in reduced food intake due to decreased pharyngeal pumping), has been repeatedly shown to extend life span by a mechanism different from mutations that reduce IIS (Houthoofd et al., 2003; Kaeberlein et al., 2006b; Lakowski and Hekimi, 1998; Lee et al., 2006). Specifically, all of these dietary restriction methods increase life span in animals lacking DAF-16. In contrast, one study found both DAF-16 and AAK-2 to be required for a specific method of dietary restriction, termed solid dietary restriction (sDR), involving maintenance of worms on solid agar plates in the presence of diluted bacterial food (Greer et al., 2007). Another study found that mutations in *daf-2* produced increased growth and stress resistance in *eat-2* mutants (Iser and Wolkow, 2007). Similarly, growth impairment normally observed in response to dietary restriction was suppressed in *daf-2* mutants (Iser and Wolkow, 2007). Thus, IIS and most forms of dietary restriction are thought to act in parallel pathways to mediate longevity in worms, but have potential to interact downstream by influencing AAK-2 and DAF-16 activity under some circumstances.

In flies, the life span on a range of food concentrations of long-lived Chico mutants, which have reduced IIS, is right-shifted relative to wild type, meaning that Chico mutants are shorter lived than controls on low food concentrations and longer lived on normal to high food concentrations (Clancy et al., 2002). This was initially taken as an indication that dietary restriction requires IIS to extend life span. However, a 2008 study found that deletion of dFOXO did not block the ability of dietary restriction to extend life span (Giannakou et al., 2008). Overexpression of dFOXO in the adult fat body partially mimicked the long-lived Chico mutants in that the mutant flies were longer lived at normal to high food concentrations (Giannakou et al., 2008). Thus IIS and dietary restriction interact when IIS is active, but IIS is not required for dietary restriction to extend life span. One possible explanation is that Drosophila IIS does not act entirely through dFOXO, but influences life span through a different mediator.

IIS shares a complex relationship with dietary restriction in mice as well. Growth hormone receptor knockout (GHRKO) mice are longer lived than wild type controls and have reduced levels of both insulin and IGF-1 (Coschigano et al., 2000; Liu et al., 2004; Zhou et al., 1997). GHRKO mice are no longer lived than wild type mice subject to dietary restriction, nor do GHRKO mice show increased longevity or improved insulin sensitivity when subjected to dietary restriction, with the exception of an increase in maximum life span in females (Al-Regaiey et al., 2007; Bonkowski et al., 2006). In contrast, dietary restriction extends the lives of mice with pituitary mutations, which are defective for production of several hormones, including growth hormone (Bartke et al., 2001). This suggests that dietary restriction and IIS may act via partially distinct pathways, although it is also possible that they act via a similar mechanism, but that neither intervention optimally activates that mechanism with respect to longevity.

Sirtuins: a complex and unresolved connection to dietary restriction

The connection between Sir2 and dietary restriction in yeast has been a source of controversy (Guarente, 2005; Kaeberlein and Powers, 2007; Kaeberlein et al., 2006a; Kennedy et al., 2005; Lamming et al., 2005). Sirtuins were first proposed as mediators of dietary restriction based on the known role of Sir2 in yeast replicative aging and the discovery that Sir2 is activated in yeast in a NAD-dependent manner (Guarente, 2000). This hypothesis was supported by early evidence that reducing glucose in the medium did not extend the replicative life span of short-lived yeast lacking *SIR2* (Lin et al., 2000). An alternative interpretation of this result is that accumulation of ERCs in the *sir2Δ* strain causes enough damage that cells die before they can respond to dietary restriction, masking the life span extension normally observed. Indeed, subsequent studies from independent labs found that Sir2 is not required for life span extension by dietary restriction under conditions where ERC accumulation is reduced (Jiang et al., 2000; Kaeberlein et al., 2004; Kaeberlein and Powers, 2007; Kaeberlein et al., 2006a; Lamming et al., 2005). More specifically, while dietary restriction does not extend life span of *sir2Δ* strains (Kaeberlein et al., 2004; Lin et al., 2000), suppression of both the short replicative life span and increased ERC accumulation by deletion of *FOB1* in a *sir2Δ* background allows robust life span extension by dietary restriction (Kaeberlein et al., 2004). Combining dietary restriction with overexpression of Sir2 or deletion of *FOB1* also results in an additive life span extension (Kaeberlein et al., 2005b). In a recent study, we found that deletion of *sir2*Δ prevents replicative life span extension in 32 different single-gene deletion strains and 4 forms of dietary restriction (discussed in detail in Chapter 4) (Delaney et al., 2011), indicating that the inability of an intervention to extend *sir2*Δ replicative life span is not generally useful in identifying the downstream mechanism. Dietary restriction has therefore been shown to control longevity via at least one Sir2-independent mechanism in yeast, and two studies have reinforced this model that dietary restriction does not act through Sir2 by showing that Sir2 activity is not enhanced in vivo by dietary restriction (Riesen and Morgan, 2009; Smith et al., 2009).

In multicellular eukaryotes the interaction between dietary restriction and sirtuins is unresolved. Reports concerning *sir-2.1* and dietary restriction in worms are conflicting, but, with the exception of one study (Wang and Tissenbaum, 2006), all support the idea that dietary restriction by a variety of methods does not require *sir-2.1* for life span extension (Greer and Brunet, 2009; Hansen et al., 2007; Kaeberlein et al., 2006b; Lee et al., 2006; Mair et al., 2009). Consistent with a model in which *sir-2.1* and dietary restriction act via distinct mechanisms, life span extension by *sir-2.1* overexpression requires *daf-16* (Tissenbaum and Guarente, 2001), while life span extension by dietary restriction does not (Lakowski and Hekimi, 1998). Thus, while the majority of evidence supports a model in which dietary restriction and

sir-2.1 act in parallel, further work will be required to determine definitively how *sir-2.1* interacts with dietary restriction in worms, if at all.

The situation in the published literature is less complicated in flies. Epistasis maps dSir2 to the same pathway as both dietary restriction and the histone deacetylase Rpd3 with respect to longevity (Rogina and Helfand, 2004), and both dietary restriction and reduced Rdp3 activity increase transcription of dSir2 (Rogina et al., 2002). Unlike the case in other organisms, the role of dSir2 in the response to dietary restriction in flies has not been studied extensively however, and would benefit from additional characterization.

The relationship between sirtuins and dietary restriction is further complicated by a recent collaborative study examining the original *sir-2.1* and dSir2 over expression constructs in worms and flies, respectively (Burnett et al., 2011). In both cases, life span extension in the sirtuin overexpression strains was found to be caused by artifacts within the strain background or the transgenic overexpression machinery. This complicates the relationship with dietary restriction, as in some cases the same strains were used to examine epistatic relationships between sirtuins and dietary restriction or other aging pathways. Additional research will be necessary to determine which of these findings can definitively be linked to overexpression of sirtuins. The details and potential implications of this study are discussed in Chapter 4.

The relationship between SIRT1 and dietary restriction in mice is complex. SIRT1 has been linked to both stress resistance and the regulation of metabolic processes, including hormone levels and fat storage, providing a potential connection to dietary restriction via diet and nutrient sensing (Guarente and Picard, 2005). While the effect of dietary restriction on longevity in mice with elevated SIRT1 levels is not known, knocking out SIRT1 in mice represses the increase in physical activity normally observed in response to dietary restriction (Chen et al., 2005a) and prevents life span extension from dietary restriction (Li et al., 2008). SirT1 mRNA and protein levels are reported to be increased in some tissues in response to dietary restriction, but there is evidence that dietary restriction also downregulates SIRT1 in some tissues. One study in mice looking specifically at the liver found that SIRT1 activity is decreased in response to dietary restriction and increased in response to high-fat diet (Chen et al., 2008). Liver-specific SIRT1 knockout mice are also partially protected from fat accumulation and have improved metabolic characteristics on a high-fat diet relative to wild type animals with similar food intake (Chen et al., 2008).

A common approach used to study the interaction between sirtuins and diet is to look at the response to pharmacological activators of sirtuins. The most common is resveratrol, a potent small-molecule activator of Sir2 found in the skin of grapes and other plants (Howitz et al., 2003). Resveratrol has been reported to increase life span in yeast (Howitz et al., 2003), worms (Viswanathan et al., 2005; Wood et al., 2004), flies (Bauer et al., 2004; Wood et al., 2004), and one short-lived species of fish

(Valenzano et al., 2006), though the findings in yeast, worms, and flies have proven difficult to replicate (Bass et al., 2007b; Kaeberlein et al., 2005c). In mice, resveratrol was protective against the health consequences of a high-fat diet (Baur et al., 2006; Lagouge et al., 2006). A potential confounding factor in studies using resveratrol is specificity. Resveratrol activates AMPK in addition to SIRT1, raising the question as to which effects are caused by increased SIRT1 activity and which are caused by increased AMPK activity (Baur et al., 2006). One recent study identified PDE4 inhibition as the probably mechanism by which resveratrol activates AMPK (Park et al., 2012). SIRT1720, a small-molecule activator of SIRT1 that does not activate AMPK and has improved potency relative to resveratrol, was identified in a small-molecule screen (Milne et al., 2007). Like resveratrol, SIRT1720 was found to protect mice fed a high-fat diet from developing obesity and insulin resistance by promoting oxidative metabolism in metabolic tissues (Feige et al., 2008). While feeding mice a high-fat diet cannot exactly be considered the opposite of dietary restriction, these studies do provide a clear link between diet and sirtuins, and high-fat diet may indeed be a more appropriate model for modern human societies. Notably, Feige et al. (2008) also observed transcriptional changes typically associated with low energy levels in response to treatment with SIRT1720, suggesting a potential link between sirtuins and dietary restriction.

TOR signaling: a conserved mediator of life span extension by dietary restriction

Among genetic pathways that regulate life span, the evidence is most consistent for TOR signaling as a mediator of the response to dietary restriction (Stanfel et al., 2009). As noted above, reduced TOR signaling is the only intervention aside from dietary restriction that extends life span in mice, flies, worms, and both yeast paradigms. The TOR signaling pathway is also a conserved nutrient responsive pathway (Kapahi and Zid, 2004) that has been observed to be inhibited, as measured through a reduction in autophagy and S6 kinase (S6K) activity, in response to dietary restriction in a variety of model organisms (Arsham and Neufeld, 2006; Bhaskar and Hay, 2007; De Virgilio and Loewith, 2006).

Findings in invertebrate models point strongly toward TOR signaling as a mediator of dietary restriction. In yeast, replicative life span extension by dietary restriction and *TOR1* deletion is non-additive (Kaeberlein et al., 2005d). Replicative life span extension by dietary restriction, deletion of *TOR1*, or deletion of *SCH9* is additive with deletion of *FOB1* and independent of *SIR2* (Kaeberlein et al., 2004; Kaeberlein et al., 2005d; Kaeberlein et al., 2006a; Tsuchiya et al., 2006), placing dietary restriction and TOR in a common pathway that is distinct from Sir2 and Fob1. In further support of this notion, the starvation-responsive GCN4 transcription factor attenuates replicative life span extension by deletion of TOR1 (Steffen et al., 2008). GCN4 and TOR signaling influence many of the same cellular processes and

GCN4 expression is primarily regulated by translation, suggesting a model in which TOR signaling or dietary restriction influences GCN4 target genes by translationally regulating GCN4 expression (Steffen et al., 2008; Valenzuela et al., 2001; Yang et al., 2000). TOR has yet to be definitively linked to dietary restriction with respect to aging in mice or the yeast chronological paradigm.

Life span extension from reduced TOR signaling and dietary restriction is similarly non-additive in worms (Hansen et al., 2007). Studies have also found that autophagy induced by reduced TOR signaling is required for dietary restriction life span extension in both worms (Hansen et al., 2008; Jia and Levine, 2007; Toth et al., 2008) and flies (Juhasz et al., 2007). A connection between dietary restriction and TOR signaling has not been tested directly in the yeast chronological paradigm, though one group has linked chronological life span extension by deletion of *TOR1* to mitochondrial respiration (Bonawitz et al., 2007). Bonawitz et al. (2007) proposed a model in which dietary restriction derepresses respiration by inhibiting TOR signaling, leading to increased mitochondrial oxygen consumption and resulting in decreased damage from reactive oxygen species and extension of chronological life span.

As with IIS and sirtuins, a role for TOR signaling in mammalian response to dietary restriction has yet to be definitively demonstrated, though indirect evidence from several studies examining phenotypes in mice treated with rapamycin provides some cause for optimism about a connection between aging, dietary restriction, and TOR signaling. For example, treatment with rapamycin prevents weight gain in both humans and rats (Rovira et al., 2008) and improves resistance to cancer, neurodegeneration (Caccamo et al., 2010; Spilman et al., 2010), and cardiac disease in mice (Gao et al., 2006; Wullschleger et al., 2006). Dietary restriction has long been known to reduce the occurrence of cancer in rodents (Ross and Bras, 1965; Tannenbaum, 1942; Weindruch and Walford, 1982; Yu et al., 1982) and has been found to suppress proteotoxicity in models of neurodegenerative diseases in nematodes (Steinkraus et al., 2008).

Several studies have also examined the role of components of the TOR signaling pathway in the context of high-fat diet. A 2008 study found that mice with an adipose-specific knockout of raptor, an essential gene and specific component of the mammalian TORC1 (mTORC1) complex, were lean, had less adipose tissue, exhibited improved insulin sensitivity, and were resistant to diet-induced obesity relative to control mice (Polak et al., 2008). Polak et al. (2008) also found increased expression of genes encoding mitochondrial uncoupling proteins and heightened energy expenditure caused by an increase in uncoupled respiration, suggesting that mTORC1 regulates energy homeostasis by controlling adipose metabolism. Whole-body knockout of S6K, which is positively regulated by mTORC1, results in mice that are lean and have improved insulin sensitivity and resistance to age- and diet-induced obesity because of increased energy expenditure (Pende et al., 2000; Um et al., 2004). Consistent with these findings, whole-body knockout of 4E-BP1 or 4E-BP2, which are both negatively regulated by mTORC1, results in

obese mice that are hypersensitive to diet-induced obesity (Le Bacquer et al., 2007). These studies indicate that reduced TOR signaling is protective against the damaging effects of eating a high-fat diet, which is consistent with a model in which the action of dietary restriction on longevity is mediated by reduced TOR signaling.

CONCLUSION

The past few decades have seen the emergence of IIS, TOR signaling, sirtuins, and dietary restriction as important and evolutionarily conserved regulators of aging and longevity. Pharmacological agents that target components of these pathways, such as resveratrol and rapamycin, are being developed and tested for aging-related activities in model organisms. Clinical trials for some of these agents are already under way for treatment of cancer and diabetes and will probably be expanded to other age-related disorders. These trials mark the first clinical benefits derived from comparative genetics of aging in model organisms.

The current state of the aging research field highlights the complex nature of the aging process and how it interacts with other aspects of an organism's biology at the molecular, cellular, organ, and evolutionary level. The identification and investigation of key genetic pathways in the determination of longevity has started to bring into focus the shape of the aging process as a whole. Ongoing efforts in aging research are focused on discovering new aging pathways, identifying the underlying molecular causes of aging that are acted upon by these pathways, understanding how aging genes and interventions interact to determine longevity and disease outcomes, and, based on findings in these areas, to developing environmental, pharmacological, and genetic interventions to delay aging and treat age related disease. This chapter has outlined out current understanding of the most widely studied organisms, interventions, and genetic pathways in aging research. The following chapters discuss complete and ongoing research related to ongoing goals in aging biology.

Chapter 2: Large-scale Approaches to Studying Aging

CHAPTER SUMMARY

Over the past decade, methodological advances in measuring and analyzing longevity has pushed the comparative study of aging to a genomic scale in worms and yeast, allowing for the first quantitative analysis of conservation of genetic factors involved in determining longevity. These studies provide insight into the scope of cellular processes that influence longevity and the conservation of longevity determinants between organisms, and have led to the identification of pharmacological agents targeting both the TOR signaling pathway and sirtuins that are now in clinical trials for the treatment of cancer and diabetes. These trials represent the beginning of efforts to translate studies of the basic biology of aging to interventions useful for fighting age-related diseases in people. This chapter reviews the advancement of large-scale technologies and methods as applied to questions within the field of aging biology, including genome-scale life span screens, genomics, proteomics, and metabolomics, and discusses insights gained with respect to novel processes involved in aging, mechanisms of longevity determination, and conservation of aging genetics.

INTRODUCTION

A great deal of effort has gone into the identification and characterization of interventions and genetic pathways that influence longevity in invertebrate models (see Chapter 1). Much of this work has been accomplished by looking at secondary age-associated phenotypes, such as stress resistance and fecundity, or by looking for genes associated with pathways already known to influence aging. This approach has yielded valuable insight and an understanding of specific pathways and processes that influence life span but does not inform with respect to the total number of genes and pathways that affect aging. Are there only a few aging genes or many? The past decade has seen the creation of an ORF deletion collection in yeast and RNAi libraries in nematodes and fruit flies, allowing for the first time the development of unbiased methods for looking at life span on a genomic-scale.

The desire to apply findings from studies in diverse organisms to human aging raises another central question answerable only by inquiry at a genomic level: to what degree are the molecular mechanisms involved in the determination of life span conserved between evolutionarily divergent organisms? Results from the first genome-wide longevity studies indicate that a large number of genes are likely to play a role in longevity and provide the first quantitative evidence for evolutionary conservation of longevity determinants. A running theme among genome-scale screens is the identification of components of the TOR signaling pathway, highlighting its importance as a conserved aging factor. This chapter discusses the transition of the aging field into genome-scale research and the implications of recent findings in the context of conserved longevity interventions. The primary focus of the chapter is genome-scale longevity screens, though other high-throughput methods, such as microarrays and proteomics, are also discussed.

WORM GENOME-WIDE LONGEVITY SCREENS

The research discussed in Chapter 1 demonstrates that life span is under genetic control and that the mechanisms of control are conserved across divergent species, at least to a degree. The next task is to determine whether the majority of genes involved in life span control are already known, or whether there are still a substantial number of longevity genes yet to be discovered. This requires the ability to look at a large fraction of the genes in a particular genome. The search for genes that influence life span entered the realm of genomics with the creation of large-scale genetic libraries for *S. cerevisiae* and *C. elegans*. These libraries are being used to screen for mutations that extend life span. Looking specifically for increased

life span is particularly important when screening at the genomic level, where the potential for identifying mutations that shorten life span independent of aging is vast.

Unlike other systems, where RNAi requires injection or transgenic expression of double stranded RNA, RNAi can be used to knock down the expression of any given ORF in *C. elegans* by simply feeding animals bacteria expressing double-stranded RNA corresponding to that ORF (Timmons and Fire, 1998). This opens the way for large numbers of worm genes to be targeted by manipulating the genetics of bacteria using well understood and easily scalable techniques without having to directly modify the worm genome. Based on this idea, two RNAi libraries were created using *E. coli* that together cover more than 90% of the ORFs in the *C. elegans* genome (Kamath et al., 2003; Rual et al., 2004). Two large-scale longevity screens (Hamilton et al., 2005; Hansen et al., 2005) and several screens targeting specific subsets of genes (Chen et al., 2007a; Curran and Ruvkun, 2007; Dillin et al., 2002; Kim and Sun, 2007; Lee et al., 2003) were performed using these libraries, resulting in the identification of 276 genes that extend life span when knocked down (Smith et al., 2008b). These two genome-wide screens represent the first unbiased approach to the discovery of novel aging genes.

The implication of the discovery of a large number of genes that influence life span in independent studies is that a substantial fraction of the genes in the *C. elegans* genome are likely to play a role in aging. The question "why so many?" brings us back to evolutionary theory (see Introduction). One implication of post-Medawar aging theory is that organisms will be selected for overall fitness and not for maximum longevity. Under this model for selection you might expect to find a large number of genes that increase life span when their expression is altered. A further extension of the relationship between fitness and longevity is that mutations that increase longevity should also have a detrimental effect on overall fitness. Indeed, long-lived *C. elegans* mutants were found to have reduced fitness relative to wild type in both a demographic survival analysis (Chen et al., 2007b) and in direct competition assays (Jenkins et al., 2004; Walker et al., 2000). Mutations resulting in enhanced longevity are also associated with reduced performance in other areas—most commonly reproduction—in worms (Apfeld and Kenyon, 1999; Van Voorhies and Ward, 1999) and flies (Buck et al., 2000; Burger et al., 2007; Marden et al., 2003; Mockett and Sohal, 2006).

YEAST GENOME-WIDE LONGEVITY SCREENS

The *S. cerevisiae* haploid deletion collection consists of approximately 4,800 yeast strains in a common strain background, each with a single non-essential gene deletion (Winzeler et al., 1999). The strategy of completely knocking out a gene removes the problems associated with variability in the

efficiency of gene knock down by RNAi experienced in the *C. elegans* RNAi screens, but has the disadvantage of excluding all essential genes. The yeast deletion collection has been used to screen for long-lived mutants in both the replicative and chronological paradigms.

Genome-scale Replicative Life Span Screening

Measurement of replicative life span is manual labor intensive and a screen of the deletion collection has been underway since the early 2000s. In an initial report covering the first 564 randomly selected single gene mutants, 13 (2.3%) were found to be long-lived, 5 of which are known to function in the TOR signaling pathway (Table 2.1) (Kaeberlein et al., 2005d). Chapter 3 provides a detailed description of the methods used in screening the yeast ORF collection for increased replicative analysis and reports the current results from this screen.

The 51 gene deletion strains reported thus far from the deletion collection screen for increased replicative life span have led to surprising advances in our understanding of the pathways modulating replicative longevity in yeast. For example, the initial analysis of 564 randomly selected deletion strains led to the hypothesis that dietary restriction is mediated primary via reduced signaling through the TOR kinase (Kaeberlein et al., 2005d). This was based on the observation that among the 13 replicatively long-lived single-gene deletion strains identified from the original 564, at least 5 are known to function in the TOR pathway (Kaeberlein et al., 2005d).

TORC1 regulates several downstream processes that may contribute to its role in aging, including protein degradation via autophagy, mitochondrial metabolism, stress response, and mRNA translation (Stanfel et al., 2009). TORC1 signaling in yeast also influences stress responsive transcription factors in a cooperative and/or redundant fashion with the PKA and the ribosomal S6 kinase ortholog, Sch9 (Hosiner et al., 2009; Pedruzzi et al., 2003; Smets et al., 2008; Swinnen et al., 2006). These transcription factors include Msn2, Msn4, Rim15, and Gis1. As a consequence, reduced TOR signaling results in a constitutive stress response. Induction of these stress responsive transcription factors appears to be particularly important for chronological life span extension, but the majority of available data suggest they play only a minimal role in modulation of replicative life span. In fact, it has been reported that triple deletion of *MSN2*, *MSN4*, and *RIM15* modestly increases replicative life span and does not prevent life span extension from deletion of *SCH9* (Fabrizio et al., 2004b). In a separate report, deletion of both *MSN2* and *MSN4* did not prevent life span extension from dietary restriction (Lin et al., 2000).

Table 2.1 Published genes for which deletion results in increased replicative life span. The genes with nematode orthologs indicated were identified as part of the worm to yeast ortholog screen for conserved longevity determinants (Smith et al., 2008b).

Yeast ORF	Yeast Gene	Nematode ORF	Nematode Gene	Function
YNR051C	BRE5			molecular function unknown
YBL087C	RPL23A			structural constituent of ribosome
YBR084C-A	RPL19A	C09D4.5	rpl-19	structural constituent of ribosome
YBR238C				molecular function unknown
YBR255W	MTC4			molecular function unknown
YBR266C	SLM6			molecular function unknown
YBR267W	REI1			sequence-specific DNA binding
YCR028C-A	RIM1			single-stranded DNA binding
YDL035C	GPR1			G-protein coupled receptor activity
YDL075W	RPL31A			structural constituent of ribosome
YDL082W	RPL13A			structural constituent of ribosome
YDR006C	SOK1			molecular function unknown
YDR110W	FOB1			ribosomal DNA (rDNA) binding
YDR268W	MSW1			tryptophan tRNA ligase activity
YDR382W	RPP2B			structural constituent of ribosome
YDR500C	RPL37B			structural constituent of ribosome
YDR523C	SPS1			protein serine/threonine kinase activity
YER017C	AFG3	Y47G6A.10	spg-7	ATPase activity
YFR032CA	RPL29			structural constituent of ribosome
YGL076C	RPL7A			structural constituent of ribosome
YGL078C	DBP3	B0511.6		ATP dependent RNA helicase activity
YGL147C	RPL9A	R13A5.8	rpl-9	structural constituent of ribosome
YGL167C	PMR1	B0365.3	eat-6	calcium-transporting ATPase activity
YGL208W	SIP2			SNF1A/AMP-activated protein kinase activity
YGR063C	SPT4	F54C4.2	spt-4	Pol II transcription elongation factor activity
YGR162W	TIF4631	M110.4	ifg-1	translation initiation factor activity
YHL002W	HSE1	C14F5.5	sem-5	protein binding
YIL002C	INP51	JC8.10	unc-26	inositol-polyphosphate 5-phosphatase activity
YIL052C	RPL34B			structural constituent of ribosome
YJL138C	TIF2	F57B9.6	inf-1	translation initiation factor activity
YJR066W	TOR1	B0261.2	let-363	protein binding
YJR094WA	RPL43B			structural constituent of ribosome
YKL056C	TMA19			molecular function unknown
YKR059W	TIF1	F57B9.6	inf-1	translation initiation factor activity
YKR072C	SIS2	Y46H3C.6		phosphopantothenoylcysteine decarboxylase activity
YLR061W	RPL22A			structural constituent of ribosome
YLR136C	TIS11	F52E1.1	pos-1	mRNA binding
YLR180W	SAM1	C06E7.1	sams-3	methionine adenosyltransferase activity
YLR262C	YPT6	T23H2.5	rab-10	GTPase activity
YLR371W	ROM2			signal transducer activity
YLR448W	RPL6B	R151.3	rpl-6	structural constituent of ribosome
YNL037C	IDH1	F43G9.1		isocitrate dehydrogenase (NAD) activity
YNL229C	URE2			transcription co-repressor activity
YNR030W	ALG12	T27F7.3		alpha-1,6-mannosyltransferase activity

Table 2.1 (continued).

Yeast ORF	Yeast Gene	Nematode ORF	Nematode Gene	Function
YOL086C	ADH1	W09H1.5		alcohol dehydrogenase activity
YOL100W	PKH2	H42K12.1	pdk-1	protein kinase activity
YOR109W	INP53	JC8.10	unc-26	inositol-polyphosphate 5-phosphatase activity
YOR136W	IDH2	F43G9.1		isocitrate dehydrogenase (NAD) activity
YOR312C	RPL20B			structural constituent of ribosome
YPL079W	RPL21B			structural constituent of ribosome
YPL101W	ELP4			Pol II transcription elongation factor activity
YGR130C*		C01G8.5	erm-1	molecular function unknown
YHR205W*	SCH9	Y47D3A.16	rsks-1	protein serine/threonine kinase activity

[a] Indicates genes published as part of the worm to yeast ortholog study but not verified in the MAT**a** background. The data in this table was compiled from numerous sources (Kaeberlein et al., 2005b; Kaeberlein et al., 2005d; Managbanag et al., 2008; Smith et al., 2008b; Steffen et al., 2008).

Among TORC1-regulated processes, control of mRNA translation appears to be the most relevant for replicative life span determination. TORC1 activity promotes mRNA translation in multiple ways, including both up-regulation of ribosomal S6 kinase and S6 kinase-independent regulation of translation initiation factors and ribosomal protein biosynthesis (Wullschleger et al., 2006). Sch9 is known to also modulate replicative life span and genetically maps to the same epistasis group as dietary restriction and TOR, consistent with a role downstream of TOR in modulating aging (Fabrizio et al., 2004b; Kaeberlein et al., 2005d).

In the initial analysis of 564 deletion strains, strains lacking two different genes coding for ribosomal large subunit proteins (*rpl31aΔ* and *rpl6bΔ*) were among the long-lived mutants (Kaeberlein et al., 2005d). While most ribosomal proteins are thought to be essential in yeast, the majority of genes encoding ribosomal proteins are present in the yeast genome in duplicate, often allowing for viable deletion of either paralog (Komili et al., 2007; McIntosh and Warner, 2007). Since this study, deletion of genes encoding 13 additional large subunit ribosomal proteins and 3 translation initiation factors (*tif1Δ*, *tif2Δ*, and *tif4631Δ*) have been found to increase replicative life span from the deletion set analysis (Table 2.1) (Steffen et al., 2008).

Genome-wide Analysis of Chronological Life Span

Chronological life span has typically been assayed by culturing cells into stationary phase in liquid synthetic defined media, maintaining the cells in the expired culture media, and periodically measuring the percent of cells still alive by diluting and plating onto a nutrient rich agar-based media (Kaeberlein, 2006). Viability is then calculated based on the number of colonies arising on the nutrient agar. Alternative methods with different culture media components have also been described. For example, some studies use glycerol as the primary carbon source rather than glucose or transfer stationary phase cells to water rather than maintaining them in expired media. All of these methods require the relatively time- and resource-consuming step of counting colony forming units in order to quantify survival of the aged cells.

Powers et al. (2006) described a high-throughput method for qualitatively measuring chronological life span of cells aged in 96-well microtiter plates. Rather than monitoring survival by determining colony forming units, Powers et al. (2006) estimated relative cell viability of the population by diluting the aging culture into rich liquid media and measuring the optical density at 600 nm (OD) following a fixed outgrowth period. All cell and liquid transfers were automated using a high-density replica pinning robot. While less quantitative than the traditional methodologies, this method offers the ability to monitor survival for several thousand strains simultaneously. As proof-of-principle, Powers et al. (2006) screened the homozygous diploid ORF deletion collection for long-lived mutants. Of the 90 longest-lived strains, 16 have been implicated in TOR signaling and nutrient uptake (Powers et al., 2006). Thus the first unbiased yeast longevity screen in both the replicative and chronological paradigms strongly implicate TOR signaling as a central regulator of aging and longevity. In both yeast aging paradigms, genetic or pharmacological inhibition of TOR signaling increases life span and is believed to mediate the life span extending benefits of dietary restriction (Table 1.2) (Fabrizio et al., 2001; Kaeberlein et al., 2005d; Powers et al., 2006). Dietary restriction can be accomplished in the chronological aging assay in a manner similar to the replicative aging assay, by reducing the glucose concentration of the growth media, or by an alternative method in which aging stationary phase cells are transferred to water. Similar to the case for replicative life span, chronological life span extension from dietary restriction is believed to be independent of Sir2. In contrast to replicative life span, the mechanism by which reduced TOR signaling and dietary restriction promote chronological life span appears to be mediated primarily by regulation of carbon metabolism, which is discussed in detail below (Bonawitz et al., 2007).

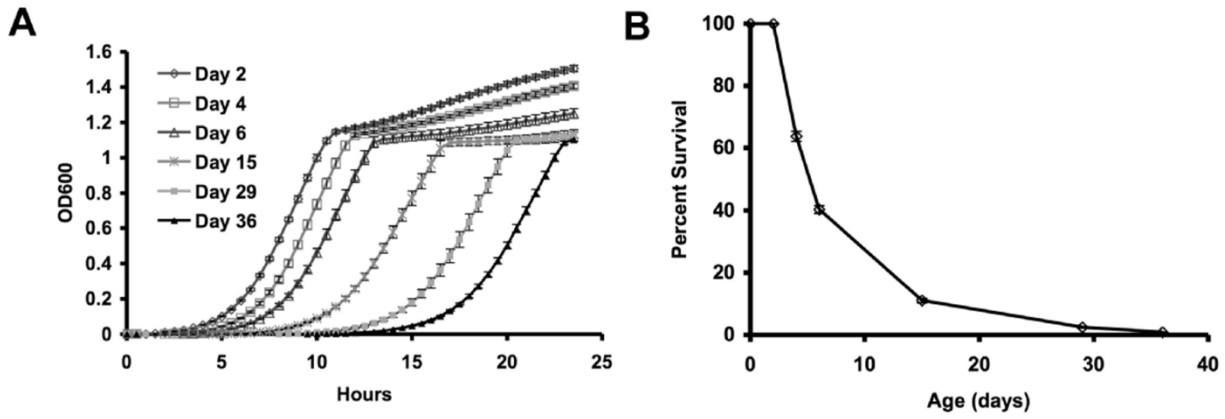


Figure 2.1 Chronological life span survival curves are calculated based on time delay for strain specific outgrowth. (A) Outgrowth curves shift rightward as stationary phase cells age. (B) Survival curves are calculated from the time shift between outgrowth curves.

Of the 16 genes identified by Powers et al. (2006) that are implicated in the TOR pathway, 5 were found to consistently extend chronological life span when subject to more stringent analysis: the nitrogen-responsive transcription factor *GLN3*, the lysine biosynthetic homo-isocitrate dehydrogenase *LYS12*, the nitrogen-responsive ammonium permeases *MEP2* and *MEP3*, and amino acid permease *AGP1*. Each of these deletion mutants shows increased glycogen accumulation characteristic of starvation, and each mutation inhibits TOR signaling by limiting amino acid uptake or synthesis (Powers et al., 2006). Powers et al. (2006) also demonstrate that pharmacological inhibitors of TOR signaling (methionine sulfoximine and rapamycin) increase chronological life span.

More recently, we modified the method described by Powers et al. (2006) to create a high-throughput assay for measuring chronological life span that allows quantitative analysis of chronological life span across the entire deletion collection (Murakami et al., 2008). This redesigned method improves the quantitative resolution by using an entire outgrowth curve to calculate residual survival rather than a single outgrowth time-point. Our studies use a Bioscreen C MBR (Growth Curves USA) machine to obtain outgrowth curves from aged cells, though any shaker/incubator/plate reader combination will suffice. To monitor viability at each age-point, 5 μL of the aging culture is inoculated into 145 μL of YPD in one well of a Bioscreen Honeycomb plate. Outgrowth of the inoculated cells takes place in the Bioscreen C MBR machine at 30°C with continuous shaking. OD is determined every 30 min for 24 h yielding highly reproducible outgrowth curves from which relative survival can be calculated. Outgrowth curves of aging cells show a distinct age-dependent rightward shift, such that the length of time required to achieve a given OD value increases with age (Figure 2.1A). A survival curve can be generated from the

Bioscreen growth data based on the estimated fraction of cells retaining viability at each time point (Figure 2.1B). The viable fraction is calculated relative to the initial time point (typically day 2) based on the rightward time shift required for outgrowth to reach a fixed OD value of 0.3 using the formula:

$$v_n = \frac{1}{2^{\left(\frac{\Delta t_n}{\delta}\right)}}$$

where v_n is the viability at time point n, t_n is the time shift between the outgrowth curves at OD = 0.3 for the initial and nth time points, and δ is the doubling time of the strain (determined by the maximal slope of the semi-log plot of OD as a function of time). We have recently developed software that will perform all calculations needed to determine chronological life span from outgrowth data, which can be accessed at http://www.sageweb.org/yoda (Olsen et al., 2010).

Ongoing studies are currently aimed at using the Bioscreen method to obtain quantitative measures of chronological life span for each single-gene deletion strain in the ORF deletion collections. During the initial phase of these studies, the effect of media composition on chronological life span was also explored. As previously observed (Fabrizio et al., 2005; Smith et al., 2007a), dietary restriction by lowering the glucose content of the initial culture media from 2 to 0.5% (or lower) significantly increased chronological life span (Murakami et al., 2008). Surprisingly, increasing the amino acid content of the media also increased chronological life span (Murakami et al., 2008). This response to high amino acid abundance does not appear to be directly related to amino acid metabolism, but instead reflects the pro-longevity effects of inducing the osmotic stress response.

While exploring the possible mechanisms by which dietary restriction might increase chronological life span, it was observed that cells cultured in low glucose media do not acidify their cultures to the same extent as cells grown in 2% glucose (Burtner et al., 2009). Standard growth media for chronological aging experiments initially has a pH of about 4.5. Within a few days, the expired media reaches a pH of approximately 3.0 when the starting glucose concentration is 2%, does not change significantly when the starting glucose concentration is 0.5%, and becomes alkalinized to about pH 6.0 when the starting glucose concentration is 0.05%. Interestingly, buffering the pH of cells grown in 2% glucose media at 6.0 is sufficient to extend chronological life span in a manner comparable to cells grown in un-buffered 0.05% media. Further experiments demonstrated that the causative factor underlying these observations is acetic acid, which is produced by chronologically aging cells during back-fermentation of ethanol and is known to induce an apoptotic-like response in yeast cells (Herker et al., 2004). An important additional conclusion from the studies of Burtner et al. (2009) is that many of the previously known mutations that increase chronological life span can be explained by either (1) reduced production

of acetic acid during growth into stationary phase or (2) increased resistance to acetic acid (Table 2.2). By modifying the chronological life span procedure it may be possible to minimize the cell non-autonomous effects of organic acid secretion during fermentation.

In a more recent study, a candidate-gene approach was taken to measure chronological life for four sets of deletion collection strains: (1) a randomly selected set of strains, (2) strains lacking yeast homologs of genes reported to extend *C. elegans* life span, (3) strains reported to be replicatively long-lived, and (4) strains identified in a genome-wide screen for decreased acidification of the culture medium (Burtner et al., 2011). Neither the set of *C. elegans* homologs nor the set of replicatively long-lived strains was found to be enriched for chronologically long-lived strains as compared to the randomly selected set, suggesting that yeast chronological aging is not mechanistically similar to either aging in worms or replicative life span in yeast. Notably, the strain set selected based on increased media acidification was significantly enriched for strains with increased chronological life span. This finding supports a model in which media acidification plays a primary role in chronological aging under conditions commonly used in chronological life span assay and is consistent with the idea that acetic acid is a primary molecular cause of chronological aging.

Table 2.2 Increase in chronological life span related to acetic acid can be caused either by reduced acetic acid production or increased acetic acid resistance.

Condition or strain yielding increased chronological life span	Reduced acetic acid production	Increased acetic acid resistance
non-fermentable carbon source	X	
water	X	
high osmolarity		X
sch9Δ	X	X
ras2Δ		X
tor1Δ	X	
HAP4 overexpression	X	
ADH1 overexpression	X	
adh2Δ	X	
yca1Δ		X

The past few years have seen the emergence of a competitive-survival strategy for identifying genes involved in chronological aging. In this strategy, the ~4,800 strains from the yeast deletion collection are pooled in a common chronological aging culture. Portions of the culture are taken at different time points and allowed to grow for a specified amount of time before harvesting DNA. Microarray or deep sequencing is used to determine the relative abundance of each deletion strain in the aged culture using two unique sequences built into each deletion mutant. Strains enriched in the chronologically aged culture are considered putatively chronologically long-lived and confirmed using standard single-strain chronological life span techniques. Novel chronological longevity genes were reported based on this approach in three recent publications (Fabrizio et al., 2010; Gresham et al., 2011; Matecic et al., 2010).

Fabrizio et al. (2010) screened the deletion collection in standard synthetic media, while both Gresham et al. (2011) and Matecic et al. (2010) used media depleted for specific nutrients (leucine or phosphate, and glucose, respectively). Long-lived strains included mutants for genes acting in a variety of processes, including amino acid biosynthesis, purine biosynthesis, Golgi trafficking, lipid biosynthesis and processing, and heat resistance. Strikingly, all three studies, as well as the previously mentioned candidate gene study by Burtner et al. (2011), identified multiple chronologically short-lived strains with mutations in genes related to mitochondrial function. Respiratory capability has previously been reported to be required for chronological survival (Bonawitz et al., 2007). Fabrizio et al. (2010), Gresham et al. (2011), and Matecic et al. (2010) also all identified multiple short-lived autophagy mutants, suggesting that the ability of a cell to degrade cellular components is important for long-term survival in a non-dividing state.

Notably, numerous novel determinants of chronological life span were identified in all three competitive-survival screens (Fabrizio et al., 2010; Gresham et al., 2011; Matecic et al., 2010) as well as the candidate gene approach reported by Burtner et al. (2011). This suggests that many genes involved in chronological aging have yet to be identified. The screen of each individual strain from the deletion collection for increased chronological life span that is currently underway is anticipated to identify many of these unknown genes.

QUANTITATIVE EVIDENCE FOR CONSERVED MECHANISMS OF LONGEVITY CONTROL

Early applications of genomics to longevity have provided the first glimpse of the true scope of the aging landscape and identified potential key players in the aging process, but the degree to which mechanisms that control longevity are generally conserved between evolutionarily disparate organisms has only recently begun to be addressed. Unbiased genome-wide analyses of longevity in yeast and worms provide the first opportunity to address in a quantitative manner the degree of conservation of aging determinants between evolutionarily disparate organisms. This directly impacts the question of relevance to human aging. On the evolutionary timeline, yeast and nematodes are separated by approximately 1.5 billion years, while nematodes and humans are separated by only approximately 1 billion years (Wang et al., 1999). Thus, if we can identify genes that play a conserved role in modulating life span between yeast and nematodes, a subset is likely to play a similar role in mammalian aging as well (Figure 2.2).

Results from genome-wide longevity screens were leveraged to provide the first quantitative evidence for conservation of longevity control between *S. cerevisiae* and *C. elegans* (Smith et al., 2008b). Smith et al. (2008b) measured replicative life span for single gene deletion strains in yeast corresponding to each of the 276 worm genes identified in the RNAi longevity screens. First, yeast genes were selected based on protein homology using a two-tiered approach. Yeast orthologs for each worm gene were identified using a high-stringency modified BLASTp reciprocal best match criterion, allowing selection of two yeast genes for a single worm gene when two yeast paralogs had BLASTp scores within 10% of each other. Up to 6 yeast homologs were then selected for each worm gene, requiring at least 20% protein sequence identity and at least 10% amino acid alignment. In total, 264 yeast homologs were selected for analysis, of which 76 met the high-stringency requirements.

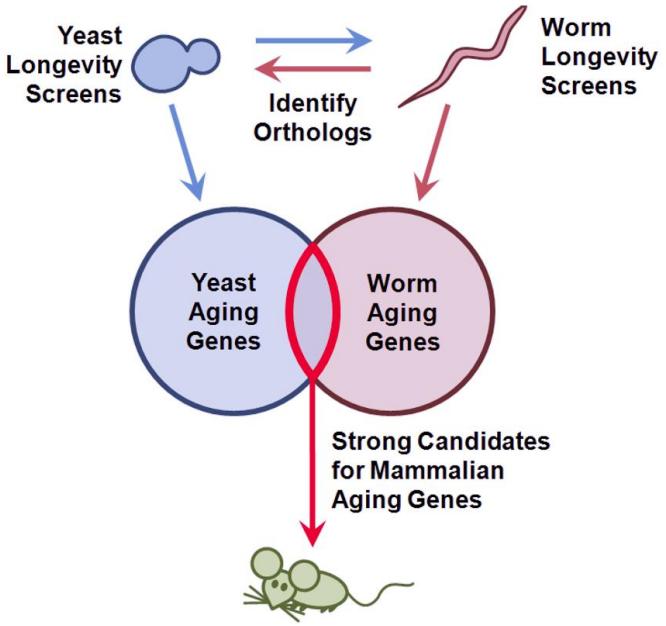


Figure 2.2. A genome-wide, multi-organism approach to studying genes involved in aging. Cross-examining orthologs of longevity genes between evolutionarily divergent organisms, such as yeast and worms, allows the identification of genes that play a conserved role in longevity determination. These genes are strong candidates for aging genes in diverse organisms, such as mammals.

Replicative life span analysis identified 25 (9.5%) long-lived mutants from the set of 264 analyzed (Smith et al., 2008b). Of the 76 orthologs that met the high-stringency requirement, 11 (14.5%) were long-lived. This is a substantial enrichment for longevity determinants as compared to the expected 2.3% (3.4% if only yeast genes with potential worm orthologs are considered) as estimated by the unbiased screen of 564 genes (Kaeberlein et al., 2005d), demonstrating evolutionary conservation of genes that control aging between yeast and worms.

The nature of the aging models used in the comparison makes this finding particularly striking. Yeast replicative life span is a measure of mitotic aging. In contrast, cells in the adult *C. elegans* are completely post-mitotic with the exception of the germline. Such genetic conservation between mitotic and non-mitotic aging is not intuitively obvious. Of the 25 genes identified by Smith et al. (2008b), 15 have clear human orthologs, adding further weight to the possibility of conservation from yeast and worm to humans.

The most notable feature of the conserved longevity factors identified by Smith et al. (2008b) is the substantial enrichment for genes that code for proteins involved in regulating mRNA translation. Among the 25 ortholog pairs, only 2 were previously known to modulate aging in both yeast and worms: *TOR1*/*let-363* and *SCH9*/*rsks-1*. *SCH9* and *rsks-1* are homologs of mammalian ribosomal S6 kinase,

which functions downstream of TOR signaling to modulate mRNA translation initiation (Pan et al., 2007; Urban et al., 2007). Excluding *TOR1*/*let-363* itself, 6 of the 10 remaining ortholog pairs in the high-stringency set can be definitively assigned functions related to mRNA translation: three ribosomal proteins of the large subunit (*RPL19A*/*rpl-19*, *RPL6B*/*rpl-6*, *RPL9A*/*rpl-9*) and three translation initiation factors (*TIF1*/*inf-1*, *TIF2*/*inf-1*, *TIF4631*/*ifg-1*). As outlined previously, all of these factors are thought to act in a single conserved longevity pathway (Figure 1.4).

Further support for this hypothesis has recently been provided by the identification of the nutritionally regulated Gcn4 transcription factor as a potential downstream mediator of life span extension in response to reduced TOR signaling and altered mRNA translation (Kaeberlein et al., 2005d; Steffen et al., 2008). Gcn4 induces expression of amino acid biosynthetic genes in response to amino acid starvation (Hinnebusch, 2005) and plays a role in a variety of cellular processes including autophagy, ER stress response, organelle biosynthesis, and induction of mitochondria transport carrier proteins (Jia et al., 2000; Natarajan et al., 2001; Patil et al., 2004). Steffen et al. (2008) found that deletion of *GCN4* partially blocked the life span extension resulting from dietary restriction, deletion of *TOR1*, deletion of *SCH9*, or deletion of an RPL. Furthermore, Gcn4 activity was specifically upregulated in two long-lived RPL mutants (*rpl20bΔ* and *rpl31aΔ*) but not in mutants lacking paralogs of these genes that are not long lived (*rpl20aΔ* and *rpl31bΔ*), demonstrating that the increase in Gcn4 activity is linked to the increase in life span (Steffen et al., 2008).

Cellular levels of Gcn4 are primarily controlled by translation and protein degradation, as opposed to transcription (Hinnebusch, 2005), and both RPL mutations and reduced TOR signaling have previously been shown to induce Gcn4 activity (Cherkasova and Hinnebusch, 2003; Foiani et al., 1991; Kubota et al., 2003; Martin-Marcos et al., 2007; Valenzuela et al., 2001). Cellular levels of Gcn4 are translationally regulated by four small inhibitory upstream ORFs (uORFs) in the 5' leader region of the *GCN4* gene (Hinnebusch, 2005). The mechanism of regulation is thought to involve relative availability of the large and small ribosome subunits. Specifically, when 60S ribosomal subunit levels are low, ternary complexes containing initiation factors and 40S ribosomal subunits are proposed to more frequently scan through the inhibitory uORFs before interacting with 60S subunits, increasing translation of *GCN4* (Steffen et al., 2008). Thus, while reducing availability of 60S ribosomal subunits reduces overall translation, translation of the *GCN4* transcript specifically increases, supporting a model where reduced mRNA translation influences longevity by differentially modifying translation of specific mRNA targets. This mechanism for translation inhibition may extend to other genes involved in controlling life span and several yeast genes are known to contain inhibitory uORFs, including *HAP4* and *CLN3* (Vilela and McCarthy, 2003; Zhang and Dietrich, 2005), which are involved in nutrient response. As previously noted, overexpression of *HAP4* increases replicative life span (Lin et al., 2002).

A model placing TOR, S6 kinase, protein synthesis, and a Gcn4-like transcription factor in a linear pathway controlling life span may be overly simplistic on the broader stage of public mechanisms of longevity control. While both TOR signaling and TOR-regulated protein synthesis factors modulate life span in both yeast and worms (Smith et al., 2008b), longevity epistasis studies in *C. elegans* map *let-363* (TOR), but not *rsks-1* (S6K) and translation initiation factors, to the same pathway as dietary restriction (Hansen et al., 2007). The results of Hansen et al. (2007) suggest that dietary restriction extends life span by reduced protein translation via TOR signaling, while knockdown of S6 kinase and other protein synthesis factors may act through a different mechanism.

Notably absent from the set of 25 conserved longevity ortholog pairs, not to mention any the genome-wide longevity screens in worms, are genes known to function in the same pathway as *SIR2*/*sir-2.1* (Curran and Ruvkun, 2007; Dillin et al., 2002; Hamilton et al., 2005; Hansen et al., 2005; Lee et al., 2003; Smith et al., 2008b). This lack of evidence for Sir2-related conservation of longevity control may reflect the limited understanding of upstream and downstream factors involved in *SIR2*/*sir-2.1*-mediated longevity control. It may also be due to the apparently dissimilar mechanisms by which Sir2 orthologs modulate longevity in different organisms (Figure 1.3).

EMERGING HIGH-THROUGHPUT STRATEGIES FOR STUDYING AGING

Genome-scale screens for increased longevity have proven a valuable tool for identifying new aging pathways. With the possible exception of yeast chronological aging, the methods for quantifying life span remain time consuming and manual labor intensive. The development of high-throughput survival assays would substantially increase the potential for large-scale longevity screens of genetic libraries, environmental conditions, or pharmacological agents. Alternatively, techniques to generate large populations of aged individuals would allow biochemical approaches to identifying age-associated changes in physiology and molecular markers. Several groups are taking steps to address these technical problems.

With respect to worms, survival is typically judged by manually prodding individual worms and looking for a response. To generate a complete survival curve, all individual animals are typically examined every 2-3 days for the duration of the experiment. Genetic screens typically side step this problem by examining the number of worms surviving to a certain time point and performing follow up experiments to generate complete survival data for genes that show a potential for increased life span based on the single point. One group sought to develop an alternative means by which to judge when an animal dies that might be modified for automation or rapid manual scoring. They developed a method in

which survival is evaluated using SYTOX green fluorescent nucleic acid dye, which is excluded in live worms but is easily taken up dead worms (Gill et al., 2003). An automated worm sorter and a microplate fluorometer were combined to automatically sort and assess fluorescence of individual worms. Gill et al. (2003) tested the method in thermal and oxidative stress survival assays, and demonstrated that resulting survival characteristics were similar to those measured by a traditional manual assessment, though at much higher throughput (11,520 worms/hour for the automated system, as compared to 600 worms per hour for an experience investigator). While the stress survival assays were carried out over the course of approximately one day, the method can in principal be scaled to apply to a full standard worm life span. The potential for toxicity to the SYTOX dye may be one complicating factor that would have to be examined for application to full-scale life span experiments. Gill et al. (2003) also demonstrated that the system can be used to quantify GFP expression driven by a promoter of interest, opening the door for large-scale transcriptional expression screens.

In the context of yeast replicative aging, biochemical and genomic studies limited by the necessity of obtaining a large, relatively pure population of cells that have undergone a large number of divisions. Each time a mother cell divides it produces a virgin daughter cell that subsequently begins dividing. A dividing cell population is necessary to produce replicatively aged cells, but also produces a population in which replicatively aged cells are exceedingly rare ($\sim 1/2^n$ for n generation old cells). One technique for acquiring large numbers of replicatively aged cells employs cell sorting based on the number of fluorescently labeled bud scars and has been used in several studies (Chen and Contreras, 2007; Sinclair and Guarente, 1997; Smeal et al., 1996), but cannot produce a sufficiently large population of cells older than 12 generations to be used for microarrays or other large-scale approaches. Two additional methods have been developed to produce populations of replicatively aged cells from dividing cell populations in yeast.

One technique for extracting replicatively aged cells from a mixed population is termed counter flow centrifugation elutriation (CCE). CCE, originally invented in 1948, uses the balance between centrifugal force and counter flow drag in a spinning buffer media to separate cells of different sizes in a mixed population (Sloot et al., 1988). As yeast cells divide they also increase in size (Mortimer and Johnston, 1959; Nestelbacher et al., 2000), resulting in a population of small, young cells and large, old cells. Using CCE to select large cells from a dividing population thus results in a subset enriched for cells with advanced replicative age. This technique was employed to separate mixed yeast cell populations and compare young cells (2–3 generations) to old cells (16–18 generations) and to "old" cells lacking *DNA2-1*, a model of premature aging (8 generations) (Lesur and Campbell, 2004). Lesur and Campbell (2004) found transcription upregulation of a variety of genes involved in energy storage and environmental stress response in the aged and prematurely aged cells relative to young cells. Laun et al.

(2001) used CCE to produce produced cell populations with 30% terminally senescent cells, indicating that the population was substantially enriched for cells near the end of their replicative life span. This technique was used in a transcriptome study comparing replicatively aged cells to a population of cells driven to apoptosis by mutation of *CDC38*, which identified *MRPL25*/*AFO1* (Laun et al., 2005), a gene that encodes a mitochondrial large subunit ribosomal protein that influences longevity by interacting with *TOR1* through mitochondrial back signaling (Heeren et al., 2009). A related method combining growth synchronization and rate-zonal sedimentation in density gradients was used to produce cell populations highly enriched for cells aged up to 20 generations (Egilmez et al., 1990). This second method was applied in another transcriptome study to identify the long-lived *LAG1* deletion strain (D'Mello N et al., 1994). While an improvement on fluorescence-based cell sorting, CCE is still only able to enrich for cells aged to around 20 generations, which is still well below the median replicative life span of many of the common strains in yeast aging (typically in the low to mid 20s).

A second system, termed the Mother Enrichment Program (MEP), has recently been developed to produce populations enriched for aged mother cells (Lindstrom and Gottschling, 2009). The MEP uses Cre-lox recombination to specifically disrupt two essential genes, *UBC9* and *CDC20*, in newly formed daughter cells, thus eliminating the replicative capacity of the daughter cells without altering that of the mother. This system allows highly enriched populations of mother cells to be grown that can be purified with single-step affinity purification. MEP has promise to be a powerful tool for obtaining large quantities of replicatively aged cells for biochemical and microarray studies.

In addition to applications in young-vs.-old comparisons, the MEP has been proposed as a high-throughput method to measure replicative life span (Lindstrom and Gottschling, 2009). Since daughter cells are produced but do not continue dividing, the viability of MEP cultures is determined specifically by the replicative life span of the mother cells. The use of a plate reader system, such as that used to measure chronological life span (Murakami et al., 2008) can allow rapid, high-throughput measurement of replicative life span for strains carrying the MEP biological machinery in liquid media. While there are still technical challenges yet to overcome before MEP becomes widely used, this technique shows particular promise for the application of replicative life span drug screening, where current labor-intensive methods for measuring replicative life span limit the number of drugs that can be easily tested, and where further genetic manipulation of the strains is not necessary.

Two recent studies present a microfluidics approach to studying yeast replicative aging (Lee et al., 2012; Xie et al., 2012). In both systems, individual virgin mother cells are secured within a microfluidics chamber. Media is continuously flowed over the cells, maintaining a constant environment and washing away newly budded daughter cells. By combining the microfluidics device with continuous microscopy, budding events are automatically tracked and quantified to generate replicative life span

data. While the microfluidics technology does not yet allow for high-throughput quantification of replicative longevity, these systems do allow continuous monitoring of individual yeast cells throughout their replicative life spans. A variety of phenotypes can be simultaneously tracked, including cell cycle dynamics, organelle morphology, and expression and localization of fluorescent markers. For example, Lee et al. (2012) reported a gradual increase in both cell and vacuole size with replicative age, and a dramatic increase in cell division time in the last few divisions prior to senescence. Xie et al. (2012) identified transcriptional activity of the molecular chaperone Hsp104 to be a good predictor of the replicative life span of individual cells. Microfluidics hold a great deal of promise for both the development of automated, high-throughput systems for measuring replicative life span and for identifying important age-associated changes in cell morphology and molecular markers of aging.

MICROARRAYS, PROTEOMICS, AND METABOLOMICS

Genome-scale technologies have become standard throughout the biological sciences and have been applied to study aging over the past decade. The application of microarrays to aging generally is still in its infancy and has challenges to overcome (Melov and Hubbard, 2004). Several studies have used microarrays to look at gene expression changes between yeast populations with different age distributions. Two studies by Lin et al. (2001b) and Lesur and Campbell (2004) have attempted to compare replicatively young (1–3 generations) and old (7–8 generations and 16–18 generations, respectively) populations to identify changes in expression patterns with age. Both studies found upregulation of genes involved in gluconeogenesis and glucose storage in the older cell populations. In addition, Lesur and Campbell (2004) found an upregulation of environmental stress proteins in the older aged population. Replicative aging studies using microarrays share several challenges. The primary challenge is the aforementioned difficulty with obtaining a sufficiently pure quantity of replicatively aged cells. The second is the relatively young age of the "old" cells used in studies to date. Even 16–18 generation cells are well below the typical low-to-mid 20 generation median age of most strains commonly used to study replicative life span in yeast. Developing strategies such as the MEP have the potential to solve both of these problems going forward. A third problem involves the medium used to grow cells. The yeast cells used in most replicative aging microarray studies have been grown in liquid culture, while replicative life span is traditionally measured by microdissection of cells grown on plates. The disparate growth conditions limit the ability to correlate results from microarray studies to changes in replicative life span. Microarrays have yet to be applied to study expression changes with chronological age, though the chronological aging paradigm lacks many of the system specific problems associated with

replicative aging since pure populations of chronologically aged cells are easy to obtain in large quantities using the same media conditions used to measure chronological life span.

Similar microarray studies comparing young and old individuals found that genes involved in oxidative stress response are upregulated with age in flies (Landis et al., 2004; Pletcher et al., 2002; Zou et al., 2000), mice (Weindruch et al., 2001) and monkeys (Kayo et al., 2001), which is consistent with an observed increase in expression of oxidative stress genes in young individuals from long-lived *C. elegans* strains (McElwee et al., 2003; Murphy et al., 2003). One group used microarray analysis to compare age-associated gene expression changes between *C. elegans* and *D. melanogaster* (McCarroll et al., 2004) and found a similar age-related gene expression program involving mitochondrial metabolism and DNA repair, among others. Further studies of this type will be of interest, particularly involving comparison of gene expression patterns between invertebrates and mammals.

An alternative microarray strategy compares gene expression patterns between young, age-matched individuals with different longevity phenotypes (resulting from differences in environmental exposure, genotype, or both) with the goal of identifying genetic programs that contribute to increased life span when activated early in life. One study used this approach to compare the expression profiles of wild type yeast to three chronologically long-lived strains with mutations in *TOR1*, *SCH9*, and *RAS2* (Cheng et al., 2007a; Cheng et al., 2007b). The expression patterns implied an overall reduction in transcription in the three mutant strains as compared to wild type, as well as a down-regulation in genes involved in the TCA cycle and oxidative phosphorylation relative to genes involved in glycolysis. The *ras2Δ* strain also showed a reduced expression of genes involved in mitosis, distinguishing it from the other two long-lived mutants. In mice, this strategy demonstrated that gene expression for Ames dwarf mice is different from wild type mice subject to dietary restriction and that changes in gene expression in response to dietary restriction are different for wild type and Ames dwarf mice (Masternak et al., 2004). This is in agreement with the independent action of dietary restriction and IIS on life span in *C. elegans* (Houthoofd et al., 2003; Kaeberlein et al., 2006b; Lakowski and Hekimi, 1998; Lee et al., 2006). Similar studies have linked dietary restriction to osmotic stress and increased respiration in yeast (Kaeberlein et al., 2002; Lin et al., 2002) and to growth hormone signaling in mice (Miller et al., 2002). Microarrays have also been used to demonstrate that gene expression changes associated with dietary restriction occur quickly relative to life span in mice (Dhahbi et al., 2004), which is consistent with a rapid decrease in mortality in response to dietary restriction in flies (Mair et al., 2003) and mice (Dhahbi et al., 2004) and the observation that dietary restriction extends worm life span even when initiated late in life (Smith et al., 2008b). The ability to identify short-term changes in gene expression with potential long-term consequences on life span opens the possibility of screening for pharmacological agents that mimic the beneficial effects of dietary restriction (Kaeberlein, 2004).

A study combining the two microarray strategies compared the age-associated changes between mice fed a control diet and mice subject to dietary restriction and found that dietary restriction reversed a subset of the age-related gene expression changes (Weindruch et al., 2001). Taken together, aging microarray studies to date demonstrate their potential for identifying global changes associated with advanced age or enhanced longevity. Unfortunately, many of these early attempts at applying microarrays to aging have suffered from a myriad of technical and analytical problems, such as limited sample size or lack of rigorous statistical analysis. Nevertheless, the field remains optimistic that microarrays will be prevalent in the future of aging research and addressing the technical challenges and discussion of novel approaches to using microarrays in aging has been the topic of many reviews (Becker, 2002; Golden et al., 2006; Han et al., 2004; Melov and Hubbard, 2004; Nair et al., 2003; Werner, 2007). Technical issues aside, microarrays are inherently limited in that they are observational in nature. Another method is needed to identify genes mechanistically involved in aging.

Microarrays are currently the standard approach used to measuring transcript levels in a cell population or tissue; however, since microarrays are based on sequence-specific hybridization, they suffer from problems with background noise and cross-hybridization and can only be used to measure relative transcript abundance (Irizarry et al., 2005). Recent advances in massively parallel DNA sequencing technology allows transcript level to be analyzed by deep sequencing of reverse transcribed RNA as an alternative to microarrays. While not yet widely used, one study demonstrates the advantages of deep sequencing as compared to multiple microarrays when both technologies are applied to look for transcriptional differences in hippocampal tissue between two different mouse strains (t Hoen et al., 2008). The authors found that the deep sequencing approach identified differential transcription of more transcripts with higher precision than any of the microarrays. Deep sequencing identified transcripts with abundance spanning 4 orders of magnitude, which allowed detection of much lower abundance transcripts. Deep sequencing was also more reproducible across laboratories as compared to the microarrays, which the authors attribute to lack of cross-hybridization and lower background noise (t Hoen et al., 2008).

Microarrays and related technologies that measure the “transcriptome” of a tissue or organism can provide valuable insight into the genes that are involved in phenotypes associated with a given genetic background or biological intervention. A limitation of these techniques is that they only give indirect information about the content of proteins, metabolites, and other molecules directly involved in an organisms interaction with its environment. The detection of these molecules is the focus of up and coming fields such as proteomics, which studies the protein complement of a cell or organism, and metabolomics, which studies the array of small molecule metabolites present in an organism. One group has recently taken the first steps toward establishing these methods in yeast aging by comparing the

metabolic histories of chronologically aging yeast with and without dietary restriction using a variety of techniques to measure phenotypes ranging from protein and metabolite levels to ROS, mutation rates, and stress resistance (Goldberg et al., 2009). They conclude that yeast set up a metabolic profile prior to entering a non-proliferative state that depends on the contents of the original media and present a model suggesting how this profile might contribute to chronological aging. While proteomic and metabolomic methods have yet to be widely applied to the study of yeast aging, both fields are growing and hold promise to provide valuable insight in the future.

CONCLUSION

The advent of moderate- to high-throughput genome-scale techniques onto the aging research scene has revolutionized our understanding of the scope and mechanistic underpinnings of the processes that determine longevity. Genome-wide longevity screens in worms and yeast have identified hundreds of novel genetic aging factors and highlighted key genetic pathways, such as TOR signaling and mRNA translation. Interspecies longevity studies have demonstrated evolutionary conservation of aging for the first time. Early microarrays point to oxidative stress, mitochondrial metabolism, and DNA repair as important factors upregulated with age or by longevity interventions.

New technologies and methodologies promise improved accuracy or broader scope for current assays, such as life span analysis or transcriptome measurements, as well as entirely new realms of information, such as proteomics and metabolomics. As we begin to grasp the complete range of molecular, cellular, and intercellular processes involved in aging, the development of new techniques will be necessary to investigate the complex interactions between these systems. Large-scale approaches to aging have proven their worth and will continue to be a primary source of information going forward.

Chapter 3: The Development of a Formal System for Large-Scale Life Span Epistasis Analysis

CHAPTER SUMMARY

Genome-wide screens have identified hundreds of genes in yeast and worms, while targeted strategies in multiple models have built known genes into pathways that play a conserve role in the aging process. As key players in these conserved aging pathways continue to be uncovered and characterized using model systems, we will gain a better understanding of how they function and interact to integrate environmental signals into cellular responses that modulate aging. It has become apparent that, although longevity interventions can be mapped to genetically distinct pathways through epistasis and other types of studies, in reality most (or all) of these conserved longevity modifiers interact within cells as part of a complex network. In future studies, it will be important to consider not only which proteins play a conserved longevity role, but which interactions between longevity factors have also been conserved. Such an approach should make it possible to develop a more comprehensive picture of the overarching longevity network and may resolve lingering questions and controversies in the field while providing more effective routes toward therapies for improving human health span and longevity. Tools for analyzing interactions between multiple interventions will be necessary to develop a network-level understanding of how aging is controlled. This chapter describes the results from the first genome-wide replicative life span screen in yeast and describes a formalized system for analyzing and interpreting life span epistasis interactions, which is a first step in a strategy for building an aging gene network.

INTRODUCTION

The emergence of genome-wide life span screens in worms and yeast has led to the discovery of hundreds of genes that modify life span (reviewed in Chapter 2). In worms, RNAi libraries covering more than 90% of the ORFs in the worm genome allow high-throughput screens for a variety of phenotypes, including life span (Kamath et al., 2003; Rual et al., 2004). In yeast, the yeast ORF deletion collection, consisting of ~4,800 single-gene deletion strains, has spurred the development of high-throughput methods for measuring chronological life span (Burtner et al., 2011; Powers et al., 2006). Measurement of yeast replicative life span at the genome-scale is complicated by the manual labor intensive nature of the assay, and true high-throughput methods have yet to be developed. To deal with this problem, a moderate-throughput iterative strategy was developed to identify long-lived mutants in the yeast deletion collection by determining replicative life span for a minimum number of cells and using statistical methods to select strains for further testing (Kaeberlein et al., 2005d). A preliminary report describing replicatively long-lived strains from the first 564 strains in the ORF deletion collection identified 13 genes for which deletion extends replicative life span, of which 5 (*ROM2, RPL6B, RPL31A, TOR1,* and *URE2)* map to the TOR signaling pathway (Kaeberlein et al., 2005d). This screen was recently completed and is discussed further below.

In addition to genome-scale longevity screens, various other approaches have been employed to identify and investigate novel interventions capable of increasing life span. Screens for more easily measured secondary age-associated phenotypes, such as stress resistance, have been used to identify novel longevity determinants. Drug screens have identified pharmacological agents capable of increasing life span in worms and flies (Bauer et al., 2004; Collins et al., 2006; Lublin et al., 2011). In 2008, we used a cross-species approach to identify a set of genes that play a conserved role in longevity control by screening yeast single-gene deletion strains lacking orthologs of known worm aging genes for increased replicative life span (Smith et al., 2008b). On the environmental side, a major combined effort is underway to understand the mechanistic underpinnings of dietary restriction.

As new aging interventions are discovered and characterized they are grouped into pathways based on interactions with respect to specific phenotypes. The most prevalent of these pathways—dietary restriction, IIS, TOR signaling, and sirtuins—are detailed in Chapter 1. One implication that comes out of the investigation of the interaction between genetic interventions with respect to aging is that the commonly defined pathways are only partially distinct, and that complex interactions between pathways occur with respect to shared response to upstream signals, interaction between pathway components, and influence on overlapping sets of downstream factors. For example, TOR activity both modulates and is modulated by IIS, while dietary restriction alters signaling through both pathways.

Epistasis analysis is widely used in aging research and is a primary means by which pathway relationships are established; however, its use is often complicated by a wide range of possible outcomes and by variation in how outcomes are interpreted from one research group to another (Cordell, 2002; Gems et al., 2002). One problem in the biomedical literature results from what is meant by an "epistatic interaction". In most cases, a definition is assumed without being clearly stated. When considering a quantitative phenotype like life span, epistasis most commonly refers to a departure from additivity when two interventions are combined (Cordell, 2002). The interventions are said to be independent if the combined change in the phenotype under investigation is equivalent to adding the change resulting from applying each intervention individually. All other outcomes indicate an epistatic relationship. This relationship can be in the form of epistatic synergy (the combined change is greater than expected from adding the individual changes) or epistatic antagonism (the combined change is less than expected from adding the individual changes). By this definition, epistatic dependence (the combined change falls between the individual changes) is a subset of epistatic antagonism.

A second complication arising from epistasis is the fact that a single epistasis outcome can result from multiple models of biological interaction between two interventions (Cordell, 2002). Two examples within the aging field demonstrate this point clearly. In worms, reducing expression of the insulin/IGF-1-like receptor, DAF-2, increases life span in the context of a wild type background, but has no effect on life span in a background lacking the FOXO-family transcription factor, DAF-16 (Kenyon et al., 1993). This epistatic interaction was interpreted to show that reduced DAF-2 activity extends life span by activating DAF-16, and this relationship between DAF-2 and DAF-16 has been confirmed by observations using a variety of techniques examining multiple phenotypes. In yeast, dietary restriction by reducing the glucose concentration in the media extends replicative life span in a wild type context, but has no effect on life span when the *SIR2* gene is deleted (Kaeberlein et al., 2004; Lin et al., 2000). Again, this epistatic relationship was interpreted to show that dietary restriction extends life span by activating Sir2. Unlike the case for DAF-2 and DAF-16 in worms, when the short life span of *sir2Δ* is repressed by deletion of *FOB1*, dietary restriction robustly extends replicative life span (Kaeberlein et al., 2004; Lamming et al., 2005), demonstrating that Sir2 is not necessary for life span extension by dietary restriction. We now think that deletion of Sir2 limits life span by inducing a form of damage that accumulates with replicative age, preventing life span extension by dietary restriction but remaining independent of the mechanism by which dietary restriction extends life span (see Chapter 4). In these examples, two pairs of interventions with different biological interactions produce identical epistasis outcomes with respect to life span.

Despite these complications, epistasis analysis is a powerful tool for determining whether an interaction exists between two different interventions. As the list of genes, drugs, and environmental

manipulations that influence life span continues to grow, and the complexity of the interactions between aging pathways begins to be revealed, properly defining and interpreting epistatic interactions will become even more critical. This chapter first describes the results from the recently completed replicative life span screen of the yeast ORF deletion collection, and then presents a formal system for annotating and interpreting epistatic interactions with respect to life span phenotypes. A final section describes the application of this system to a life span epistasis dataset examining the interaction between two sets of genes previously implicated in yeast replicative aging.

RESULTS

An Iterative Strategy for Moderate-Throughput Replicative Life Span Analysis

True high-throughput methods for quantitatively measuring replicative life span in yeast have yet to be described and current methods require the relatively time consuming microdissection of daughter cells away from mother cells every 1–2 generations. At least 50 cells are typically necessary to obtain reliable replicative life span data for a single strain, with the experiment preferably performed in triplicate. The MATα deletion set contains ~4,800 strains with an average life span for the parental strain (BY4742) of approximately 26 cell divisions (Kaeberlein et al., 2005b). In order to screen the entire MATα deletion collection for replicative life span, standard methodology requires microdissection of approximately 19 million daughter cells. These factors have limited large-scale attempts at replicative life span determination and caused investigators to focus primarily on hypothesis driven or candidate gene studies of replicative aging.

To address this issue, we developed an iterative strategy for identifying replicatively long-lived single gene deletions from the haploid yeast ORF deletion collection in order to bring large-scale screens for replicative life span in to the realm of practicality (Kaeberlein et al., 2005d). This approach uses the standard microdissection method for determining replicative life span, but focuses on using smaller set sizes for each single gene deletion mutant available in the MATα deletion set. In order to minimize the effort required per strain, statistical methods were used to identify the minimum number of mother cells that needed to be assayed in order to identify 95% of mutants with a 30% or greater increase in replicative life span. The result is an iterative method in which 5 cells are initially assayed for each deletion mutant (Figure 3.1) (Kaeberlein et al., 2005d). Based on the average replicative life span of these 5 cells and that of the wild type parental strain, each mutant is given a putative longevity classification. Additional cells are assayed for strains that show potential for long replicative life span until a definitive classification can

be made. Once a deletion mutant has been definitively classified as long-lived in the MATα background, the corresponding deletion from the MAT**a** deletion collection is examined for replicative life span. Those deletions that are found to be long-lived in both haploid mating types are considered to be high-confidence modifiers of replicative life span.

The iterative approach for identifying long-lived deletion mutants was developed based on data collected as part of a large-scale analysis of genes previously reported to increase life span in different strain backgrounds (Kaeberlein et al., 2005b). From this analysis, replicative life span data was generated for greater than 10,000 cells, of which more than 500 were wild type (strain BY4742) and more than 500 were deletion strains with a mean replicative life span at least 30% greater than BY4742 (*hxk2Δ*, *gpa2Δ*, *gpr1Δ*, and *fob1Δ*). These data were used to determine the number of cells statistically required at each stage of the iterative process for genome-wide replicative life span analysis.

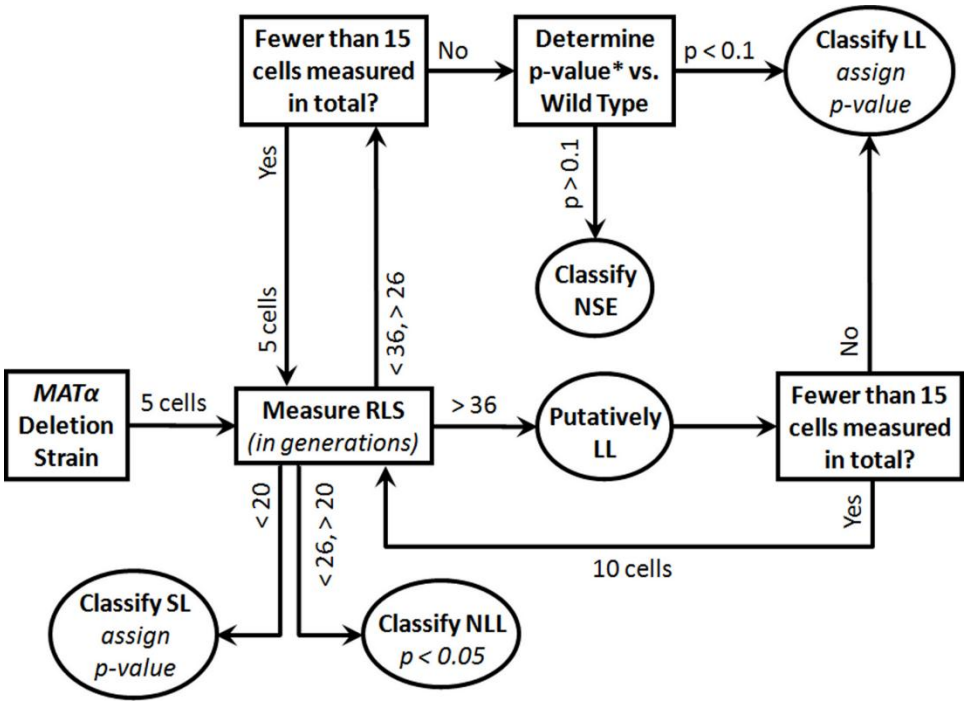


Figure 3.1 Flow diagram illustrating the iterative approach for identifying long-lived strains from the yeast deletion collection. LL – Long-Lived; NSE – No Significant Extension; NLL – Not Long-Lived; SL – Short-Lived. Mutations classified as LL by this process are subsequently verified as replicatively long-lived in the MAT**a** background. P-values are for comparison of mean replicative life span for the deletion strain in question to the cumulative probability distribution of BY4742 wild type replicative life span with n = 5, except *, which indicates a p-value for a Wilcoxon Rank-Sum test comparing replicative life span of deletion mutant to experiment matched BY4742 wild type.

The replicative life span data for wild type mother cells (N > 500) were pooled into one set and replicative life span data for the mother cells of long-lived mutants *hxk2Δ*, *gpa2Δ*, *gpr1Δ*, and *fob1Δ* (N > 500) were pooled into a second set. Probability distributions were then generated for mean life span as a function of the number of cells examined (n), when n = 3, 5, 10, 15, and 20 for WT and LLM, respectively. For example, the n = 3 distribution for wild type was generated by randomly choosing 3 data points from the pooled wild type life span set, calculating the mean of the 3 values, and repeating the process 100,000 times. A histogram was then generated for the probability that a particular mean life span is obtained for a set size n = 3, with bins of width 0.1 generations. From this numerical analysis, an iterative strategy was established in which initially analyzing the replicative life span of 5 cells per deletion strain should allow the identification of a large fraction of strains with mean replicative life span 30% longer than wild type or greater.

In the final iterative method (Figure 3.1), if the 5 cell mean replicative life span is less than 26 generations, the strain is classified as not long-lived (NLL). From the cumulative probability distribution for known long-lived strains with n = 5, this is predicted to result in misclassification of a long-lived strain less than 5% of the time (false negative rate, FNR < 0.05). If the mean life span is less than 20, the strain is classified as short-lived (SL) and a p-value is assigned based on the cumulative probability distribution for wild type cells with n = 5. If the mean life span for 5 cells is greater than 36, the strain is putatively classified as long-lived (LL) and an additional 10 cells are examined. From the cumulative probability distribution for wild type cells with n = 5, this is predicted to result in misclassification of a strain with wild type life span less than 2% of the time (false positive rate, FPR < 0.02). For the remaining strains with a 5-cell mean life span between 26 and 36, an additional 5 cells are analyzed (1 iteration) and the same criteria for classification are applied. This process is repeated until every strain is either classified as SL, NLL, or LL, or until replicative life span has been determined for a total of at least 15 cells for each unclassified strain. The replicative life span data for strains from which at least 15 mother cells have been assayed are compared against experiment-matched wild type replicative life span data using a Wilcoxon Rank-Sum test and a p-value is generated. Strains with $p \leq 0.1$ are classified as LL, and strains with $p > 0.1$ are classified as having no significant life span extension (NSE). All strains classified as LL are subsequently analyzed in the MAT**a** background by determining the replicative life span for the corresponding deletion strain contained in the MAT**a** deletion collection. In cases where strains are significantly long-lived in the MATα background, but not in the MAT**a** background, the data sets for both mating types are pooled. The pooled replicative life span data is compared against experiment-matched wild type replicative life span data using a Wilcoxon Rank-Sum test to generate a p-value. Strains with $p \leq 0.1$ remain classified as LL, but are denoted as low-stringency. Strains with pooled $p > 0.1$ are reclassified as NSE.

In practice, replicative life span analysis of the deletion set is carried out in 95-strain sets (94 deletion strains and wild type). The ORF deletion collection is packaged in 96-well plates, with most plates containing 94 strains. Replicative life span is determined for 5 cells per strain, with 12 strains (one row of the 96-well plate) analyzed per 100 mm YPD plate. All replicative life span experiments are carried out blind.

Genome-Wide Replicative Life Span Screen Identifies 239 Pro-Aging Genes

Since the preliminary report covering the first 564 genes in the deletion collection (Kaeberlein et al., 2005d), the iterative strategy described above has been used to screen the entire haploid yeast ORF deletion collection for single-gene deletion strains with increased replicative life span. Replicative life span was examined for 4,687 strains in total, of which 447 (9.5%) were initially classified as LL based on the MATα data alone. Following verification in MAT**a** background, 239 (5.1%) retained the LL designation (Table 3.1). Replicative life span extension in this set ranged from 1.3% to 47.3% relative to experiment matched wild type cells (Figure 3.2). The vast majority of strains (3,677, 78.5%) were given a final classification as either NLL or NSE, indicating that no significant extension was observed, while 771 (16.4%) were found to have decreased replicative life span. Since the iterative strategy was designed to identify replicatively long-lived strains with a low false positive rate, it is not known what false negative rate applies to the latter two categories. With respect to the long-lived set, 115 (2.5%) were classified as high-stringency based on independent statistically significant extension in MATα and MAT**a** mating types (Tables 3.1 and 3.2), while the remaining 124 (2.6%) were classified as low stringency based on a lack of statistically significant extension in MAT**a** cells alone, but significant extension when MATα and MAT**a** experiments were pooled (Table 3.1 and 3.3).

Replicatively Long-Lived Gene Set Enriched for mRNA Translation Components

Gene ontology (GO) mapping was employed to search for GO Terms enriched in the replicatively long-lived gene set. The GO Slim Mapper on the *Saccharomyces* Genome Database website (SGD, 2012a) was first applied to the 239 genes to examine the associated range of GO Terms. The three GO Term categories (component, function, and process) were independently queried. The GO Terms associated with the long-lived gene represent a wide range of cellular processes and components including aging-associated processes, such as translation, respiration, and DNA repair (Table 3.4).

Table 3.1. Replicative life span classification summary for iterative screen of the haploid yeast ORF deletion collection. LL – Long-Lived; NSE – No Significant Extension; NLL – Not Long-Lived; SL – Short-Lived. LL strains are classified as high-stringency if statistically significant replicative life span extension was observed in both mating types and low-stringency if extension was significant both in the MATα background and when the two mating types were pooled, but not in MAT**a** background alone.

		Number of Strains	% of Total
Replicative Life Span Category	SL	771	16.4
	NLL	1746	37.3
	NSE	1931	41.2
	LL	239	5.1
	Total	4687	100.0
LL Stringency	High	115	2.5
	Low	124	2.6

In order to determine whether certain processes, functions, or components were specifically enriched in the long-live gene set, the GO Term Finder was employed (SGD, 2012b). The list of 239 replicatively long-lived genes were queried for associated GO Terms in the manually-curated GO Consortium database and compared to GO Terms associated with a reference list containing the 4,768 genes screened in the replicative life span screen. The replicatively long-lived gene set was significantly enriched ($p < 0.01$) for 42 GO Terms (Table 3.5). All three GO categories are clearly enriched for genes that have been annotated to play a role in mRNA translation or related processes. Of the total set of 239 genes, 64 (25.1%) are annotated as having some role related to translation (Table 3.6). This confirms the observed enrichment for translation genes among the 13 replicatively long-lived strains in the preliminary report describing the first 564 genes screened (Kaeberlein et al., 2005d). While genes involved in various aspects of cytoplasmic translation, mitochondrial translation, or TOR signaling are represented in the replicatively long-lived gene set, the majority are specifically involved in either the structure or biogenesis of the cytoplasmic ribosome (Table 3.6).

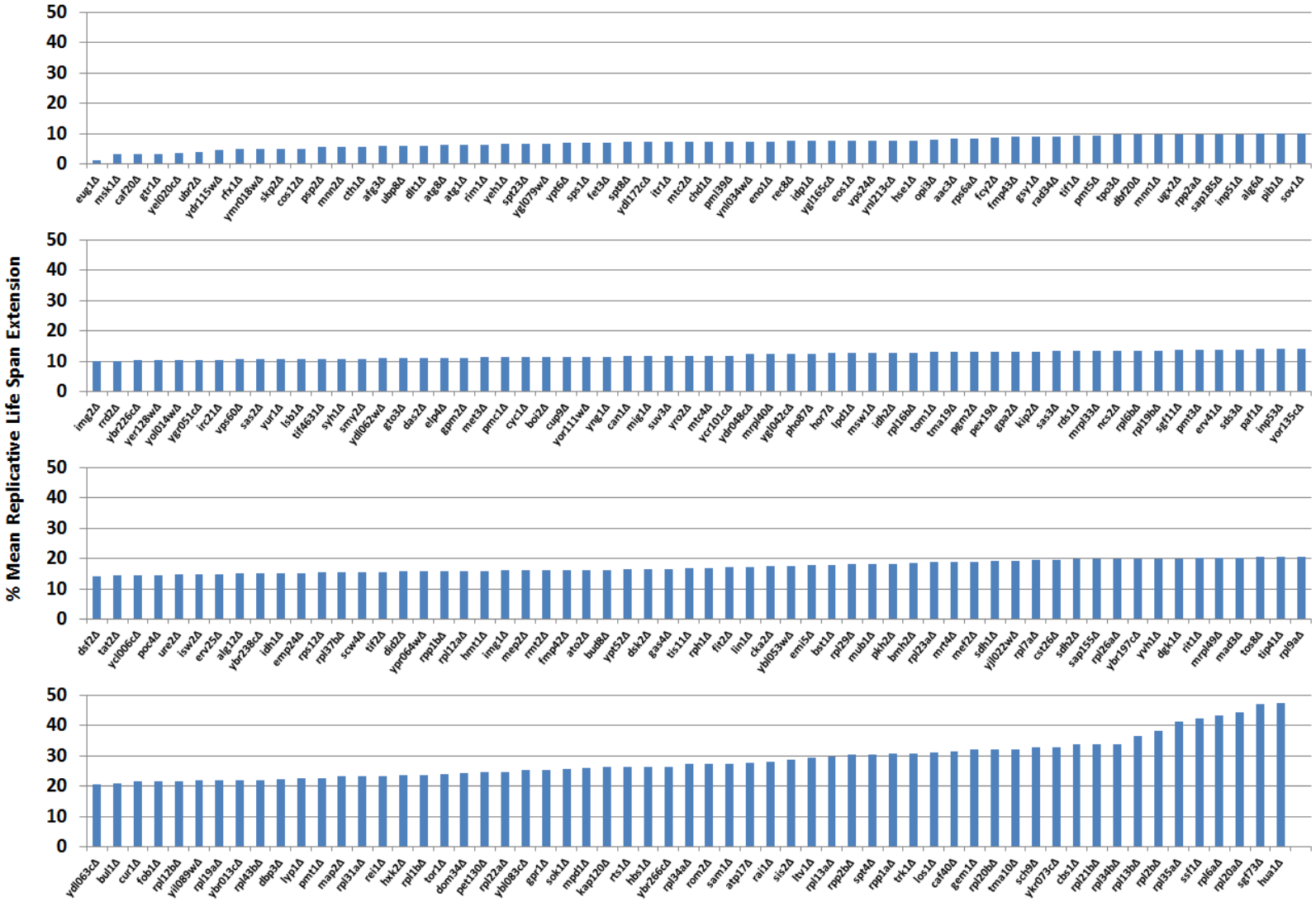


Figure 3.2. The 239 strains identified as long-lived in the iterative replicative life span screen display mean replicative life span extension ranging from 1.3% to 47.3%. All values shown are calculated based on mean replicative life span of pooled MATα and MAT**a** cells with the indicated genotype compared to that of pooled experiment matched wild type MATα and MAT**a** cells.

Table 3.2. Replicative life span data for 115 high stringency long-lived strains from the iterative replicative life span screen. The data provided is calculated from pooled MATα and MAT**a** data for each genotype and experiment matched wild type cells.

	Mutant		Wild Type				Mutant		Wild Type				Mutant		Wild Type		
		Mean		Mean				Mean		Mean				Mean		Mean	
Genotype	N	RLS	N	RLS	% Ext	Genotype	N	RLS	N	RLS	% Ext	Genotype	N	RLS	N	RLS	% Ext
aac3Δ	180	30.7	225	26.4	16.3	*mrt4Δ*	45	38.4	65	26.3	45.8	*sam1Δ*	205	33.2	281	25.7	29.3
atg8Δ	210	32.1	228	29.1	10.3	*msw1Δ*	65	32.8	65	26.3	24.6	*sap155Δ*	170	36.5	180	29.3	24.5
ato2Δ	45	36.4	65	26.9	35.6	*mub1Δ*	505	34.2	563	25.8	32.5	*sap185Δ*	124	33.5	125	27.4	22.4
bst1Δ	75	38.3	105	28.9	32.6	*ncs2Δ*	65	33.4	65	27.3	22.3	*sas3Δ*	90	28.4	130	23.8	19.4
bud8Δ	80	30.7	170	25.0	22.8	*pet130Δ*	45	37.2	65	27.2	36.6	*sch9Δ*	2000	35.9	2028	27.0	33.4
can1Δ	105	34.4	105	26.0	32.0	*pho87Δ*	65	31.4	85	25.8	21.9	*sds3Δ*	85	35.6	105	27.1	31.4
chd1Δ	145	31.1	144	27.4	13.4	*pkh2Δ*	265	31.2	302	25.8	21.1	*sgf11Δ*	125	36.6	105	29.3	25.0
cka2Δ	85	36.5	105	28.8	27.0	*pmt1Δ*	85	35.1	105	25.6	36.8	*sgf73Δ*	888	40.8	930	26.4	54.7
dbp3Δ	165	31.5	210	24.7	27.4	*pmt3Δ*	85	31.9	125	25.2	26.8	*sis2Δ*	160	36.1	185	25.7	40.8
dom34Δ	95	32.8	205	24.5	33.6	*pmt5Δ*	85	29.1	85	24.1	20.5	*sok1Δ*	984	37.1	1043	28.2	31.3
dsf2Δ	45	32.8	85	26.8	22.4	*poc4Δ*	45	33.6	85	26.3	27.6	*spt4Δ*	225	39.3	245	28.0	40.2
dsk2Δ	85	33.4	85	25.0	33.6	*rai1Δ*	45	35.3	45	23.5	50.0	*suv3Δ*	105	33.8	125	28.2	19.9
elp4Δ	145	32.1	205	27.6	16.2	*rec8Δ*	65	31.2	105	26.3	18.5	*tat2Δ*	65	33.5	105	26.4	27.0
emi5Δ	65	31.7	85	24.4	29.9	*rei1Δ*	150	37.9	170	28.0	35.6	*tif4631Δ*	255	30.5	278	25.4	20.3
emp24Δ	85	32.4	145	27.2	19.0	*rim1Δ*	165	32.0	245	27.5	16.3	*tip41Δ*	45	32.2	45	24.1	33.7
eos1Δ	145	35.0	185	28.2	24.3	*rit1Δ*	45	34.9	45	24.1	44.9	*tis11Δ*	160	32.2	185	26.1	23.3
erv41Δ	45	31.0	85	24.4	27.0	*rom2Δ*	120	32.9	170	24.9	32.5	*tma10Δ*	25	33.2	45	25.1	32.2
fit2Δ	45	32.8	45	20.7	58.9	*rph1Δ*	85	35.7	105	28.1	27.1	*tma19Δ*	105	35.7	145	28.3	26.1
fob1Δ	4379	32.8	4668	26.9	22.2	*rpl13aΔ*	185	34.7	219	25.4	36.4	*tom1Δ*	345	32.1	404	27.3	17.6
gpm2Δ	85	29.4	85	22.9	28.4	*rpl16bΔ*	108	32.6	163	27.7	17.7	*tor1Δ*	2504	32.6	2607	26.0	25.1
gpr1Δ	992	36.6	1312	28.1	30.4	*rpl19aΔ*	125	34.3	139	26.4	29.7	*ubp8Δ*	125	35.1	145	26.8	31.0
gsy1Δ	125	30.7	125	25.7	19.2	*rpl20bΔ*	1623	36.4	1638	26.3	38.5	*ubr2Δ*	419	38.4	438	28.6	34.2
gto3Δ	74	30.8	125	25.4	21.3	*rpl21bΔ*	185	36.9	205	24.7	49.5	*ybl053wΔ*	40	32.7	40	24.2	35.2
hor7Δ	83	34.0	145	26.6	27.7	*rpl22aΔ*	385	36.6	384	26.5	38.3	*ybl083cΔ*	50	34.8	70	24.8	40.5
hse1Δ	260	29.2	281	26.3	10.9	*rpl23aΔ*	130	34.0	134	26.0	31.1	*ybr197cΔ*	65	34.1	105	25.7	32.3
hxk2Δ	665	32.7	874	26.1	25.3	*rpl29Δ*	110	32.1	114	24.4	31.5	*ybr238cΔ*	193	31.4	220	26.1	20.6
idh2Δ	715	30.9	884	26.8	15.2	*rpl31aΔ*	928	34.9	998	26.8	30.0	*ybr266cΔ*	130	37.3	140	26.2	42.3
img2Δ	65	29.6	85	23.4	26.4	*rpl34bΔ*	147	35.4	213	25.7	37.5	*ydl062wΔ*	85	34.0	105	25.9	31.6
inp51Δ	490	31.7	505	27.9	13.4	*rpl37bΔ*	160	30.0	184	23.6	26.9	*ydl063cΔ*	45	33.9	85	26.0	30.4
isw2Δ	225	33.0	245	27.8	18.5	*rpl43bΔ*	105	32.5	119	24.2	34.4	*ydr115wΔ*	25	32.0	45	25.7	24.4
kip2Δ	85	33.0	145	28.0	18.1	*rpl6bΔ*	365	31.5	434	26.8	17.5	*yer128wΔ*	45	30.0	45	25.0	20.0
los1Δ	365	31.1	445	23.3	33.3	*rpl7aΔ*	132	31.0	168	25.9	19.4	*ygl165cΔ*	25	30.8	45	25.1	22.8
lpd1Δ	75	31.5	84	23.2	35.8	*rpl9aΔ*	185	31.4	219	24.8	26.9	*yjl022wΔ*	65	33.3	105	25.8	29.0
ltv1Δ	45	31.6	45	20.1	57.0	*rpp1bΔ*	65	32.9	119	27.1	21.5	*ykr073cΔ*	45	38.2	85	24.3	57.1
lyp1Δ	45	35.2	65	26.1	35.0	*rpp2bΔ*	55	34.9	59	23.2	50.2	*yor135cΔ*	75	29.9	160	25.0	19.6
map2Δ	130	32.5	130	25.2	28.6	*rps12Δ*	45	32.6	55	24.2	34.9	*ypt52Δ*	45	32.1	45	20.3	58.4
met3Δ	85	31.5	105	26.6	18.5	*rrd2Δ*	85	36.9	85	28.1	31.3	*yro2Δ*	145	32.3	185	27.2	18.6
mnn2Δ	125	30.5	185	26.4	15.4	*rts1Δ*	45	37.7	85	27.3	38.4	*yur1Δ*	65	31.2	125	26.1	19.3
mpd1Δ	45	33.1	45	19.8	67.3												

Table 3.3. Replicative life span data for 124 low stringency long-lived strains from the iterative replicative life span screen. The data provided is calculated from pooled MATα and MAT**a** data for each genotype and experiment matched wild type cells.

	Mutant		Wild Type		
		Mean		Mean	
Genotype	N	RLS	N	RLS	% Ext
afg3Δ	510	25.5	545	24.1	6.0
alg12Δ	245	31.7	304	27.6	15.0
alg6Δ	45	30.4	85	27.6	10.1
atg1Δ	724	28.8	801	27.1	6.4
atp17Δ	55	31.8	105	24.9	27.6
bmh2Δ	115	30.6	115	25.7	18.7
boi2Δ	145	31.5	205	28.3	11.5
bul1Δ	65	32.9	105	27.2	20.8
caf20Δ	445	28.4	505	27.5	3.4
caf40Δ	64	32.1	65	24.5	31.4
cbs1Δ	55	31.6	125	23.6	33.7
cos12Δ	325	28.9	265	27.6	5.0
cst26Δ	150	32.8	245	27.4	19.6
cth1Δ	100	28.9	165	27.4	5.7
cup9Δ	125	29.0	145	26.0	11.5
cur1Δ	45	31.5	85	26.0	21.4
cyc1Δ	125	29.3	125	26.3	11.4
das2Δ	100	29.4	145	26.5	11.1
dbf20Δ	80	30.2	125	27.6	9.6
dgk1Δ	45	32.8	65	27.3	20.0
did2Δ	125	31.6	125	27.3	15.8
dlt1Δ	184	31.0	285	29.2	6.2
eno1Δ	125	27.0	145	25.1	7.5
erv25Δ	50	32.1	70	28.0	14.9
eug1Δ	125	31.1	145	30.7	1.4
fcy2Δ	85	29.1	85	26.8	8.6
fet3Δ	50	27.9	50	26.1	7.1
fmp42Δ	45	31.5	45	27.1	16.2
fmp43Δ	80	30.4	100	27.9	9.0
gas4Δ	40	32.4	45	27.7	16.6
gem1Δ	45	31.7	45	24.0	32.1
gpa2Δ	815	31.9	1152	28.2	13.2
gtr1Δ	45	25.5	45	24.7	3.4
hbs1Δ	65	35.5	105	28.0	26.4
hmt1Δ	65	29.1	85	25.1	16.0
hua1Δ	45	30.8	85	20.9	47.3
idh1Δ	524	32.7	665	28.4	15.1
idp1Δ	345	28.4	425	26.4	7.7
img1Δ	45	33.1	65	28.5	16.0
inp53Δ	355	32.4	409	28.4	14.2
irc21Δ	45	29.7	85	26.9	10.6
itr1Δ	125	29.6	125	27.6	7.3
kap120Δ	95	33.7	145	26.7	26.2
lin1Δ	65	30.9	80	26.4	17.1
lsb1Δ	180	29.6	205	26.7	10.7
mad3Δ	45	31.5	85	26.2	20.3
mef2Δ	85	32.4	105	27.3	19.0
mep2Δ	125	30.6	125	26.4	16.1
mig1Δ	70	26.8	110	24.0	11.8
mnn1Δ	65	30.5	65	27.8	9.7
mrpl33Δ	105	28.0	125	24.7	13.5
mrpl40Δ	45	33.3	45	29.6	12.3
mrpl49Δ	45	34.7	45	28.9	20.2
msk1Δ	85	28.5	105	27.6	3.2
mtc2Δ	25	29.4	45	27.4	7.3
mtc4Δ	149	30.5	270	27.3	11.9
opi3Δ	80	30.3	85	28.0	8.1
paf1Δ	65	33.3	105	29.2	14.0
pex19Δ	124	31.7	145	28.0	13.2
pgm2Δ	125	31.3	125	27.6	13.2
pib1Δ	365	31.0	384	28.1	10.1
pmc1Δ	270	30.6	250	27.5	11.4
pml39Δ	45	29.2	45	27.2	7.4
psp2Δ	70	31.4	70	29.7	5.5
rad34Δ	45	32.3	45	29.6	9.2
rds1Δ	283	29.0	225	25.6	13.4
rfx1Δ	285	28.0	285	26.7	4.8
rmt2Δ	65	30.6	65	26.3	16.1
rpl12aΔ	84	30.1	99	26.0	16.0
rpl12bΔ	82	33.8	82	27.8	21.6
rpl13bΔ	82	34.7	93	25.4	36.6
rpl19bΔ	220	32.4	294	28.5	13.5
rpl1bΔ	85	31.1	99	25.2	23.7
rpl20aΔ	80	35.8	120	24.8	44.4
rpl26aΔ	100	31.4	119	26.2	19.8
rpl2bΔ	80	34.3	100	24.8	38.3
rpl34aΔ	40	34.2	40	26.9	27.4
rpl35aΔ	80	35.0	120	24.8	41.2
rpl6aΔ	100	35.5	100	24.8	43.1
rpp1aΔ	42	37.4	42	28.6	30.7
rpp2aΔ	95	26.4	99	24.1	9.7
rps6aΔ	155	30.1	175	27.7	8.5
sas2Δ	362	31.8	425	28.7	10.7
scw4Δ	45	27.8	45	24.0	15.6
sdh1Δ	65	32.8	65	27.5	19.2
sdh2Δ	65	32.1	65	26.8	19.7
skp2Δ	245	30.3	285	28.9	4.9
smy2Δ	45	30.8	85	27.8	10.9
sov1Δ	80	31.3	105	28.4	10.2
sps1Δ	125	33.0	125	30.9	7.1
spt23Δ	45	28.1	85	26.3	6.8
spt8Δ	165	31.5	145	29.4	7.2
ssf1Δ	85	34.0	200	23.9	42.2
syh1Δ	85	30.4	145	27.4	10.9
tif1Δ	415	28.2	494	25.8	9.2
tif2Δ	320	31.2	404	27.0	15.7
tos8Δ	135	30.5	165	25.3	20.5
tpo3Δ	125	28.8	125	26.2	9.6
trk1Δ	40	28.9	70	22.1	30.7
ugx2Δ	45	30.8	65	28.0	9.7
ure2Δ	85	30.4	125	26.5	14.7
vps24Δ	125	29.7	125	27.6	7.8
vps60Δ	125	32.2	125	29.1	10.6
ybr013cΔ	45	35.1	85	28.7	22.0
ybr226cΔ	50	30.1	80	27.3	10.3
ycl006cΔ	60	29.5	150	25.8	14.5
ycr101cΔ	45	30.5	85	27.2	11.9
ydl172cΔ	45	30.9	85	28.8	7.3
ydr048cΔ	65	31.7	105	28.2	12.3
yeh1Δ	45	30.4	65	28.5	6.7
yel020cΔ	45	29.0	45	27.9	3.7
ygl042cΔ	65	29.2	70	26.0	12.4
ygl079wΔ	105	30.0	110	28.1	6.9
ygr051cΔ	45	31.1	85	28.2	10.5
yil089wΔ	55	35.9	65	29.5	21.9
ymr018wΔ	130	30.3	150	28.9	4.9
yng1Δ	45	27.3	45	24.4	11.5
ynl034wΔ	45	31.6	65	29.4	7.5
ynl213cΔ	45	31.6	65	29.3	7.8
yol014wΔ	85	31.3	65	28.4	10.4
yor111wΔ	115	30.8	220	27.6	11.5
ypr064wΔ	45	30.5	105	26.4	15.8
ypt6Δ	185	30.5	225	28.5	7.0
yvh1Δ	150	29.2	205	24.4	20.0

Table 3.4. GO terms associated with the 239 genes identified by the replicative life span screen.

GO Category	GO Id	GO Term	# of Genes in Long-Lived Set	% of Genes in Long-Lived Set
Component:	5737	cytoplasm	121	50.6
	16020	membrane	48	20.1
	5634	nucleus	47	19.7
	5840	ribosome	45	18.8
	5739	mitochondrion	29	12.1
	5575	cellular component unknown	24	10
	5886	plasma membrane	15	6.3
	5783	endoplasmic reticulum	14	5.9
	5694	chromosome	12	5
	12505	endomembrane system	11	4.6
	5740	mitochondrial envelope	10	4.2
	5773	vacuole	9	3.8
	5624	membrane fraction	7	2.9
	5794	Golgi apparatus	6	2.5
	5730	nucleolus	6	2.5
	30427	site of polarized growth	4	1.7
	5933	cellular bud	4	1.7
	5618	cell wall	4	1.7
	5856	cytoskeleton	3	1.3
	16023	cytoplasmic membrane-bounded vesicle	3	1.3
	5938	cell cortex	2	0.8
	5777	peroxisome	1	0.4
	5815	microtubule organizing center	1	0.4
	5576	extracellular region	1	0.4
	other	other	5	2.1
	n/a	not annotated	34	14.2
Function:	3674	molecular function unknown	72	30.1
	5198	structural molecule activity	41	17.2
	3735	structural constituent of ribosome	28	15.9
	22857	transmembrane transporter activity	14	5.9
	3723	RNA binding	14	5.9
	16491	oxidoreductase activity	12	5
	3677	DNA binding	10	4.2
	16887	ATPase activity	10	4.2
	16757	transferase activity, transferring glycosyl groups	10	4.2
	16301	kinase activity	9	3.8
	16874	ligase activity	7	2.9
	30234	enzyme regulator activity	6	2.5
	16791	phosphatase activity	6	2.5
	16746	transferase activity, transferring acyl groups	5	2.1
	16853	isomerase activity	5	2.1
	988	protein binding transcription factor activity	5	2.1
	3924	GTPase activity	5	2.1
	8135	translation factor activity, nucleic acid binding	4	1.7
	4386	helicase activity	4	1.7
	1071	nucleic acid binding transcription factor activity	4	1.7
	8233	peptidase activity	3	1.3
	8168	methyltransferase activity	3	1.3
	8289	lipid binding	3	1.3
	19899	enzyme binding	2	0.8
	3729	mRNA binding	2	0.8
	16765	transferase activity, transferring alkyl or aryl (other than methyl) groups	2	0.8
	30674	protein binding, bridging	2	0.8
	19843	rRNA binding	2	0.8
	42393	histone binding	2	0.8
	16829	lyase activity	2	0.8
	8134	transcription factor binding	2	0.8
	4871	signal transducer activity	2	0.8
	16798	hydrolase activity, acting on glycosyl bonds	1	0.4
Function (cont.)	8565	protein transporter activity	1	0.4
	32182	small conjugating protein binding	1	0.4
	4518	nuclease activity	1	0.4
	16810	hydrolase activity, acting on carbon-nitrogen (but not	1	0.4
	16779	nucleotidyltransferase activity	1	0.4
	43167	ion binding	1	0.4
	8092	cytoskeletal protein binding	0	0
	51082	unfolded protein binding	0	0
	30533	triplet codon-amino acid adaptor activity	0	0
	30555	RNA modification guide activity	0	0
	other	other	12	5
	n/a	not annotated	4	1.7
Process:	8150	biological process unknown	42	17.6
	2181	cytoplasmic translation	33	13.8
	6366	transcription from RNA polymerase II promoter	18	7.5
	7005	mitochondrion organization	15	6.3
	5975	carbohydrate metabolic process	14	5.9
	6325	chromatin organization	14	5.9
	6091	generation of precursor metabolites and energy	14	5.9
	42273	ribosomal large subunit biogenesis	14	5.9
	42221	response to chemical stimulus	13	5.4
	278	mitotic cell cycle	13	5.4
	70647	protein modification by small protein conjugation or removal	12	5
	16570	histone modification	11	4.6
	55085	transmembrane transport	10	4.2
	6520	cellular amino acid metabolic process	10	4.2
	70925	organelle assembly	10	4.2
	23052	signaling	10	4.2
	6605	protein targeting	9	3.8
	6486	protein glycosylation	9	3.8
	6629	lipid metabolic process	9	3.8
	18193	peptidyl-amino acid modification	9	3.8
	45333	cellular respiration	9	3.8
	32543	mitochondrial translation	9	3.8
	6401	RNA catabolic process	8	3.3
	51169	nuclear transport	8	3.3
	71554	cell wall organization or biogenesis	8	3.3
	42255	ribosome assembly	8	3.3
	51186	cofactor metabolic process	8	3.3
	6811	ion transport	8	3.3
	6364	rRNA processing	8	3.3
	33043	regulation of organelle organization	7	2.9
	6468	protein phosphorylation	7	2.9
	6873	cellular ion homeostasis	7	2.9
	6360	transcription from RNA polymerase I promoter	7	2.9
	6974	response to DNA damage stimulus	6	2.5
	6414	translational elongation	6	2.5
	7124	pseudohyphal growth	6	2.5
	43543	protein acylation	6	2.5
	48193	Golgi vesicle transport	6	2.5
	51726	regulation of cell cycle	5	2.1
	16197	endosome transport	5	2.1
	70271	protein complex biogenesis	5	2.1
	6260	DNA replication	5	2.1
	51321	meiotic cell cycle	5	2.1
	7059	chromosome segregation	5	2.1
	43934	sporulation	5	2.1

Table 3.4 (continued).

GO Category	GO Id	GO Term	# of Genes in Long-Lived Set	% of Genes in Long-Lived Set
Process: (cont.)	15931	nucleobase-containing compound transport	5	2.1
	8033	tRNA processing	5	2.1
	51603	proteolysis involved in cellular protein catabolic process	5	2.1
	6354	transcription elongation, DNA-dependent	5	2.1
	55086	nucleobase-containing small molecule metabolic process	5	2.1
	9451	RNA modification	4	1.7
	6353	transcription termination, DNA-dependent	4	1.7
	6310	DNA recombination	4	1.7
	6979	response to oxidative stress	4	1.7
	6970	response to osmotic stress	4	1.7
	8213	protein alkylation	4	1.7
	51052	regulation of DNA metabolic process	4	1.7
	9311	oligosaccharide metabolic process	4	1.7
	48285	organelle fission	4	1.7
	6417	regulation of translation	4	1.7
	7010	cytoskeleton organization	3	1.3
	6397	mRNA processing	3	1.3
	6413	translational initiation	3	1.3
	8380	RNA splicing	3	1.3
	42274	ribosomal small subunit biogenesis	3	1.3
	6281	DNA repair	3	1.3
	31399	regulation of protein modification process	3	1.3
	7114	cell budding	3	1.3
	7033	vacuole organization	3	1.3
	6383	transcription from RNA polymerase III promoter	3	1.3
	16050	vesicle organization	3	1.3

GO Category	GO Id	GO Term	# of Genes in Long-Lived Set	% of Genes in Long-Lived Set
Process: (cont.)	16050	vesicle organization	3	1.3
	51049	regulation of transport	3	1.3
	6897	endocytosis	3	1.3
	6865	amino acid transport	3	1.3
	6352	transcription initiation, DNA-dependent	2	0.8
	746	conjugation	2	0.8
	6997	nucleus organization	2	0.8
	10324	membrane invagination	2	0.8
	48308	organelle inheritance	2	0.8
	42594	response to starvation	2	0.8
	910	cytokinesis	2	0.8
	6457	protein folding	2	0.8
	6470	protein dephosphorylation	2	0.8
	51604	protein maturation	2	0.8
	1403	invasive growth in response to glucose limitation	2	0.8
	6418	tRNA aminoacylation for protein translation	2	0.8
	61025	membrane fusion	2	0.8
	43144	snoRNA processing	1	0.4
	54	ribosomal subunit export from nucleus	1	0.4
	8643	carbohydrate transport	1	0.4
	9408	response to heat	1	0.4
	7031	peroxisome organization	1	0.4
	6887	exocytosis	0	0
	6869	lipid transport	0	0
	902	cell morphogenesis	0	0
	32200	telomere organization	0	0
	32196	transposition	0	0
	48284	organelle fusion	0	0
	6766	vitamin metabolic process	0	0
	6497	protein lipidation	0	0
	other	other	4	1.7
	n/a	not annotated	2	0.8

Table 3.5. Gene ontology terms significantly enriched in replicatively long-lived gene set. The "Long-Live" gene set includes the 239 genes identified in the low-stringency group, and the "Reference" gene set includes the 4,687 total nonessential genes examined in the replicative life span screen.

			% Genes in Set			Translation
GO Category	GO Id	GO Term	Long-Lived	Reference	P-value	Associated
Component:	22625	cytosolic large ribosomal subunit	13.0	1.4	2.77E-21	√
	15934	large ribosomal subunit	15.5	2.3	5.40E-20	√
	5840	ribosome	18.9	4.9	5.08E-14	√
	22626	cytosolic ribosome	13.9	2.7	2.02E-13	√
	44391	ribosomal subunit	16.4	3.9	7.14E-13	√
	30529	ribonucleoprotein complex	21.8	6.8	1.26E-12	√
	44445	cytosolic part	14.3	3.3	9.26E-12	
	5829	cytosol	17.6	6.1	2.27E-08	
	43228	non-membrane-bounded organelle	29.0	13.7	3.01E-08	
	43232	intracellular non-membrane-bounded organelle	29.0	13.7	3.01E-08	
	32991	macromolecular complex	39.5	23.1	6.40E-07	
	44464	cell part	75.2	59.8	3.24E-05	
	5623	cell	75.2	59.8	3.24E-05	
	44424	intracellular part	67.2	52.5	0.0003	
	5622	intracellular	67.6	53.1	0.0003	
	43229	intracellular organelle	59.2	45.2	0.0008	
	43226	organelle	59.2	45.2	0.0009	
	5737	cytoplasm	50.8	37.9	0.0034	
	44446	intracellular organelle part	42.4	30.3	0.0047	
	44422	organelle part	42.4	30.3	0.0048	
	44444	cytoplasmic part	45.4	33.4	0.0080	
Function:	3735	structural constituent of ribosome	16.0	3.8	9.22E-13	√
	5198	structural molecule activity	17.2	5.2	4.7E-10	
Process:	2181	cytoplasmic translation	13.9	2.7	8.70E-13	√
	44267	cellular protein metabolic process	39.5	19.1	2.96E-11	√
	19538	protein metabolic process	39.5	19.7	2.52E-10	√
	6412	translation	19.7	6.3	2.94E-10	√
	34645	cellular macromolecule biosynthetic process	36.1	18.3	9.55E-09	
	9059	macromolecule biosynthetic process	36.1	18.4	1.24E-08	
	42273	ribosomal large subunit biogenesis	5.9	0.6	1.50E-08	√
	10467	gene expression	35.3	18.8	3.23E-07	
	44260	cellular macromolecule metabolic process	51.3	33.5	3.19E-06	
	44249	cellular biosynthetic process	41.6	25.1	5.05E-06	
	43170	macromolecule metabolic process	51.7	34.4	9.80E-06	
	9058	biosynthetic process	41.6	25.5	1.31E-05	
	27	ribosomal large subunit assembly	3.4	0.3	0.0001	√
	71826	ribonucleoprotein complex subunit organization	5.5	1.2	0.0017	√
	44238	primary metabolic process	58.8	44.3	0.0019	
	42254	ribosome biogenesis	8.0	2.6	0.0048	√
	44237	cellular metabolic process	61.3	47.8	0.0073	
	22613	ribonucleoprotein complex biogenesis	8.8	3.1	0.0084	√
	70925	organelle assembly	4.2	0.8	0.0091	

Table 3.6. Genes identified in the iterative replicative life span screen with a defined role in mRNA translation or TOR signaling. Gene in this table were selected from the replicatively long-live gene set based on their annotation with at least one GO Term indicated as "Translation Associated" in Table 3.5, as well as known components of the TOR signaling pathway.

Category	Structure or Function	Gene
Ribosom Structure and Processing	Large Subunit	*RPL1B, RPL2B, RPL6A, RPL6B, RPL7A, RPL9A, RPL12A, RPL12B, RPL13A, RPL13B, RPL16B, RPL19A, RPL19B, RPL20A, RPL20B, RPL21B, RPL22A, RPL23A, RPL26A, RPL29, RPL31A, RPL34A, RPL34B, RPL35A, RPL37B, RPL43B*
	Small Subunit	*RPS6A, RPS12*
	Stalk	*RPP1A, RPP1B, RPP2A, RPP2B*
	Biogenesis	*DBP3, LTV1, MRT4, RAI1, REI1, SPT4, SSF1, YDL063C, YVH1*
	rDNA Silencing	*PAF1*
Mitochondrial Translation	Ribosomal Protein, Large subunit	*IMG1, MRPL33, MRPL40, MRPL49, YDR115W*
	Aminoacyl-tRNA Synthetase	*MSK1, MSW1*
	Translation Elongation Factor	*MEF2*
	Protein Assembly/Degredation	*AFG3*
	Translational Activator of COB mRNA	*CBS1*
TOR Signaling	Nutrient responsive kinase	*TOR1, SCH9*
	GDP-GTP exchange factor for Rho1p	*ROM2*
	Regulator of nitrogen catabolite repression	*URE2*
Other	Translation Initiation Factor	*TIF1, TIF2, TIF4631*
	Translation Machinery Associated	*TMA10, TMA19*
	Cap Associated Factor	*CAF20*
	Methionine AminoPeptidase	*MAP2*
	Nuclear mRNA Export	*TOM1*

A formal notation system for life span epistasis

Many of the genes identified in the screen are clearly associated with translation processes and are likely to map the TOR signaling pathway (Tables 3.5 and 3.6). While this enrichment further promotes the central role for translation in longevity determination, a wide range of genes with diverse roles in the cell were identified (Table 3.4). Some of these genes have not yet been characterized, or do not have a defined role in the biology of the cell. As the role of these genes in the aging process begins to be characterized, developing and understanding the interaction between individual genes and the central aging pathways will become increasingly important.

In order to facilitate a relatively large number of simultaneous epistasis comparisons, and to address some of the inherent problems with epistasis discussed in the introduction to this chapter, we developed a formal notation system for epistatic interactions (Figure 3.3). The primary goal of this system is to define a standard notation and identify an initial interpretation for a given pattern of interaction between two interventions that play a role in aging. While this chapter primarily discuses genetic manipulations, any genetic, environmental, or pharmacological intervention that alters life span can be analyzed using this system.

At the most basic level, epistasis analysis examines the relationship between two interventions with respect to a phenotype of interest—life span in this case. For the purposes of this chapter, two generic interventions, x and y, will be considered mutations that reduce expression of genes X and Y. Five relationships are considered: (A) the percent change in life span resulting from x in the context of an unmodified control background (WT → x); (B) the percent change in life span resulting from x in a background subject to y (y → xy); (C) the percent change in life span resulting from y in the context of an unmodified control background (WT → y); (D) the percent change in life span resulting from y in a background subject to x (x → xy); and (E) the percentage change in life span resulting from the combined interventions xy in the context of an unmodified control background (WT → xy) (Figure 3.3A,B). P-values for each relationship are calculated using the Wilcoxon Rank Sum test and used to indicate whether a given comparison shows a significantly increased life span (+), decreased life span (-), or no statistically significant effect (N). By considering the set of comparisons as a whole, each epistatic interaction can be described as a four- or five-symbol pattern (Figure 3.3C). The final relationship, E, is only useful in distinguishing between a few rare cases and is excluded in portions of the following discussion.

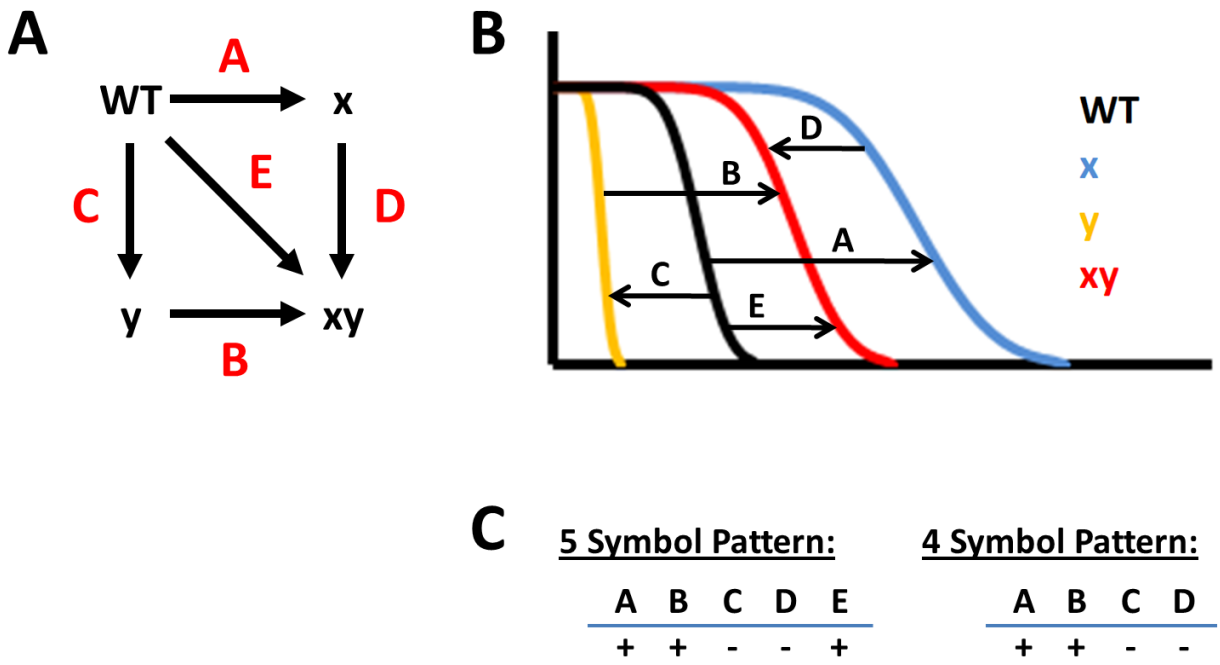


Figure 3.3. A formalized system of notation for life span epistasis relationships. Formalized epistasis notation denotes the percent change in life span (A-E) between WT, intervention x, intervention y, and combined intervention xy (A). For example, a given set of life span data (B) can be represented as a four- or five-symbol pattern indicating the relationships between each tested condition (C).

The pattern defines a basic system of notation that can be applied to any life span epistasis data set. For our purposes, we consider only comparisons where at least one of the interventions increases life span. In addition to the basic pattern, the relative values of A and B are important in further defining patterns in certain situations. For example, consider the pattern "+ + N N", in which x extends life span and y has no effect on life span. If the effect of x is identical in the untreated background and the background subject to y (A = B), the interventions would most often be considered independent. In contrast, if x produces a smaller effect in the context of y than in a control context (B < A), the common interpretation would be that x is partially dependent on y. The reverse relationships (C and D) are also important but redundant, since the identical consideration can be achieved by reversing the positions of x and y. Using the epistasis pattern and the relationship between A and B, a complete set of possible interactions can be defined (Table 3.7). When only considering situations in which at least one intervention increases life span, 20 interactions are possible. There are additional patterns that are either not logically possible (e.g. "+ - + +") or represent datasets with insufficient data to completely resolve at least one relationship that does not show a statistically significant life span difference (e.g. "+ N N N").

Table 3.7. Epistasis patterns. The formalized epistasis system defines 20 unique epistasis patterns assuming that at least one of the interventions being tested increases life span. Each category is determined by the four symbol pattern and the change in life span resulting from intervention x in the wild type (A) or y (B) background. 11 patterns are either impossible or require additional data to resolve at least one relationship showing no statistically significant extension (N). Representative life span curves are shown, as is the simplest genetic model capable of explaining the epistasis pattern, including the necessary relationship between degrees of influence. Genetic models assume that interventions x and y specifically reduces activity of genes X and Y, respectively.

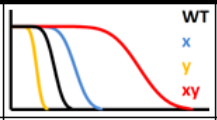
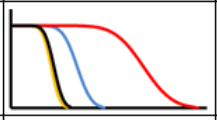
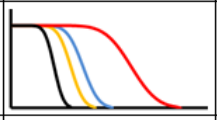
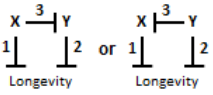
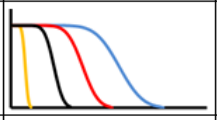
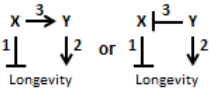
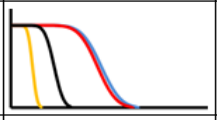
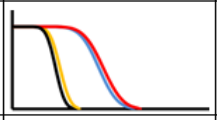
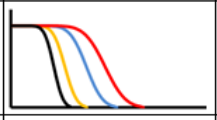
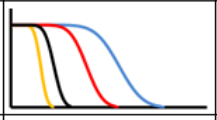
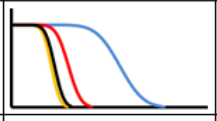
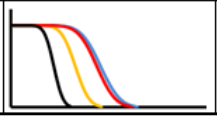
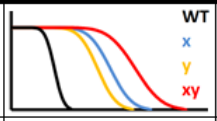
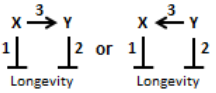
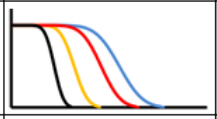
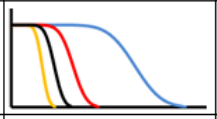
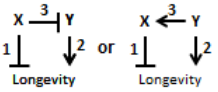
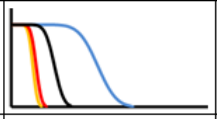
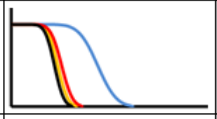
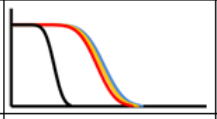
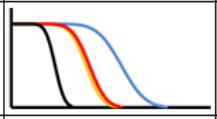
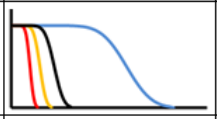
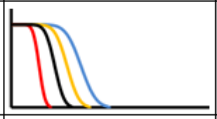
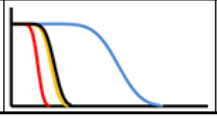
#	Pattern A B C D	A/B Relation	Dependence Category	Life Span Order	Life Span Curves	Simplest Genetic Model	Model Relationship
1	+ + - +	0<A<B	Synergy	y < WT < x < xy			2 - 3 < 1
2	+ + N +	0<A<B	Synergy	WT = y < x < xy			1 = 2 - 3
3	+ + + +	0<A<B**	Synergy	WT < x, y < xy			1 ≥ 2 > 3 or 1 < 2 - 3
4	+ + - -	0<A<B**	Synergy	y < (WT, xy) < x			2 + 3 < 1 or n/a
5	+ + - N	0<A<B	Synergy	y < WT < x = xy			1 < 2
6	+ + N N	0<A=B	Independence	WT = y < x = xy			n/a
7	+ + + +	0<A=B**	Independence	WT < x, y < xy			2 ≤ 1
8	+ + - -	0<A=B**	Independence	y < (WT, xy) < x			n/a
9	+ + N -	0<B<A	Partial Dependence	WT = y < xy < x			2 = 1 - 3
10	+ + + N	0<B<A	Partial Dependence	WT < y < x = xy			1 < 2

Table 3.7 (continued).

#	Pattern A B C D	A/B Relation	Dependence Category	Life Span Order	Life Span Curves	Simplest Genetic Model	Model Relationship
11	+ + + +	0<B<A**	Partial Dependence	WT < y ≤ x < xy			2 ≤ 1
12	+ + + -	0<B<A	Partial Dependence	WT < y < xy < x			2 < 1 - 3
13	+ + - -	0<B<A**	Partial Dependence	y < (WT, xy) < x			n/a or 2 > 1 - 3
14	+ N - -	0=B<A	Dependence*	y = xy < WT < x			1 ≤ 2
15	+ N N -	0=B<A	Dependence	WT = y = xy < x			1 = 2
16	+ N + N	0=B<A	Dependence	WT < x = y = xy			1 = 2
17	+ N + -	0=B<A	Dependence	WT < y = xy < x			
18	+ - - -	B<0<A	Antagonism	xy < y < WT < x			2 + 3 > 1
19	+ - + -	B<0<A	Antagonism	(WT, xy) < (y, x)			1 < 2 + 3; 2 < 4 + 1
20	+ - N -	B<0<A	Antagonism	xy < WT = y < x			1 < 2 + 3; 4 + 1 = 2

Impossible Patterns	A	B	C	D
	+	N	+	+
	+	N	N	+
	+	N	N	N
	+	N	-	N
	+	-	+	+
	+	-	+	N
	+	-	N	+
	+	-	N	N
	+	-	-	+
	+	-	-	N
	+	N	-	+

Based on the four symbol pattern and the relationship between A and B, each epistatic relationship can be assigned to a tentative epistasis category. A genetic model describing the simplest genetic interaction between x and y capable of producing the observed epistasis pattern was developed for each relationship (Table 3.7). The categories cover the range of possible epistatic interactions, including independence (the presence of one intervention does not alter the effect of the other on life span), dependence (the influence of one intervention on life span is completely prevented by the second intervention), partial dependence (the influence of one intervention on life span is partially prevented by the second intervention), synergy (the combined intervention produces a greater effect on life span than expected from simply adding the separate effects), and antagonism (the combined intervention produces a life span that is shorter than that produced by either of the individual interventions). We emphasize that both the category assignment and genetic model should be considered tentative. As noted in the introduction to this chapter, multiple models can produce a single epistasis pattern and biological systems are often far more complex than the simple models presented in Table 3.7. However, these models and categories provide an initial direction for further investigation, particularly in cases when the biological function of a novel longevity determinant is not known.

As an example, consider the two relationships discussed in the introduction. In the relationship between either DAF-2 and DAF-16 in worms, or the relationship between dietary restriction and Sir2 in yeast, the intervention that normally increases life span in a wild type context (reduced DAF-2 activity or dietary restriction) has no effect when the potential downstream mediator (DAF-16 or Sir2) is absent (Kaeberlein et al., 2004; Kenyon et al., 1993; Lin et al., 2000). Both cases produce the identical pattern: number 14, "+ N N –" (Table 3.7). In both cases, complete dependence is assumed as an initial model. Upon further examination, numerous lines of evidence support this initial model for the DAF-2 and DAF-16 and the relationship is confirmed. In the case dietary restriction/Sir2, repressing ERC formation in *sir2*Δ cells by deleting *FOB1* allows dietary restriction to robustly extend life span and the initial model is rejected in favor of a more complex model capable of explaining the all available data.

For a more complex example, consider the relationship between Sch9 and dietary restriction in yeast. Both interventions extend replicative life span in wild type cells, with deletion of *SCH9* imparting a greater replicative life span extension than dietary restriction. When dietary restriction is applied to *sch9*Δ cells, the replicative life span is similar to dietary restriction alone, falling between wild type and *sch9*Δ (Kaeberlein et al., 2005d). If we assign deletion of *SCH9* to "intervention x" and dietary restriction "intervention y", this produces epistatic pattern "+ N + -" (Table 3.7). This pattern implies some degree of partial dependence between the two interventions, but cannot be explained by a simple linear model. One possible explanation of this interaction is that both interventions alter activity of the same downstream factor to different degrees. In this model, the activity of the downstream factors is optimized

for maximal replicative life span in *sch9*Δ cells, but only partially optimized in response to dietary restriction. When the two interventions are combined, the activity of the downstream factors is increased beyond the optimal value and the replicative life span is short relative to deletion of *SCH9* alone. Additional information is necessary to make a definitive statement about the interaction between these two interventions.

Both examples illustrate the complexity inherent in interpreting epistatic interactions between two interventions with respect to a quantitative trait, such as life span. When applied to a large number of interactions simultaneously, the complications inherent in the interaction portrayed in the first example become irrelevant. In situations where life span is limited by a particular intervention, such as deletion of *SIR2*, individual epistatic interactions may be difficult to interpret. However, when you look at a variety of life span extending interventions applied in a *sir2*Δ background, the majority will fail to increase replicative life span (see Chapter 4), rendering *sir2*Δ largely uninformative about the molecular mechanism by which a particular intervention increases replicative life span. In the rare case where an intervention can increase replicative life span of a strain lacking *SIR2*, it is likely doing so by reducing ERC accumulation.

Application of Epistasis System to 77 Gene Interactions

One goal of developing a systematic notation for epistatic interactions was to establish a method for consistently examining large numbers of epistatic interactions simultaneously in order to gain insight into the interactions between sets of interventions. We applied this system to replicative life span data for 77 interactions between two sets of genetic mutations known to impact replicative life span (Table 3.8). The first set consists of eight single-gene deletions and one single-copy overexpression construct that have previously been reported to increase replicative life span. The second set consists of 10 single-gene and 1 double-gene deletion in factors previously identified as downstream mediators of replicative life span extension. Specific interactions were excluded in cases where construction of the double mutant strain proved difficult (e.g. many of the combinations with *rpl31a*Δ and *gpa2*Δ) or where replicative life span data from independently constructed strains for the same combination was conflicting. The rationale behind selecting these sets of mutations was that by looking at a range of downstream regulators of replicative life span in the context of different long-lived mutants, previously uncharacterized points of interaction might be identified between aging pathways. In addition, identifying key downstream regulators that display differential interactions between known aging pathways will be useful in placing novel longevity interventions into (or out of) these pathways.

Table 3.8. Epistasis analysis of 77 genetic interactions. Epistasis patterns (A) and categories (B) for interactions between 9 replicatively long-lived mutants and 11 potential downstream mediators of replicative life span.

A

	SIR2OE					*fob1Δ*					*tor1Δ*					*sch9Δ*					*rpl20bΔ*					*rpl31aΔ*					*gpr1Δ*					*gpa2Δ*					*sok1Δ*				
	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E	A	B	C	D	E
gcn4Δ	+	+	N	-	+	+	N	N	-	N	+	+	+	N	+	+	N	+	-	N	+	+	N	-	+	+	N	N	-	N	+	+	N	N	+						+	+	N	N	+
	***	*		***	***	***			***		***	***	**		***	***		***	***		***	***		***	*	***			***		***	**			***						***	***			***
gcn2Δ	+	+	+	N	+	+	+	N	-	+						+	+	N	-	+	+	+	N	N	+	+	+	N	N	+	+	+	+	N	+	+	N	-	+	+	+	N	N	+
	***	***	***		***	***	**		**	***						***	***		**	***	***	***			***	*	**			**	***	**	*		***	***		*	*	*	***	***			***
yap1Δ	+	+	-	-	N	+	N	-	-	-	+	N	-	-	N	+	-	-	-	-	+	N	-	-	-	+	-	N	-	-	+	N	-	-	-	+	N	-	-	-	+	N	-	-	-
	***	***	***	***		***		***	***	***	***		*	***		***	***	*	***	***	***		***	***	***	***	***		***	***		***		***	***	***	***		**	***	*	***		*	***	*
hmo1Δ	+	N	-	-	-	+	+	-	-	-	+	N	-	-	-	+	N	-	-	-	+	N	-	-	-	+	-	-	-	-	+	+	-	-	-						+	+	-	-	-
	***		***	***	***	***	**	***	***	***	***		***	***	***	***		***	***	***	*		***	***	***	***		***	***	***	***		***	***	***	***						***	***	***	***	***
atg1Δ	+	+	N	-	+	+	+	N	N	+						+	N	N	-	N	+	N	+	N	+																+	+	+	N	+
	***	**		*	***	***	***			***						***			***		***		**		***																***	***	*		***
atg13Δ	+	+	N	N	+	+	-	N	-	N						+	+	N	-	+						+	N	+	N	+	+	+	N	+	+							+	+	N	N	+
	***	**			***	***	*		***							***	***		**	***						**		**		**	***	**		***								***	**			***
pnc1Δ											+	+	N	-	+	+	+	N	N	+	+	+	N	N	+	+	-	N	-	-	+	+	N	N	+						+	+	N	N	+
											***	**		***	*	***	***			***	***	***			***	*	***		***	***	***		***								***	***			***
hap4Δ	+	-	-	-	-						+	+	-	-	+	+	+	N	N	+	+	+	N	N	+						+	+	N	N	+	+	N	N	-	N	+	N	N	-	N
	***	**	***	***	***						***	***	***	**	***	***	***			***	***	***			***						***	***			***	***			**		***			***	
hac1Δ	+	+	+	N	+	+	+	+	N	+	+	+	+	N	+	+	+	N	N	+	+	+	+	N	+						+	+	N	N	+	+	N	N	-	+	+	N	N	-	+
	***	*	**		***	***	**	**		***	***	**	*		***	***	***			***	***	***	*		***						***	***			***	**	***		*	***	***			***	*
maf1Δ	+	+	N	N	+	+	+	+	-	+	+	N	+	N	+	+	+	N	-	+	+	+	+	N	+																				
	***	***			***	***	**	*	*	***	***		*		**	***	*		***	**	***	*	*		***																				
msn2Δ msn4Δ	+	N	+	-	+	+	+	N	N	+	+	N	+	N	+	+	N	N	-	N	+	N	+	-	+						+	+	N	N	+						+	N	N	-	N
	***		***	***	***	***	***			***	***		***		***	***			***		***		*	**	**						***	**			**						***			***	

B

	SIR2OE	*fob1 Δ*	*tor1Δ*	*sch9Δ*	*rpl20bΔ*	*rpl31aΔ*	*gpr1Δ*	*gpa2Δ*	*sok1Δ*
gcn4Δ	Partial Dependence	Dependence	Partial Dependence	Dependence	Partial Dependence	Dependence	Independence		Independence
gcn2Δ	Partial Dependence	Partial Dependence		Partial Dependence	Independence	Independence	Partial Dependence	Dependence	Independence
yap1Δ	Independence	Antagonism	Antagonism	Dependence	Antagonism	Antagonism	Antagonism	Antagonism	Antagonism
hmo1Δ	Antagonism	Partial Dependence	Antagonism	Antagonism	Antagonism	Dependence	Independence		Independence
atg1Δ	Partial Dependence	Independence		Dependence	Dependence				Partial Dependence
atg13Δ	Independence	Antagonism		Partial Dependence		Dependence	Partial Dependence		Independence
pnc1Δ			Independence	Independence	Independence	Antagonism	Independence		Independence
hap4Δ	Dependence		Independence	Independence	Independence		Independence	Dependence	Dependence
hac1Δ	Partial Dependence	Partial Dependence	Partial Dependence	Independence	Partial Dependence		Independence	Synergy	Dependence
maf1Δ	Independence	Partial Dependence	Dependence	Partial Dependence	Partial Dependence				
msn2Δ msn4Δ	Dependence	Independence	Dependence	Dependence	Dependence		Independence		Dependence

Description of Long-lived Mutants

The set of replicatively long-lived strains includes genes associated with three pathways linked to replicative aging: sirtuins, TOR signaling, and PKA signaling. A brief description of the selected mutations is provided below, and more detailed descriptions of each pathway are provided in Chapter 1.

Sirtuins (*SIR2OE, fob1*Δ). Overexpression of *SIR2* and deletion of *FOB1* extend replicative life span (Kaeberlein et al., 1999). Sir2 was originally thought mediate replicative life span extension by dietary restriction (Lin et al., 2000), but is now thought to act independently (Kaeberlein et al., 2004). Instead, we now think that deletion of *SIR2* broadly limits replicative life span by upregulating ERC formation, a process that is inhibited by deletion of *FOB1* (see Chapter 4). Deletion of *FOB1* decreases rDNA recombination, limiting ERC formation, and suppresses the short replicative life span of cells lacking *SIR2* (Defossez et al., 1999; Kaeberlein et al., 1999).

*TOR signaling (tor1*Δ*, sch9*Δ*, rpl20b*Δ*, rpl31a*Δ*).* Tor1 and Sch9 are two central nutrient responsive kinases in the TOR signaling pathway that influences many downstream processes. Reduced TOR signaling is thought to increase replicative life span, at least in part, by regulating mRNA translation. In this vein, deletion of genes encoding large subunit ribosomal proteins has been shown to increase life span and interact with TOR signaling, and two *RPL* deletions are included in this set (*rpl20b*Δ and *rpl31*Δ) (Steffen et al., 2008).

*PKA signaling (gpr1*Δ*, gpa2*Δ*, sok1*Δ*).* Gpr1 is the yeast PKA, a third nutrient-responsive kinase that influences a variety of cellular processes, and reduced PKA signaling is associated with increased replicative life span (Lin et al., 2000). Gpa2 is a G protein that regulates PKA signaling activity in response to nutrients (Colombo et al., 1998). Overexpression of *SOK1* rescues growth defects in mutants with reduced PKA signaling (Ward and Garrett, 1994), while deletion increases replicative life span (Managbanag et al., 2008).

Description of Potential Downstream Mediators

The set of potential downstream mediators of replicative life span regulation include transcription factors involved in stress, respiration, starvation, and ribosomal protein production, a nicotinamidase, and proteins involved in autophagy.

*Starvation-responsive factors (gcn2*Δ*, gcn4*Δ*).* Gcn4 is a transcription factor that regulates a variety of cellular processes including purine biosynthesis, autophagy, organelle biosynthesis, ER stress response, and activation of mitochondrial transport carrier proteins (Jia et al., 2000; Natarajan et al., 2001;

Patil et al., 2004). Gcn4 responds to amino acid starvation by activating genes involved in amino acid biosynthesis, and is translationally regulated by the presence of four small uORFs in the *GCN4* mRNA (Hinnebusch, 2005). Extension of replicative life span by deleting *TOR1, SCH9,* or an *RPL* at least partially requires *GCN4* (Steffen et al., 2008). Gcn2 is required for increased Gcn4 activity in response to reduced TOR signaling (Cherkasova and Hinnebusch, 2003), amino acid starvation (Hinnebusch, 2005), or glucose limitation (Yang et al., 2000). In contrast, deletion of an *RPL* activates Gcn4 and increases replicative life span in a Gcn2-independent manner (Foiani et al., 1991; Martin-Marcos et al., 2007; Steffen et al., 2008).

*Stress-responsive transcription factors (yap1*Δ*, hac1*Δ*, msn2*Δ *msn4*Δ*)*. Yap1 is a transcription factor that is activated in response to oxidative stress (Hirata et al., 1994). Activity of Yap1 is regulated, at least in part, through nuclear import control (Kuge et al., 1997). Similar to *GCN4*, the *YAP1* mRNA contains a single uORF, although the role it plays in translational regulation is not known (Vilela and McCarthy, 2003). Hac1 activates genes involved in the unfolded protein response (UPR) in response to ER stress. The UPR is thought to be an important process in aging in multiple species (Chen et al., 2009; Kaeberlein and Kennedy, 2008; Naidoo, 2009), and reduced ER stress is one potential mechanism through which reduced translation may positively impact longevity. Msn2 and Msn4 are largely redundant transcription factors that respond to a range of different stresses in yeast—heat shock, osmotic shock, oxidative stress, low pH, glucose starvation, sorbic acid, and ethanol—by binding STRE elements and activating gene expression (Causton et al., 2001; Estruch, 2000; Estruch and Carlson, 1993; Gasch et al., 2000). Nuclear localization of Msn2 is promoted by dietary restriction or reduced TOR signaling (Medvedik et al., 2007) and inhibited by PKA signaling (Gorner et al., 1998). Life span extension by either dietary restriction or TOR inhibition by rapamycin is prevented by deletion of both *MSN2* and *MSN4* (Medvedik et al., 2007).

Respiration transcription factor (*hap4*Δ*).* Hap4 is the regulatory subunit of the Hap2/3/4/5 glucose-repressed transcription factor that globally regulates the expression of respiratory genes (Forsburg and Guarente, 1989). Deletion of *HAP4* results in poor growth on nonfermentable carbon sources (Kitanovic and Wolfl, 2006) and partially suppresses replicative life span extension by two forms of dietary restriction (reducing glucose or limiting amino acids) (Wang et al., 2010). Overexpression of *HAP4* extends replicative life span in a manner that is non-additive with dietary restriction by reducing media glucose (Lin et al., 2002), suggesting that dietary restriction extends replicative life span, at least in part, by activating respiratory metabolism. Like *YAP1*, the *HAP4* mRNA contains a single uORF with an unknown role in regulation (Forsburg and Guarente, 1989).

*Ribosomal biogenesis transcription factors (hmo1*Δ*, maf1*Δ*).* Hmo1 and Maf1 are transcription factors involved in the transcription of the rDNA and genes encoding ribosomal subunit proteins.

Ribosomes are highly abundant in growing yeast cells, which expend a large fraction of their energy output on ribosome biogenesis (Warner, 1999). The replicative life span screen identified a large number of genes involved in ribosome structure or biogenesis, indicating an important role for control of ribosome production and function in yeast aging (Table 3.6). Hmo1 is a chromatin associated high mobility group (HMG) family member required for TORC1-regulated Pol I-mediated transcription of the rDNA and Pol II-mediated transcription of genes encoding ribosomal proteins (Berger et al., 2007; Kasahara et al., 2007). Maf1 is required to mediate transcription of genes involved in TORC1- and Sch9-dependent ribosome biogenesis by all three polymerases (Huber et al., 2009; Johnson et al., 2007; Lee et al., 2009).

Nicotinamidase (pnc1Δ). Sir2 activity requires NAD^+ and is inhibited by nicotinamide. Pnc1 promotes Sir2 activity by converting nicotinamide to nicotinic acid, which is then recycled into NAD^+. Overexpression of *PNC1* increases replicative life span in a manner attributed to decreased Sir2 inhibition (Anderson et al., 2003).

Autophagy factors (atg1Δ, atg13Δ). Autophagy is a highly conserved process in which cells recycle internal components or organelles via the lysosome. Autophagy is thought to be mechanistically important to aging or age-related pathology in several model organisms (Cuervo et al., 2005; Hansen et al., 2008; Jia and Levine, 2007; Ventruti and Cuervo, 2007). Autophagy is upregulated in response to reduced TOR or PKA signaling (Noda and Ohsumi, 1998; Yorimitsu et al., 2007). Atg1 is a serine/threonine kinase required for induction of autophagy. Atg13 is a regulatory subunit of a complex formed with Atg1 upon initiation of autophagy. TOR activity inhibits autophagy initiation by reducing the affinity of Atg13 for Atg1. Both *atg1*Δ and *atg13*Δ cells are defective for autophagy (Funakoshi et al., 1997; Matsuura et al., 1997; Straub et al., 1997; Tsukada and Ohsumi, 1993) and provide a system in which to test the requirement of autophagy for a given intervention.

Examination of Genetic Interactions within Pathways

The previously noted complication that multiple models can result in the same epistatic relationship between two interventions means that hard conclusions cannot be drawn directly from epistasis data. One useful application of a large number of interactions between sets of genes is the ability to identify potential differences between factors that are acting within a pathway. The following sections highlight interesting interactions of this type from the data presented in Table 3.8

SIR2OE *and* fob1Δ *Display Distinct Interaction Patterns with Downstream Mediators*

Sir2 and Fob1 are often thought of as components of a similar aging pathway in yeast. While they clearly both impact replicative aging by influencing ERC formation via modulation of rDNA recombination (Defossez et al., 1999; Kaeberlein et al., 1999; Sinclair and Guarente, 1997), *fob1*Δ is not thought to impact replicative life span by upregulating Sir2 activity. Furthermore, while *fob1*Δ suppresses the short replicative life span of *sir2*Δ cells, *sir2*Δ *fob1*Δ cells have a replicative life span similar to wild type, while *fob1*Δ are replicatively long-lived (Defossez et al., 1999; Kaeberlein et al., 1999). This is despite the fact that both *sir2*Δ *fob1*Δ and *fob1*Δ cells have substantially reduced ERC levels compared to wild type cells (Defossez et al., 1999; Kaeberlein et al., 1999). This suggests that Sir2 has an impact on replicative life span that is separate from its interaction with Fob1 and ERCs.

The pattern of interactions observed between *SIROE* and *fob1*Δ cells and the downstream mediators is consistent with this idea. While similar dependence relationships are observed for *SIR2OE* and *fob1*Δ in a few cases (partial or complete dependence on *GCN2* and *GCN4*; partial dependence on *HAC1*), each intervention appears to require a different set of the remaining factors to realize its full effect on replicative life span. Replicative life span by *SIR2* overexpression is at least partially dependent on *ATG1, HAP4, HAC1,* and *MSN2/4*, but independent of *YAP1, ATG13,* and *MAF1*. In contrast, the full replicative life span extension from deletion of *FOB1* requires *YAP1, ATG13,* and *MAF1* but not *ATG1, HAP4, HAC1*. These patterns suggest that Sir2 and Fob1 may impact replicative life span through largely non-overlapping mechanisms.

TOR Signaling Components Show Subtle Differences in Interactions with GCN2, PNC1, *and* HAC1

Replicative life span phenotypes associated with the four members of the TOR signaling pathway (*TOR1, SCH9, RPL20B, RPL31A*) show similar patterns of interaction with most of the potential downstream mediators: partial or complete dependence on *GCN4, MAF1*, *MSN2/4*, and autophagy proteins, and dependence on or antagonism with *YAP1* and *HMO1*. Three interactions that may highlight interesting differences between TOR components are *GCN2, PNC1,* and *HAC1*. While replicative life span extension in all for TOR mutants was at least partially dependent on *GCN4, GCN2* is partially required for replicative life span extension in *sch9*Δ cells but unnecessary in *rpl*Δ cells. This is consistent with the previous observation that *RPL* knockdown is capable of activating Gcn4 in a Gcn2-independent manner, while other methods of reducing TOR signaling require Gcn2 for Gcn4 activation (Foiani et al.,

1991; Martin-Marcos et al., 2007; Steffen et al., 2008). Unfortunately, different *tor1*Δ *gcn2*Δ strains display differences in replicative life span that have yet to be resolved.

While *tor1*Δ*, sch9*Δ*,* and *rpl20b*Δ extended replicative life span independently of *PNC1*, *RPL31A* displayed an antagonistic relationship with *PNC1*. Particularly intriguing is the different interactions observed between the two ribosomal subunits with respect to *PNC1*. This may suggests differential NAD^+-dependent regulation of specific ribosomal subunits or point to specific extra-ribosomal functions of one of the subunits. An examination of replicative life span for additional *RPL* mutants in a *pnc1*Δ context will be of interest going forward.

Replicative life span extension via either *tor1*Δ or *rpl20b*Δ was partially dependent on *HAC1*, while *SCH9* displayed an independent interaction. This suggests that the unfolded protein response may be mechanistically related to aspects of the TOR signaling pathway involving control of ribosome biogenesis, but not Sch9 activity.

PKA Signaling Genes Show Distinct Patterns of Interaction with Downstream Factors

Similar to the case for *SIR2OE* and *fob1*Δ, *gpr1*Δ*, gpa2*Δ, and *sok1*Δ each display a distinct set in interaction with downstream factors. In particular, replicative life span extension in *sok1*Δ cells appears to require stress responsive (*hac1*Δ and *msn2/4*Δ) and respiratory (*hap4*Δ) factors, while *gpr1*Δ requires Gcn2. The few interactions that were able to be defined for *gpa2*Δ seem to show similarities with *gpr1*Δ in some cases (inability to fully extend replicative life span in the absence of *GCN2*, but able to achieve extension in the absence of *HAC1*), and with *sok1*Δ in another (dependence on *HAP4*). The difference between Gpr1 and Sok1 are not entirely surprising, since the link between Sok1 and PKA signaling is tenuous. Too few interactions are available for Gpa2 to make a clear argument for a stronger similarity to either Gpr1 or Sok1, though the link to PKA signaling is well established and additional interactions might be expected to mimic Gpr1.

Identification of Useful Downstream Mediators of Replicative Life Span

A primary goal of examining epistatic interactions across multiple potential downstream mediators of replicative life span and multiple defined aging pathways was to identify downstream factors that might be useful for placing novel factors into different pathways. For this purpose, the following sections identify specific factors that show distinct interactions with one or more aging pathways.

Combination of Gcn2 and Gcn4 May Differentiate Ribosomal Proteins and PKA Signaling

One interesting result from the interaction screen was the fact that replicative life span extension through both *SIR2OE* and *fob1*Δ, which are not linked to reduced translation, were at least partially dependent on *GCN2* and *GCN4*. This suggests that there may be non-translation mediated regulation of these factors that is relevant to aging and indicate a direction for further investigation.

Furthermore, interactions with Gcn2 and Gcn4 may prove useful in distinguishing between pathways. While *SIR2OE, fob1*Δ*, tor1*Δ*,* and *sch9*Δ are all at least partially dependent on both factors (with the exception of the interaction between Tor1 and Gcn2, for which data is not available) other gene groups show distinct interactions. Replicative life span extension by deletion of an *RPL* appears to be dependent on *GCN4* but not *GCN2*, while reduced PKA signaling through deletion of *GPR1* or *GPA2* displayed extension dependent on *GCN2*, and, for *gpr1*Δ, independent of *GCN4*. Replicative life span extension by deletion of *SOK1* is unique among the interventions tested in its independence from both factors.

Deletion of YAP1 *Prevents Replicative Life Span Extension in Most Cases*

In 8 of the 9 interactions investigated, deletion of *YAP1* prevented replicative life span extension, and in 7 of these 8 resulted in an antagonistic shortening of replicative life span. This is similar to the pattern observed when *SIR2* is deleted (see Chapter 4). In the case of Sir2, we propose a model in which deletion limits replicative life span by inducing toxic ERC accumulation early in the replicative life span. Yap1 is required for the expression of anti-oxidant genes in response to oxidative stress (Temple et al., 2005; Toone and Jones, 1999) and cells lacking Yap1 are sensitive to multiple forms of oxidative stress (Lopez-Mirabal et al., 2007; Schnell et al., 1992; Wemmie et al., 1994). The inability of 8 interventions to increase *yap1*Δ replicative life span may highlight a general importance for oxidative stress resistance in aging. Like *sir2*Δ cells, *yap1*Δ cells may have a replicative life span limited by an increased accumulation of cellular damage—in this case oxidative.

Interestingly, overexpression of *SIR2* appears to increase replicative life span in a manner independent from *YAP1*. This might suggest that *SIR2OE* cells either have a reduced production of reactive oxygen species or increased oxidative stress resistance by a Yap1-independent mechanism. Counter intuitively, *SIR2EO* replicative life span extension is prevented in the absence of the respiratory transcription factor, Hap4, suggesting that *SIR2OE* requires activation of respiration to increase life span.

Deletion of *TOR1, SCH9,* or *RPL20B* shows the opposite pattern, extending life span in the absence of Hap4 but not extending in the absence of Yap1. While an increase in respiratory genes would seem to imply an increase in reactive oxygen species production, an alternative model is one in which increased respiratory machinery improves efficiency of the electron transport chain, decreasing the creation of reactive oxygen species for the same level of energy production. Further investigation is required to determine the underlying molecular cause of these interactions. In either case, deletion of *YAP1* may prove useful for identifying interventions that increase replicative life span by activating Sir2.

Hmo1 Independence Distinguishes PKA Signaling

Similar to deletion of *YAP1*, deletion of *HMO1* produced an antagonistic interaction with many of the longevity interventions tested, and partial or complete dependence elsewhere. A clear exception to this patter is PKA signaling. Both *gpr1*Δ and *sok1*Δ increased replicative life span in cells lacking *HMO1*. Hmo1 may therefore be useful in identifying interventions that interact with PKA signaling as compared to sirtuins or TOR signaling. Among the non-PKA interventions, only *fob1*Δ was able to increase replicative life span in the absence of Hmo1, though not to the same degree as in a wild type background. Since *fob1*Δ cells are clearly not behaving in the same manner as *SIR2OE* cells with respect to interactions with downstream mediators, this may suggest a link between deletion of *FOB1* and reduced PKA signaling.

Autophagy Factors Display Differential Interacting Patterns

Atg1 and Atg13 are the catalytic and regulatory components, respectively, of a complex required for the initiation of autophagy. Intuitively, this suggests that they should display similar patterns of interaction among the longevity interventions. In contrast, in three of the four cases where an intervention was examined in the context of both *atg1*Δ cells and *atg13*Δ cells (*SIR2OE, fob1*Δ, and *sok1*Δ), a different epistatic relationship emerges for Atg1 and Atg13. In each case the intervention is shown to be independent of one autophagy factor, but at least partially dependent on the other. Only one intervention (*sch9*Δ) showed at least partial dependence on both. This may indicate that autophagy is important for a wide range of interventions, but that regulation occurs through different autophagy factors. The difference between these and other longevity factors in the context of aging warrants further study.

DISCUSSION

This chapter reports the results from a recently completed replicative life span screen of the yeast haploid ORF deletion collection, describes a formalized system for analyzing and interpreting life span epistasis data, and applies this system to a set of 77 gene interactions between a set of replicatively long-lived yeast mutants and a set of potential downstream mediators of replicative life span.

The complete replicative life span screen examined 4,687 genotypes in total, each lacking a different non-essential ORF. A preliminary report describing the first 564 genes in this set identified 13 (2.3%) strains as replicatively long-lived (Kaeberlein et al., 2005d). The complete study confirms this initial report, identifying 115 (2.5%) genotypes as replicatively long-lived, when only strains that displayed significant extension in both mating types were considered. The close agreement in the percentage of replicatively long-lived mutants identified between the initial report and final screen is remarkable and indicates that the iterative method is a consistent means for identifying long-lived strains. The screen identified an additional 124 (2.6%) strains for which the original classification of the MATα version of the genotype as long-lived was not confirmed in the MAT**a** background, but for which significant extension was still reached when the mating types were pooled. This low-stringency set of strains retains the low false positive rate for the screening method, which was determined without reference to the confirmation in the MAT**a** background, and excludes genotypes for which the MAT**a** replicative life span was low enough to prevent the pooled set from achieving significance.

For comparison, it is worth noting that the percent of deletions conferring increased replicative life span (~2%) is about twice the percentage of genes that increase life span when expression is reduced via RNAi in *C. elegans* (~1% for pooled data from all reported RNAi screens) (Smith et al., 2007b). This may represent an intrinsic difference in the fraction of genes involved in aging in the two organisms, though there are also two possible method-based explanations for the discrepancy. First, the yeast genome was screened using deletion mutants and therefore excludes essential genes, while the nematode screens used RNAi. Differences in the fraction of genes that influence longevity among essential and nonessential genes would therefore bias the yeast screens. Second, numerous independent worm RNAi longevity screens have shown a remarkable lack of overlap in the genes identified as long-lived (Curran and Ruvkun, 2007; Dillin et al., 2002; Hamilton et al., 2005; Hansen et al., 2005; Lee et al., 2003). This is indicative of a high false-negative rate for *C. elegans* RNAi longevity screens and suggests that many worm longevity genes have yet to be discovered.

In addition to the frequency of replicatively long-live strains observed in the deletion collection, the complete screen also confirms the significant enrichment for genes involved in mRNA translation and TOR signaling that was highlighted in the initial report (Kaeberlein et al., 2005d) (Tables 3.5 and 3.6). Of

the 24 (~10%) longest-lived strains in the complete data set, 17 (70.8%) are involved in aspects of translation and TOR signaling. The screen further reinforces the idea that mRNA is a central process in determining longevity.

Importantly, the iterative method identified numerous genes previously known to impact replicative longevity, including the aforementioned TOR signaling components (*TOR1*, *SCH9*, *URE2*, *ROM2*, and many *RPLs*), PKA signaling components (*GPR1*, *GPA2*, and *SOK1*), *FOB1*, and *HXK2*, to highlight a few prominent examples (Figure 3.2; Table 3.2). This demonstrates that the iterative method is a robust means to identify strains with increased replicative life span. In addition to previously known longevity genes, the replicative life span screen identified a substantial number of novel aging factors. While many of these genes have a defined function in the yeast cell, the molecular role of others has not yet been characterized. As more novel longevity genes are discovered and begin to be characterized, tools for examining the interaction between many genes and identifying sets that act via similar mechanisms will become invaluable. The wide use of epistasis analysis for examining these types of relationships and the inherent problems associated with interpreting epistatic relationships were primary motivations in our development of a formalized system for analyzing and interpreting epistatic relationships between genes with respect to longevity.

In addition to the growing number of interventions capable of increasing life span, numerous factors have been identified that mediate these interventions. A primary endpoint in developing a system for analyzing epistatic data was to identify mediating factors that display differential interactions with common aging pathways such as TOR signaling and sirtuins. In this we were partially successful. Some factors, like Gcn2 and Gcn4, Yap1, and Hmo1, show patterns of interaction that may be useful in making a preliminary prediction as to whether a novel longevity factor is acting in a manner related to a known pathway or intervention (Table 3.8). Deletion of *YAP1* appears to be the most directly useful for specifically distinguishing interventions that activate Sir2, but also the most narrowly applicable. For Gcn2 and Gcn4 or Hmo1, each produced a distinct pattern with respect to at least one defined aging pathway. Unfortunately, each case presented at least one exception in an interaction with a different pathway, rendering any predictions tenuous. For all of the other potential mediators, the interaction patterns were too complex to make clear distinctions between pathways.

Examining specific interactions within the grid reveals some interesting points. The striking difference between *SIR2* overexpression and deletion of *FOB1* with respect the majority of interaction is one example. While Fob1 and Sir2 are clearly linked with respect to their impact on ERC formation and rDNA recombination, the two interventions show different patterns with respect to which stress response and autophagy factors are important for replicative life span extension (Table 3.8). Similarly, the complete set of interactions clearly separates deletion of *SOK1* from reduced PKA signaling through

deletion of *GPR1*. More subtle differences can be observed between members of the TOR signaling pathways, most notably the requirement of Gcn2 for full extension by deletion of *SCH9* but not by deletion of an *RPL*, and the requirement of Pnc1 for replicative life span extension by deletion of *RPL31A* but not *RPL20B*.

Looking at the interaction grid as a whole reveals a few interesting trends. First, synergistic relationships are nearly non-existent among the interactions tested. This may be indicative of a broader trend in the regulation of the aging process. Alternatively, it may simply be an artifact of the way the initial interaction grid was designed, since all of the interactions examined were between an intervention known to increases life span and a potential mediator for which deletion prevents extension of replicative life span in at least one context. Perhaps more striking is the relatively few examples of independence. In the context of several pathways that are thought to be at least partially distinct, one might expect interactions with a pool of potential downstream mediators to be independent in most cases. Instead, approximately two thirds of the 77 interactions displayed partial dependence, complete dependence, or antagonism (Table 3.8), suggesting a large degree interdependence between pathways.

The overarching message from applying our epistasis system to the set of 77 interactions is a further confirmation that the processes and genetic pathways that influence aging are not independent, but interrelated in complex ways. At least three pathways are known to modulate life span in diverse organisms: IIS, sirtuins and TOR signaling. While these pathways are at least partially independent, they interact both upstream, by responding to similar environmental queues, and downstream, by influencing overlapping sets of downstream targets to regulate complex processes such as metabolism or growth. Regulatory feedback and direct interactions between components within each pathway add to the complexity. Modifying the action of even a single gene that only directly plays a role in a single pathway can potentially alter the contributions to the mortality of many other pathways (Kennedy, 2008). As our understanding of this complexity progresses, so should our analytical tools develop to incorporate and take advantage of that complexity. Networking strategies based on known biological interactions have successfully been employed to identify novel longevity factors (Managbanag et al., 2008). Furthermore, based on a set of simple rules applied epistatic interactions (Avery and Wasserman, 1992), strategies have been developed to build gene interaction networks using phenotypes other than longevity, even in the context of relatively small number of interacting gene pairs (Carter et al., 2012; Carter et al., 2007). Based on these examples, epistatic interactions with respect to life span and other age-associated phenotypes can, in principle, be used to build complex models of gene interaction in the context of aging.

EXPERIMENTAL PROCEDURES

Strains and Media

All strains used in this study were derived from the parent strains of the haploid yeast ORF deletion collections (Winzeler et al., 1999), BY4742 (MATα *his3*Δ *leu2*Δ *lys2*Δ *ura3*Δ) and BY4741 (MAT**a** *his3*Δ *leu2*Δ *met15*Δ *ura3*Δ). The MATα and MAT**a** haploid ORF deletion collection and parental strains were obtained from Research Genetics. Single-deletion strains were either taken directly from one of the ORF deletion collection or constructed by replacing the entire ORF of the indicated gene with a selectable marker using standard PCR-base protocols as previously described (Sikorski and Hieter, 1989) and verified by PCR. Double mutant strains were produced by mating haploid mutants with the desired mutations, sporulating the resulting heterozygous diploid strain, selecting haploids with the desired markers via tetrad analysis, and verifying mutations by PCR.

Replicative Life Span Analysis

Replicative life span assays were performed as previously described (Kaeberlein et al., 2004; Steffen et al., 2009). Strains were maintained and all experiments carried out on YEPD agar plates (1% yeast extract, 2% Bacto-Peptone, 2% agar, 2% glucose). Statistical significance was determined using the Wilcoxon Rank Sum test.

Gene Ontology

The GO Slim Mapper (SGD, 2012a) and Go Term Finder (SGD, 2012b) on the *Saccharomyces* Genome Database web page were used to generate GO term associations and GO term enrichments for the strains identified in the replicative life span screen, respectively. Both tools query the GO Consortium yeast database. The p-values for the enrichment test are calculated based on a hypergeometric distribution with a Bonferroni multiple testing correction.

Chapter 4: A Critical Analysis of the Role of Sirtuins in Longevity in Three Organisms

CHAPTER SUMMARY

A role for sirtuins in aging was originally identified based on the observation that overexpression of *SIR2* increases replicative life span in yeast. The sirtuin aging field expanded dramatically in the years following the initial discovery: worm and fly strains transgenically overexpressing Sir2 orthologs were reported to be long-lived, mammalian SIRT1 was linked to a variety of age-associated phenotypes, and resveratrol and other small molecules were reported to activate Sir2 and increase life span. Since their initial debut on the aging scene, sirtuins have been a steadily growing source of controversy, beginning with a continuing debate over the role of sirtuins in dietary restriction. More recently, the ability of resveratrol and other small molecules to both activate sirtuins and increase life span proved difficult to verify independently, raising questions with regard to the life span extension reported in existing transgenic models. This chapter describes a recent collaborative study revisiting the transgenic overexpression of Sir2 orthologs in worms and flies. In each case, overexpression of sirtuins was ruled out as the causative factor of life span extension. In yeast, deletion of *SIR2* prevents replicative life span extension by dietary restriction. This chapter describes a second study in yeast extending this observation to 32 different life span-extending mutations and four methods of dietary restriction. In every case, deletion of *SIR2* prevented replicative life span extension; however, replicative life span extension was restored when both *SIR2* and *FOB1* were deleted in several cases, demonstrating that *SIR2* is not directly required for replicative life span extension. These findings indicate that suppression of the *sir2*Δ life span defect is a rare phenotype among longevity interventions and suggest that *sir2*Δ cells senesce rapidly by a mechanism distinct from that of wild type cells. They also demonstrate that failure to observe life span extension in a short-lived background, such as cells or animals lacking sirtuins, should be interpreted with caution.

INTRODUCTION

Sirtuins are a widely studied class of protein deacetylases that have been linked to longevity regulation and various other age-associated phenotypes in yeast, worms, flies, and mice. Transgenic overexpression of sirtuins is reported to extend life span in yeast (Kaeberlein et al., 1999), worms (Berdichevsky et al., 2006; Tissenbaum and Guarente, 2001; Viswanathan et al., 2005), and flies (Rogina and Helfand, 2004). Resveratrol, a plant-derived polyphenol, and other small molecules have been reported to increase life span and activate sirtuins in several species (Howitz et al., 2003; Milne et al., 2007; Wood et al., 2004). More recently, attempts to reproduce both the life span extension and sirtuin activation using these molecules by multiple independent groups have proven unsuccessful (Bass et al., 2007b; Beher et al., 2009; Borra et al., 2005; Kaeberlein et al., 2005c; Pacholec et al., 2010).

In yeast, a conclusive answer has not yet been reached to the question of what downstream mechanisms mediate replicative life span extension by dietary restriction. Two non-mutually exclusive models have been proposed: increased sirtuin activity and reduced TOR signaling resulting in altered mRNA translation (Kaeberlein et al., 2005d; Medvedik et al., 2007; Steffen et al., 2008). Dietary restriction may activate Sir2 by either elevating NAD levels through increased respiration (Lin et al., 2002) or by increasing transcription of *PNC1* in an Msn2/4 dependent manner. *PNC1* is necessary for the full life span extension from dietary restriction (Anderson et al., 2003; Lin et al., 2004) and encodes an enzyme that deaminates nicotinimide, which otherwise inhibits Sir2. Contrary to the idea of sirtuins as mediators of dietary restriction, *SIR2* is not required for the replicative life span extension caused by dietary restriction (Table 12.1). Specifically, dietary restriction does not increase replicative life span in the short-lived *sir2Δ* background (Kaeberlein et al., 2004; Lin et al., 2000), but when the short life span of *sir2Δ* is repressed by deletion of *FOB1*, dietary restriction robustly extends replicative life span (Kaeberlein et al., 2004; Lamming et al., 2005). One study found that, in the absence of Fob1, other sirtuins (such as Hst2) are activated by dietary restriction to repress ERC formation (Lamming et al., 2005), though independent attempts to reproduce this result have been unsuccessful for unknown reasons (Kaeberlein et al., 2004; Tsuchiya et al., 2006). Two recent studies found that dietary restriction does not alter transcriptional silencing at the rDNA (Riesen and Morgan, 2009; Smith et al., 2009), indicating that if dietary restriction does act through Sir2 to extend life span, increased rDNA silencing is not the mediating factor. Interestingly, rDNA recombination was decreased by dietary restriction despite the lack of change in rDNA silencing and degree of reduction was similar in both wild type yeast and strains lacking *SIR2*.

An alternate model places dietary restriction and Sir2 in separate pathways with respect to replicative life span. In this model, ERC levels limit replicative life span in yeast lacking *SIR2*, such that

all cells die from ERC toxicity before the beneficial effects of dietary restriction can be realized (Kaeberlein et al., 2004). Removing ERCs as a limiting factor by deleting *FOB1* or overexpressing *SIR2* allows an even greater extension of replicative life span in response to dietary restriction than observed in wild type cells (Kaeberlein et al., 2004). If this model is correct, deletion of *SIR2* should generally limit replicative life span and prevent extension by interventions that are unable to prevent ERC formation or reduce ERC toxicity.

This chapter describes two recent studies examining the role of sirtuins in the aging process in three common aging models. The first demonstrates that life span extension in published worm and fly strains overexpressing sirtuins is caused by specific factors in the strain backgrounds unrelated to the increased sirtuin activity (Burnett et al., 2011). The second demonstrates that deletion of *SIR2* blocks replicative life span extension resulting from a diverse range of single-gene deletions and multiple forms of dietary restriction, supporting the hypothesis that deletion of *SIR2* generally limits replicative life span (Delaney et al., 2011).

RESULTS

Reported Life Span Extension by sir-2.1 *Overexpression in Worms Results from Unidentified Background Mutations*

Life span extension by overexpression of *sir-2.1* in *C. elegans* was originally reported using the LG100 strain, which contains the high-copy transgenic array *geIn3[sir-2.1 rol-6(su1006)]* (Tissenbaum and Guarente, 2001). In order to verify that life span extension in this strain resulted from the *geIn3* array, we outcrossed the LG100 strain to wild type (N2) worms 5 times, following the roller (Rol) phenotype induced by *rol-6(su1006)*. While LG100 worms were clearly long-lived relative to wild type and *rol-6(su1006)* control lines, the outcrossed LG100 worms slightly short lived (Figure 4.1A; Table 4.1), indicating that the *geIn3*, and thus *sir-2.1* overexpression, was not the cause of life span extension in the LG100 strain.

Burnett et al. (2011) further verified that SIR-2.1 protein levels were elevated in the outcrossed *geIn3* strains, and that RNAi targeting *sir-2.1* failed to abrogate life span extension in the LG100 strain, all of which support the conclusion that over expression of *sir-2.1* was not causing the increased life span in this set of strains. Instead, life span extension followed a neuronal dye-filling (Dyf) resulting from an unidentified mutation that segregated independently from the *geIn3* array during outcrossing (Burnett et al., 2011). LG100 is not the only *sir-2.1* overexpressing strain that has been reported to increase life span.

Burnett et al. (2011) further confirmed that outcrossing the low-copy transgenic *sir-2.1* overexpression strain, NL3909 (Guarente, 2007), abrogated life span extension. Finally, RNAi targeting *sir-2.1* failed to repress life span extension in a third strain, DR1786, carrying the *mDp4* duplication that includes the *sir-2.1* locus (Tissenbaum and Guarente, 2001). The outcrossing experiments using the LG100 and NL3909 strains were each independently verified by at least two research groups.

More recently, the Guarente lab at the Massachusetts Institute of Technology has independently constructed and outcrossed a new set of strains expressing the *geIn3* array. These strains do appear to have increased life span, though to a much lesser degree than the LG100 strain (Figure 4.1B; Table 4.1).

Reported Life Span Extension from dSir2 *Overexpression in Flies Results from Components of the Overexpression Machinery*

In flies, low-level overexpression of *dSir2* using a GAL4-UAS binary system (Brand and Perrimon, 1993) in which a EP-UAS-*dSir2* construct is driven by a ubiquitous tubulin-GAL4 driver, was reported to extend life span (Rogina and Helfand, 2004). Burnett et al. (2011) reexamined this system by crossing the EP-UAS-*dSir2*/*tubulin*-GAL4 transgenes into the white Dahomy strain background, and creating a novel *dSir2* overexpression line with higher *dSir2* expression using a UAS-*dSir2-Myc9*/*tubulin*-GAL4 transgene pair. Both overexpression systems increase life span relative to wild type flies; however, expression of the *tubulin*-GAL4 driver alone resulted in a similar degree of life span extension (Burnett et al., 2011). Thus, similar to the case for worms, life span extension in flies overexpressing *dSir2* results from artifacts in the background, and not the increased presence of sirtuins.

Deletion of SIR2 *Prevents Replicative Life Span Extension from 32 Single-Gene Deletions and 4 Forms of Dietary Restriction*

In yeast, deletion of *SIR2* blocks replicative life extension from dietary restriction by reducing media glucose, or from deletion of *GPA2* or *HXK2*, two genetic mimics of dietary restriction, but not in a strain lacking the rDNA replication fork block protein, *FOB1* (Kaeberlein et al., 2004). We hypothesized that this pattern resulted from a replicative life span limiting increase in ERC levels in strains lacking *SIR2* that is repressed when *FOB1* is also deleted. If this is the case, deletion of *SIR2* should generally limit replicative life span extension except in cases where the replicative life span-extending intervention is also able to repress either ERC accumulation or prevent the resulting damage. To examine the influence

of deleting *SIR2* on replicative life span extension more generally, we generated 30 double mutant strains in which a replicative life span-extending deletion was combined with deletion of *SIR2*. We also tested three additional methods of dietary restriction involving growth on alternative carbon sources (ethanol, glycerol, or raffinose). Strikingly, none of these interventions resulted in a significant replicative life span extension relative to *sir2*Δ cells (Figures 4.2 and 4.3; Table 4.2).

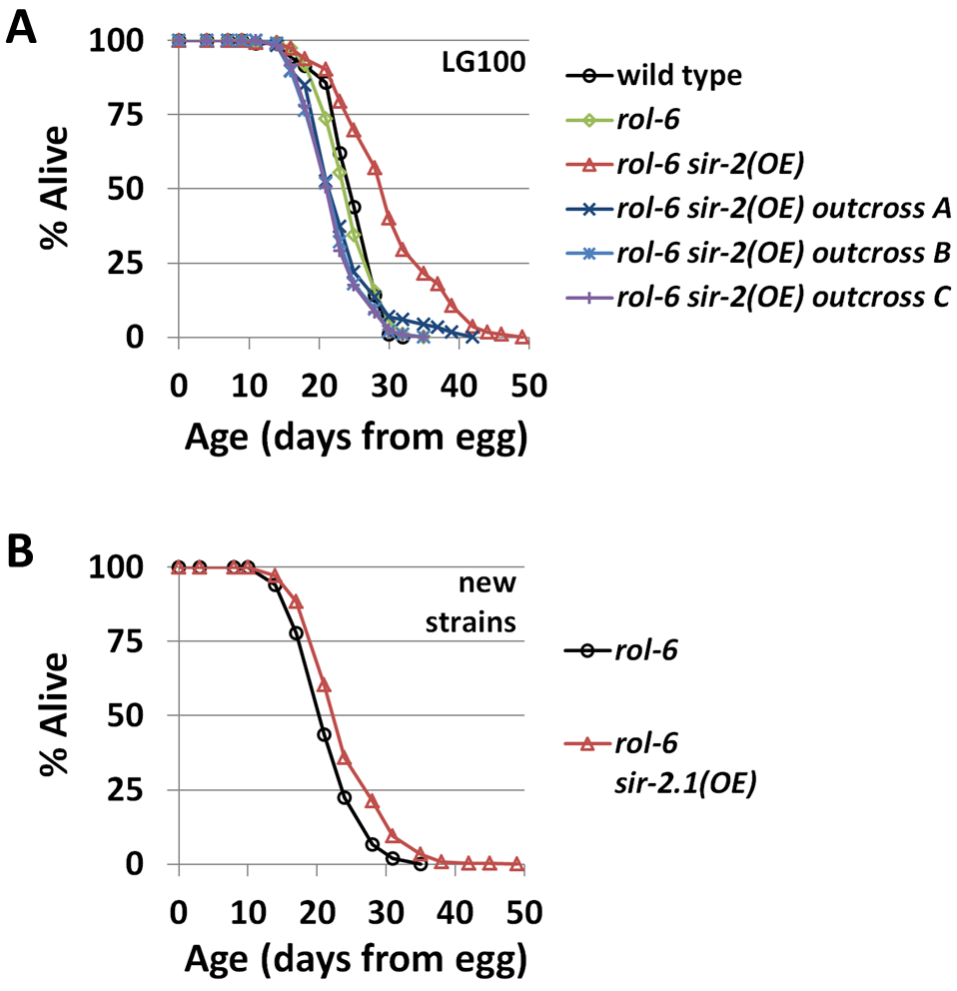


Figure 4.1. Outcrossing the LG100 *sir-2.1* overexpression strain eliminates life span extension. (A) The increased life span extension observed in the LG100 strain (*rol-6 sir-2.1(OE)*) relative to N2 (wild type) or CL2099 (*rol-6*) is eliminated when the strain is outcrossed (6x) to N2. Strains from three independent outcrosses are shown. (B) Newly constructed and outcrossed (8x) *sir-2.1(OE)* worm strains show a slight increase in life span relative to *rol-6* controls. The *rol-6* data shown is pooled for high-copy *geIs101[rol-6(su1006)]* transgenic control strains MV395, MV396, MV397, and MV398. The *rol-6 sir-2.1(OE)* data shown is pooled for high-copy *geIn3[sir-2.1 rol-6(su1006)]* transgenic strains MV391, MV392, MV393, and MV394.

Table 4.1. Summary of worm life span data presented in this study.

Genotype				Control				
Name	Median Life Span	Mean Life Span	N	Name	Median Life Span	Mean Life Span	N	Ranksum P-Value
rol-6	25	24.7	110	wild type	25	25.2	105	0.241
rol-6 sir-2.1(OE)	30	30.3	112	wild type	25	25.2	105	6.28E-09
rol-6 sir-2.1(OE) 6x outcrossed A	23	23.6	118	wild type	25	25.2	105	7.11E-05
rol-6 sir-2.1(OE) 6x outcrossed B	23	22.5	106	wild type	25	25.2	105	1.46E-06
rol-6 sir-2.1(OE) 6x outcrossed C	23	22.5	121	wild type	25	25.2	105	1.44E-07
rol-6 sir-2.1(OE)	30	30.3	112	rol-6	25	24.7	110	3.25E-10
rol-6 sir-2.1(OE) 6x outcrossed A	23	23.6	118	rol-6	25	24.7	110	0.004
rol-6 sir-2.1(OE) 6x outcrossed B	23	22.5	106	rol-6	25	24.7	110	1.29E-04
rol-6 sir-2.1(OE) 6x outcrossed C	23	22.5	121	rol-6	25	24.7	110	2.74E-05
rol-6 sir-2.1(OE) 6x outcrossed A	23	23.6	118	rol-6 sir-2.1(OE)	30	30.3	112	5.31E-14
rol-6 sir-2.1(OE) 6x outcrossed B	23	22.5	106	rol-6 sir-2.1(OE)	30	30.3	112	1.56E-16
rol-6 sir-2.1(OE) 6x outcrossed C	23	22.5	121	rol-6 sir-2.1(OE)	30	30.3	112	4.70E-18
rol-6 low-copy sir-2.1(OE)	24	24.9	398	rol-6	21	22.4	358	1.33E-08

Deletion of FOB1 *Restores Replicative Life Span Extension by 8 Single-Gene Deletions and All 4 Forms of Dietary Restriction in a* sir2Δ *Background*

One possible interpretation of these data is that each of the replicative life span-extending interventions acts upstream of Sir2 and increases replicative life span by promoting Sir2 activity. To examine this hypothesis, nine of the gene deletions and all four forms of dietary restriction were examined in a strain background in which the increased ERC levels resulting from s*ir2*Δ are repressed by deletion of *FOB1*. In eight of these cases, replicative life span was significantly extended relative to *sir2*Δ *fob1*Δ cells (Figure 4.4A; Table 4.3). In fact, most interventions increases replicative life span to a greater degree in the *sir2*Δ *fob1*Δ background than in the wild type background (Table 4.3), demonstrating that *SIR2* is not absolutely required for replicative life span extension in all cases. In contrast to each of these other interventions, replicative life span extension resulting from deletion of *SAS2* was prevented in the *sir2*Δ *fob1*Δ background. This pattern supports a model in which *SIR2* is mechanistically involved in enhancing replicative longevity in cells lacking *SAS2*, but not in the majority of interventions for which replicative life span extension is prevented by the deletion of *SIR2*.

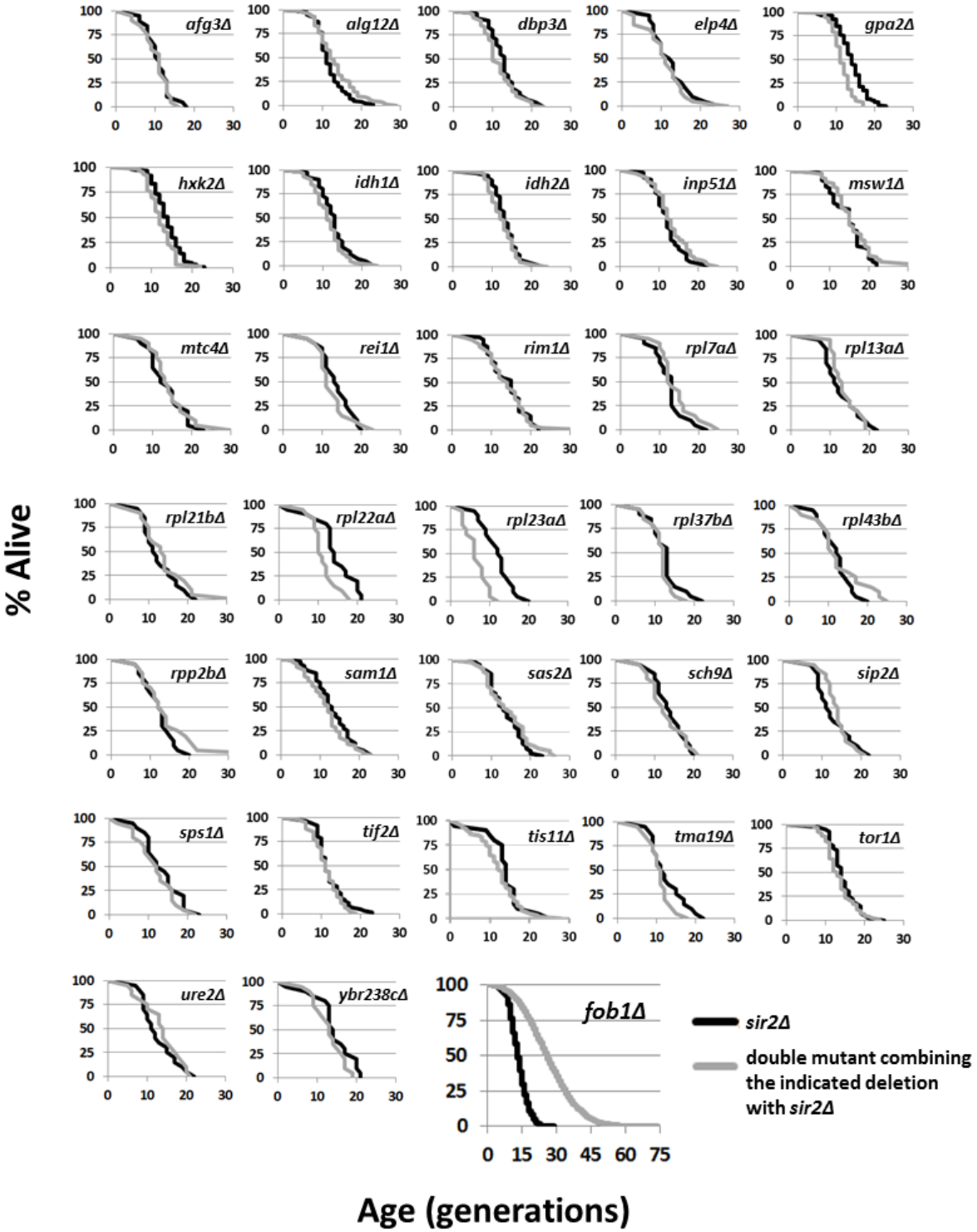


Figure 4.2. Single gene deletions that extend replicative life span in wild type cells do not extend replicative life span of *sir2*Δ cells. Replicative survival curves are provided for 33 double mutant strains combining a known long-lived gene deletion with deletion of *SIR2*.

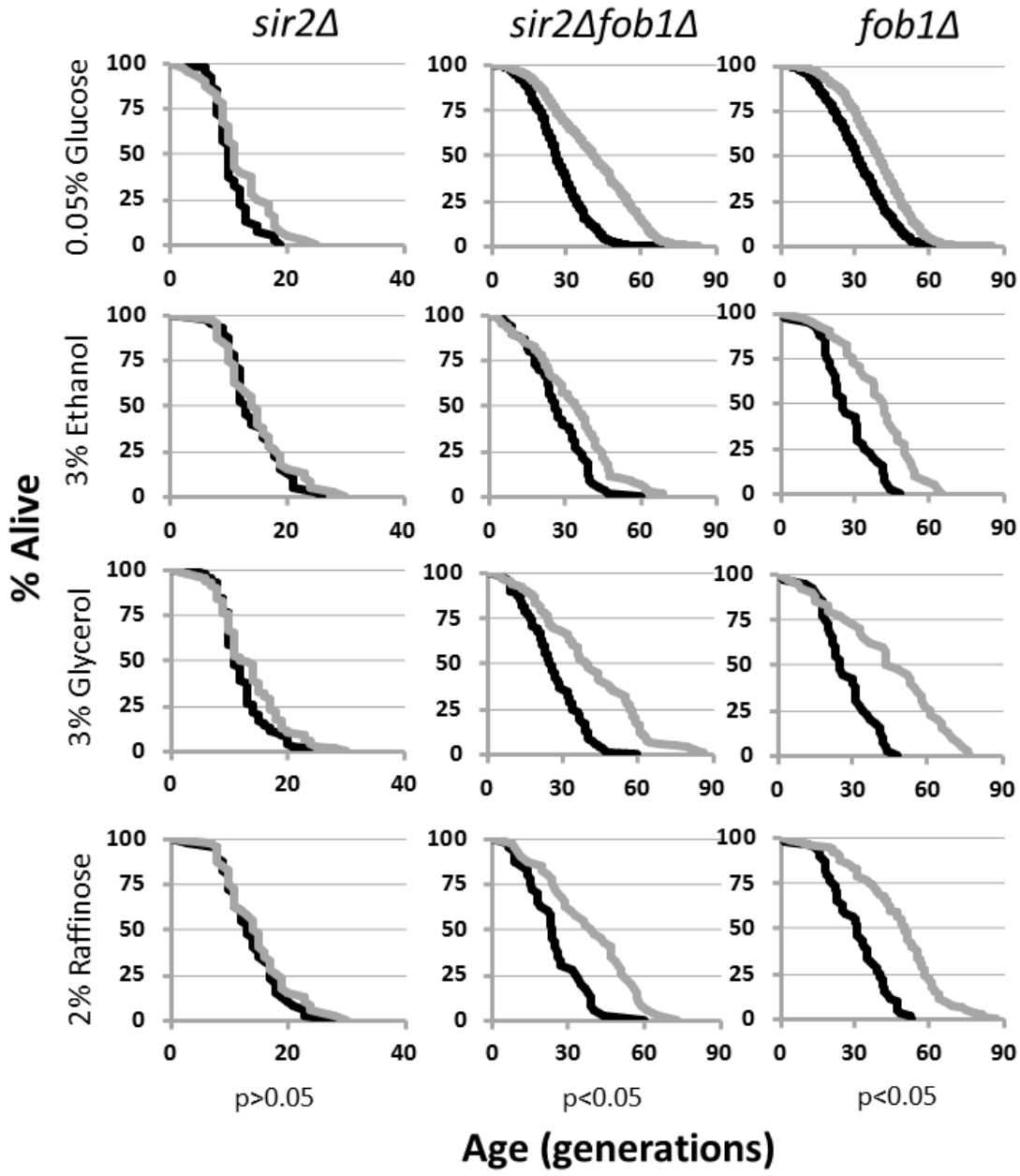


Figure 4.3. Multiple forms of dietary restriction extend replicative life span in a Sir2- and Fob1-independent manner. All four forms of dietary restriction were able to extend replicative life span of *sir2*Δ *fob1*Δ cells and *fob1*Δ cells but not *sir2*Δ cells.

Table 4.2. Summary of replicative life span data presented in this chapter.

Double Deletion				Experiment-matched Control				Ranksum
Name	Mean RLS	Median RLS	N	Name	Mean RLS	Median RLS	N	P-Value
afg3 sir2	10.3	11	20	sir2	10.9	11	19	0.757
alg12 sir2	13.6	12	100	sir2	12.6	12	99	0.284
dbp3 sir2	11.6	10	60	sir2	13.0	13	40	0.064
elp4 sir2	11.4	11	20	sir2	12.7	12.5	20	0.552
fob1 sir2	26.6	26	2250	sir2	13.5	13	628	2.8E-174
gpa2 sir2	11.5	11	79	sir2	14.2	14	100	1.4E-07
hxk2 sir2	12.4	12	60	sir2	13.8	14	110	0.005
idh1 sir2	11.5	11	60	sir2	13.0	13	40	0.066
idh2 sir2	12.6	12.5	60	sir2	13.6	13	40	0.144
inp51 sir2	13.1	12.5	60	sir2	12.2	12	39	0.304
msw1 sir2	15.9	15	60	sir2	14.6	15	60	0.417
mtc4 sir2	14.6	13.5	20	sir2	13.6	13	20	0.598
rei1 sir2	12.5	11	20	sir2	13.7	13.5	20	0.234
rim1 sir2	14.0	14	40	sir2	14.2	15	40	0.773
rpl7a sir2	14.3	12.5	20	sir2	12.6	13	20	0.298
rpl13a sir2	13.7	13	20	sir2	12.8	11.5	20	0.245
rpl21b sir2	14.2	13.5	20	sir2	12.8	11.5	20	0.516
rpl22a sir2	11.3	10.5	20	sir2	14.5	14	20	0.007
rpl23a sir2	6.6	6	20	sir2	12.1	12.5	20	4.2E-05
rpl37b sir2	11.5	12	20	sir2	12.6	13	20	0.365
rpl43b sir2	12.9	11.5	20	sir2	12.1	12.5	20	0.925
rpp2b sir2	14.6	12.5	20	sir2	12.1	12.5	20	0.499
sam1 sir2	11.6	12	60	sir2	13.3	13	40	0.120
sas2 sir2	13.5	13	40	sir2	13.5	14.1	40	0.847
sch9 sir2	12.8	12	20	sir2	13.7	13.5	20	0.433
sip2 sir2	13.9	14	20	sir2	12.8	11.5	20	0.224
sps1 sir2	11.9	12	20	sir2	13.6	13	20	0.358
tif2 sir2	11.2	11	60	sir2	12.2	11	60	0.326
tis11 sir2	12.2	12.5	60	sir2	14.1	14	20	0.105
tma19 sir2	10.7	11	20	sir2	12.8	11.5	20	0.239
tor1 sir2	13.5	13	80	sir2	14.6	14	40	0.073
ure2 sir2	13.6	14	20	sir2	12.8	11.5	20	0.457
ybr238c sir2	13.1	13	20	sir2	14.5	14	20	0.245

Table 4.2 (continued).

Single Deletion				Experiment-matched Control				Ranksum
Name	Mean RLS	Median RLS	N	Name	Mean RLS	Median RLS	N	P-Value
afg3	30.8	31	125	WT	28.3	29	145	0.041
alg12	32.4	33	145	WT	26.9	26	205	2.4E-06
dbp3	31.5	31	120	WT	24.3	25	165	6.1E-08
elp4	34.5	37	45	WT	25.6	24	65	1.2E-05
fob1	33.4	34	3899	WT	26.7	26	3918	2.8E-130
gpa2	32.0	32	675	WT	28.0	28	933	6.2E-12
hxk2	33.1	31	645	WT	26.0	25	824	3.3E-25
idh1	33.5	34	285	WT	26.5	26	445	5.4E-16
idh2	30.7	31	515	WT	26.6	26	605	1.9E-09
inp51	30.9	31	350	WT	26.8	27	345	5.5E-07
msw1	34.0	31	45	WT	28.6	28	45	0.029
mtc4	33.9	32.5	30	WT	25.0	25	90	1.0E-04
rei1	35.7	33	150	WT	28.4	27.5	150	4.0E-06
rim1	29.0	30	65	WT	25.5	26	85	0.024
rpl7a	32.6	32	87	WT	26.2	25	81	0.004
rpl13a	34.4	34	165	WT	25.7	25	199	4.5E-12
rpl21b	37.7	39	125	WT	24.4	24	145	5.8E-20
rpl22a	35.8	38	285	WT	27.1	27	204	9.0E-15
rpl23a	35.3	35	85	WT	27.7	27	89	2.9E-05
rpl37b	32.2	31	60	WT	24.8	23	59	8.7E-05
rpl43b	34.4	36	65	WT	25.2	25	79	1.6E-05
rpp2b	35.6	36	35	WT	23.2	21	39	0.001
sam1	33.0	33	165	WT	23.9	23	241	2.3E-13
sas2	32.3	33	263	WT	28.1	27	285	1.6E-05
sch9	36.3	38	1780	WT	27.0	27	1788	7.9E-129
sip2	31.1	30	125	WT	26.5	26	225	7.6E-05
sps1	31.5	29	45	WT	25.4	25	45	0.009
tif2	32.4	32.5	220	WT	26.5	26	284	6.8E-10
tis11	31.8	31	100	WT	25	26	125	7.4E-06
tma19	33.4	34	45	WT	26.1	26	65	0.002
tor1	33.2	34	1984	WT	26.3	26	2008	8.1E-85
ure2	30.0	31	40	WT	24.4	25	80	0.001
ybr238c	30.8	30	150	WT	25.8	26	180	2.4E-05

Table 4.2 (continued).

Deletion/Condition				Experiment-matched Control				Ranksum
Name	Mean RLS	Median RLS	N	Name	Mean RLS	Median RLS	N	P-Value
dbp3 sir2 fob1	32.4	33	40	sir2 fob1	24.3	23.5	20	0.003
gpa2 sir2 fob1	51	55	80	sir2 fob1	30.5	30	180	2.7E-17
hxk2 sir2 fob1	46	48	190	sir2 fob1	29.9	30	220	2.8E-22
rei1 sir2 fob1	41.2	40	80	sir2 fob1	29.9	28	20	0.004
rpl6b sir2 fob1	35.4	34.5	20	sir2 fob1	17.8	17.5	58	7.2E-07
rpl19a sir2 fob1	38.1	43	20	sir2 fob1	17.8	17.5	58	3.0E-07
rpl31a sir2 fob1	37.2	38	160	sir2 fob1	26.1	26	100	1.2E-10
sas2 sir2 fob1	23.9	24	198	sir2 fob1	25.5	25	179	0.151
sch9 sir2 fob1	37.2	37.5	40	sir2 fob1	26.125	28.5	40	0.008
-								
gpa2 fob1	48.6	50	80	fob1	33.5	36	160	1.7E-06
hxk2 fob1	43.8	41.5	20	fob1	31.5	34.5	20	0.017
rpl31a fob1	46.1	48	40	fob1	28.9	29	20	1.3E-04
sch9 fob1	45.7	48	120	fob1	30.4	28	100	7.6E-10
sir2								
0.05% glucose	10.6	10	40	sir2 2% glucose	12.1	11	40	0.133
3% glycerol	12.2	11	80	sir2 2% glucose	13.6	12.5	80	0.133
3% ethanol	14.4	13	40	sir2 2% glucose	15	14.5	40	0.897
2% raffinose	13.9	13	40	sir2 2% glucose	15	14.5	40	0.950
sir2 fob1								
0.05% glucose	40.3	41	630	sir2 fob1 2% glucose	26.7	26	549	4.9E-44
3% glycerol	41	39.5	120	sir2 fob1 2% glucose	26.1	25.5	100	1.3E-08
3% ethanol	33.3	34.5	80	sir2 fob1 2% glucose	27	26	60	0.013
2% raffinose	38.5	39.5	40	sir2 fob1 2% glucose	24.7	24	40	2.7E-04
fob1								
0.05% glucose	39	40	1371	fob1 2% glucose	31.1	31	1592	2.4E-50
3% glycerol	44.6	45.5	40	fob1 2% glucose	27.2	25	40	1.1E-04
3% ethanol	39.6	41.5	40	fob1 2% glucose	27.2	25	40	5.6E-05
2% raffinose	47.7	50	60	fob1 2% glucose	30.5	31	60	9.2E-10

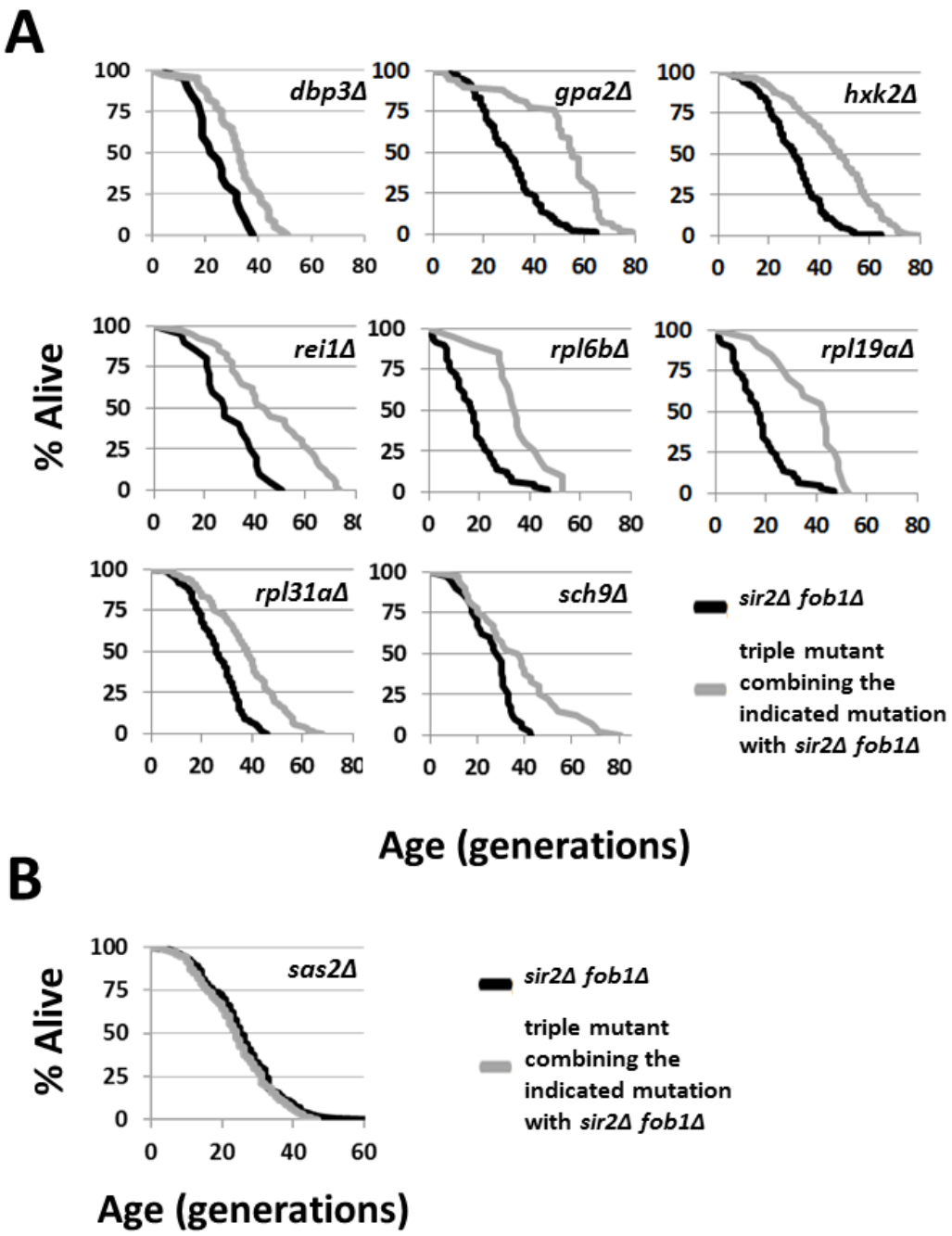


Figure 4.4. Most long-lived mutants extend replicative life span of *sir2*Δ *fob1*Δ cells. Eight long-lived mutations extend *sir2*Δ *fob1*Δ cells (A), but *sas2*Δ does not (B).

Table 4.3. Percent extension in replicative life span resulting from each gene deletion.

Deletion/	% Mean RLS Change				% Median RLS Change			
Condition	WT	sir2	fob1	sir2 fob1	WT	sir2	fob1	sir2 fob1
afg3	8.8	-5.5			6.9	0.0		
alg12	20.4	7.9			26.9	0.0		
dbp3	29.6	-10.8		33.3	24.0	-23.1		40.4
elp4	34.8	-10.2			54.2	-12.0		
fob1	25.1	97.0			30.8	100.0		
gpa2	14.3	-19.0	45.1	67.2	14.3	-21.4	38.9	83.3
hxk2	27.3	-10.1	39.0	53.8	24.0	-14.3	20.3	60.0
idh1	26.4	-11.5			30.8	-15.4		
idh2	15.4	-7.4			19.2	-3.8		
inp51	15.3	7.4			14.8	4.2		
msw1	18.9	8.9			10.7	0.0		
mtc4	35.6	7.4			30.0	3.8		
rei1	25.7	-8.8		37.8	20.0	-18.5		42.9
rim1	13.7	-1.4			15.4	-6.7		
rpl6b				98.9				97.1
rpl7a	24.4	13.5			28.0	-3.8		
rpl13a	33.9	7.0			36.0	13.0		
rpl19a				114.0				145.7
rpl21b	54.5	10.9			62.5	17.4		
rpl22a	32.1	-22.1			40.7	-25.0		
rpl23a	27.4	-45.5			29.6	-52.0		
rpl31a			59.5	42.5			65.5	46.2
rpl37b	29.8	-8.7			34.8	-7.7		
rpl43b	36.5	6.6			44.0	-8.0		
rpp2b	53.4	20.7			71.4	0.0		
sam1	38.1	-12.8			43.5	-7.7		
sas2	14.9	0.0		-6.3	22.2	-7.8		-4.0
sch9	34.4	-6.6	50.3	42.5	40.7	-11.1	71.4	31.6
sip2	17.4	8.6			15.4	21.7		
sps1	24.0	-12.5			16.0	-7.7		
tif2	22.3	-8.2			25.0	0.0		
tis11	27.2	-13.2			19.2	-10.7		
tma19	28.0	-16.4			30.8	-4.3		
tor1	26.2	-7.5			30.8	-7.1		
ure2	23.0	6.2			24.0	21.7		
ybr238c	19.4	-9.7			15.4	-7.1		

rDNA Recombination is Not Decreased and rDNA Silencing is Not Increased in 5 Long-Lived Mutants

One role of Sir2 in the cell is to silence the rDNA locus, preventing rDNA recombination. If each of the examined single-gene deletions were increasing replicative life span by increasing Sir2 activity, an increase in silencing at the rDNA and a decrease in rDNA recombination should be observed in response to each deletion. At least five long-lived deletion mutants show no indication of enhanced Sir2 activity in vivo, by either of these measures (Figure 4.5). A similar lack of increased Sir2 activity has been previously reported in cells subjected to dietary restriction (Kaeberlein et al., 2005c; Riesen and Morgan, 2009; Smith et al., 2009). Interestingly, deletion of *TOR1* caused a significant decrease in rDNA recombination, but this effect was independent of *SIR2* (Figure 4.5A).

DISCUSSION

This chapter describes two recent studies addressing technical and conceptual problems related to the role of sirtuins in aging. In the first, we show that the reported life span extension observed in worm and fly strains overexpressing sirtuins results from background artifacts and not from increased sirtuin activity. In the second, we demonstrate that deletion of *SIR2* in yeast prevents replicative life span extension from the majority of life span-extending interventions.

The first study will have several likely consequences. The first is a reexamination of the role of sirtuins as a conserved aging pathway. In yeast, replicative life span extension by overexpression of *SIR2* is not under dispute, but this study raises the question as to what role sirtuins play in aging in both worms and flies. There is some hope of positive resolution. As indicated, the novel *geIn3* strains in worms show an increase in life span (Figure 4.1B), even if the degree of extension is not as great as in the original reports using the LG100 strain. Furthermore, in *daf-2(e1370)* mutants, deletion of *sir-2.1* reproducibly increases life span (Berdichevsky et al., 2006; Burnett et al., 2011), suggesting that sirtuins may play a pro-aging role in worms under some circumstances. We have also observed that worm longevity interventions are widely dependent on temperature, including caffeine (see Chapter 5), hypoxia mutants (Leiser et al., 2011), ribosomal proteins, p53/*cep-1* mutants, and others (our unpublished data). Altered *sir-2.1* expression may impart a greater influence on longevity at a different temperature.

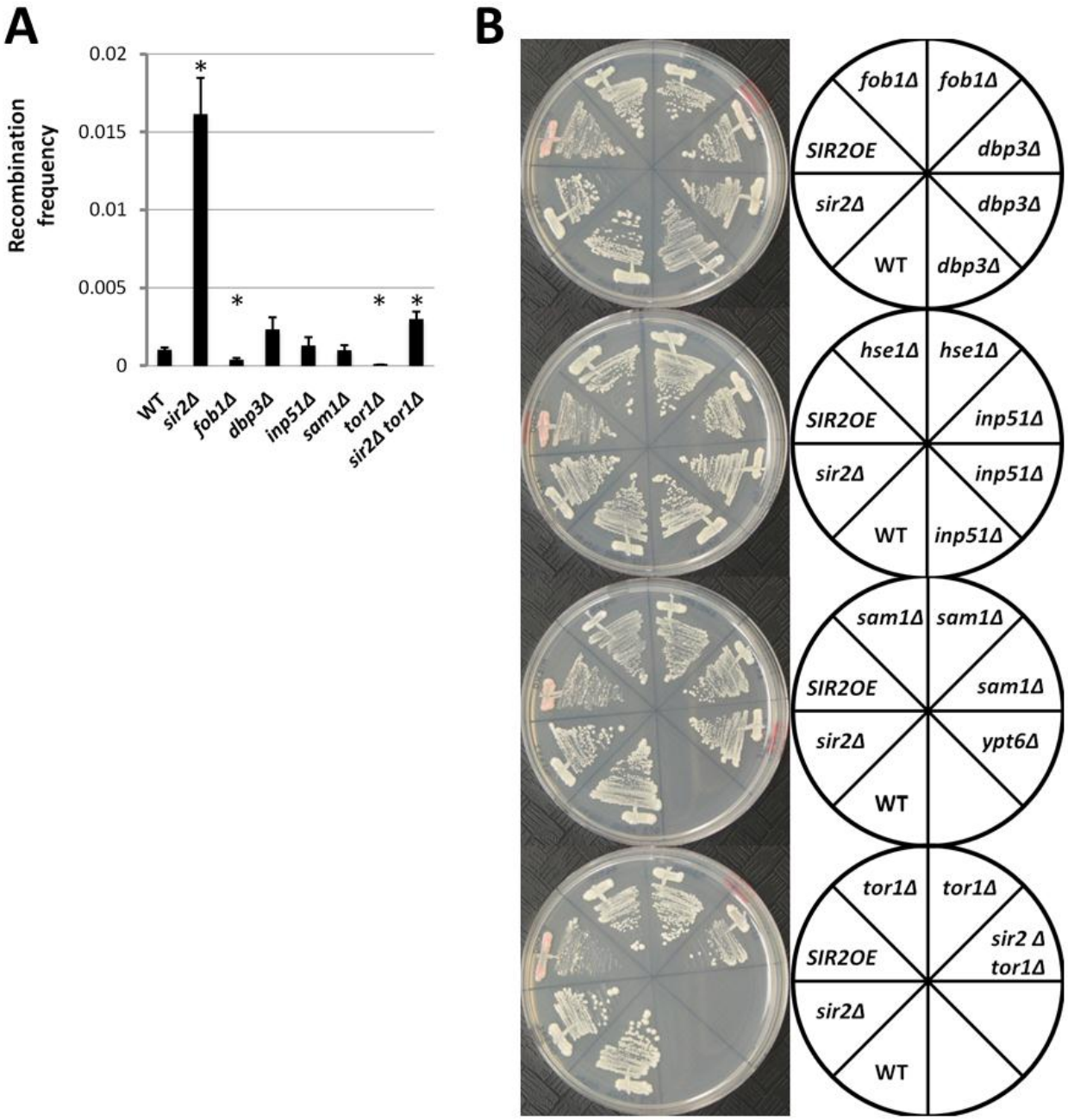


Figure 4.5. Five long-lived strains do not display increased rDNA silencing or decreased rDNA recombination. (A) Long-lived mutants do not display decreased recombination at the rDNA locus, with the exception of *tor1*Δ, which does so independently of *SIR2*. rDNA recombination is increased by deletion of *SIR2* and decreased by deletion of *FOB1* (*P < 0.05 compared to wild type). (B) Long-lived mutants do not display increased rDNA *ADE2* silencing, as indicated by red colony coloration. rDNA silencing is increased by overexpression of *SIR2*, and decreased by deletion of *SIR2*.

The discovery that the original worm and fly strains were not long-lived due to increased sirtuin activity may also help to clarify some aspects of our understanding of sirtuin biology. As noted in Chapter 1, the proposed mechanisms by which increased sirtuin activity influence longevity are different for different model organisms. The sirtuin aging pathways in worms and flies (Figure 1.3) were established, in part, using the overexpression strains discussed in this chapter. Assuming that robust long-lived strains with altered sirtuin activity can be reestablished, the current models describing these longevity pathways will have to be reevaluated in both organisms.

A second outcome of this study is to highlight the importance of improvements and standardization in techniques used to generate and examine novel strains in both worms and flies. The original sirtuin overexpression studies in both organisms were carried out using the standard techniques of the time. The worm study highlights the importance of thorough backcrossing following construction of transgenic animals and the use of secondary techniques to verify the underlying cause of interesting phenotypes, such as applying RNAi against an overexpressed gene to verify that the resulting phenotype can be reversed by specifically reducing the relevant gene expression. The result in flies highlights the importance of using a complete set of controls, including individual components of multi-part overexpression systems.

The yeast study primarily adds support for a model in which the majority of longevity interventions, including dietary restriction, increase replicative life span by a Sir2-independent mechanism, and deletion of *SIR2* limits replicative life span by increasing cellular damage, likely in the form of ERC accumulation. An alternative explanation for these data is that loss of *SIR2* alters aging such that molecular processes that do not limit replicative life span in wild type cells become limiting in *sir2*Δ cells. Sir2 has multiple functions, including repression of ERC formation (Kaeberlein et al., 1999), enhancing global rDNA stability and silencing (Gottlieb and Esposito, 1989; Smith and Boeke, 1997), promoting asymmetric inheritance of damaged proteins (Aguilaniu et al., 2003), and maintaining telomeric chromatin during aging (Dang et al., 2009). Our observation that only deletion of *FOB1* is sufficient to suppress the short replicative life span of *sir2*Δ cells suggests that (i) the primary replicative life span-limiting defect in *sir2*Δ cells is likely related to rDNA instability and (ii) none of the 32 deletions tested that slow aging in wild type cells are able to overcome this defect. One prior study reported that overexpression of Hsp104 could also suppress the short replicative life span of *sir2*Δ cells (Erjavec et al., 2007), raising the possibility that accumulation of damaged proteins in *sir2*Δ mother cells may also contribute to the reduced longevity.

Importantly, we do not propose that all of the 32 long-lived single gene deletion mutants examined here necessarily act via Sir2-independent mechanisms. For example, deletion of *SAS2*, a histone

acetyltransferase known to antagonize Sir2 effects on chromatin (Dang et al., 2009), extends wild type replicative life span but fails to extend the replicative life span of *sir2*Δ *fob1*Δ cells (Figure 4.4B). Thus, both functional and genetic evidence suggest that Sas2 likely acts in the same longevity pathway as Sir2.

This study also provides a clear demonstration of the challenges associated with interpreting longevity epistasis data discussed in Chapter 3. In particular, the failure of a longevity intervention to extend life span in a short-lived background may not be informative regarding the mechanism of life span extension in the wild type context. In the absence of strong evidence indicating that the life span shortening is caused by acceleration of the wild type aging process, caution is warranted when interpreting these types of data.

EXPERIMENTAL PROCEDURES

Genes and strains

A list of yeast genes and strains examined in this study are provided in Tables 4.4 and Table 4.5, respectively. All strains were derived from the parent strains of the haploid yeast ORF deletion collections (Winzeler et al., 1999), BY4742 (MATα *his3*Δ *leu2*Δ *lys2*Δ *ura3*Δ) and BY4741 (MAT**a** *his3*Δ *leu2*Δ *met15*Δ *ura3*Δ). The MATα and MAT**a** haploid ORF deletion collection and parental strains were obtained from Research Genetics. Single-deletion strains were either taken directly from one of the ORF deletion collection or constructed by replacing the entire ORF of the indicated gene with a selectable marker using standard PCR-base protocols as previously described (Sikorski and Hieter, 1989) and verified by PCR. Double mutant strains were produced by mating haploid mutants with the desired mutations, sporulating the resulting heterozygous diploid strain, and selecting haploids with the desired markers via tetrad analysis. *SIR2* double mutant strains were produced by replacing the entire *SIR2* ORF with *LEU2* in the background of interest. All genotypes were verified by PCR.

Worm strains used in this study are listed in Table 4.6 and were obtained from the *Caenorhabditis* Genetics Center, or from the laboratory of Dr. Leonard Guarente (Massachusetts Institute of Technology, Boston, MA, USA).

Table 4.4. Genes discussed in this chapter.

Mutant	Likely Pathway	Gene Description
afg3	-	Component, with Yta12p, of the mitochondrial inner membrane m-AAA protease that mediates degradation of misfolded or unassembled proteins and is also required for correct assembly of mitochondrial enzyme complexes
alg12	-	Alpha-1,6-mannosyltransferase localized to the ER; responsible for the addition of the alpha-1,6 mannose to dolichol-linked Man7GlcNAc2, acts in the dolichol pathway for N-glycosylation
dbp3	60S ribosome	Putative ATP-dependent RNA helicase of the DEAD-box family involved in ribosomal biogenesis
elp4	-	Subunit of Elongator complex, which is required for modification of wobble nucleosides in tRNA; required for Elongator structural integrity
fob1	SIR2/rDNA	Nucleolar protein that binds the rDNA replication fork barrier (RFB) site; required for replication fork blocking, recombinational hotspot activity, condensin recruitment to RFB and rDNA repeat segregation; related to retroviral integrases
gpa2	CR	Nucleotide binding alpha subunit of the heterotrimeric G protein that interacts with the receptor Gpr1p (PKA pathway), has signaling role in response to nutrients; green fluorescent protein (GFP)-fusion protein localizes to the cell periphery
hxk2	CR	Hexokinase isoenzyme 2 that catalyzes phosphorylation of glucose in the cytosol; predominant hexokinase during growth on glucose; functions in the nucleus to repress expression of HXK1 and GLK1 and to induce expression of its own gene
idh1	-	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase, which catalyzes the oxidation of isocitrate to alpha-ketoglutarate in the TCA cycle
idh2	-	Subunit of mitochondrial NAD(+)-dependent isocitrate dehydrogenase, which catalyzes the oxidation of isocitrate to alpha-ketoglutarate in the TCA cycle; phosphorylated
inp51	-	Phosphatidylinositol 4,5-bisphosphate 5-phosphatase, synaptojanin-like protein with an N-terminal Sac1 domain, plays a role in phosphatidylinositol 4,5-bisphosphate homeostasis and in endocytosis; null mutation confers cold-tolerant growth
msw1	-	Mitochondrial tryptophanyl-tRNA synthetase
mtc4	-	Protein of unknown function, required for normal growth rate at 15 degrees C; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm in a punctate pattern; mtc4 is synthetically sick with cdc13-1
rei1	60S ribosome	Cytoplasmic pre-60S factor; required for the correct recycling of shuttling factors Alb1, Arx1 and Tif6 at the end of the ribosomal large subunit biogenesis; involved in bud growth in the mitotic signaling network
rim1	-	Single-stranded DNA-binding protein essential for mitochondrial genome maintenance; involved in mitochondrial DNA replication
rpl7a	60S ribosome	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl7Bp and has similarity to E. coli L30 and rat L7 ribosomal proteins; contains a conserved C-terminal Nucleic acid Binding Domain (NDB2)

Table 4.4 (continued).

Mutant	Likely Pathway	Gene Description
rpl13a	60S ribosome	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl13Bp; not essential for viability; has similarity to rat L13 ribosomal protein
rpl21b	60S ribosome	Protein component of the large (60S) ribosomal subunit, nearly identical to Rpl21Ap and has similarity to rat L21 ribosomal protein
rpl22a	60S ribosome	Protein component of the large (60S) ribosomal subunit, has similarity to Rpl22Bp and to rat L22 ribosomal protein
rpl23a	60S ribosome	Protein component of the large (60S) ribosomal subunit, identical to Rpl23Bp and has similarity to E. coli L14 and rat L23 ribosomal proteins
rpl37b	60S ribosome	Protein component of the large (60S) ribosomal subunit, has similarity to Rpl37Ap and to rat L37 ribosomal protein
rpl43b	60S ribosome	Protein component of the large (60S) ribosomal subunit, identical to Rpl43Ap and has similarity to rat L37a ribosomal protein
rpp2b	60S ribosome	Ribosomal protein P2 beta, a component of the ribosomal stalk, which is involved in the interaction between translational elongation factors and the ribosome; regulates the accumulation of P1 (Rpp1Ap and Rpp1Bp) in the
sam1	-	S-adenosylmethionine synthetase, catalyzes transfer of the adenosyl group of ATP to the sulfur atom of methionine; one of two differentially regulated
sas2	SIR2/rDNA	Histone acetyltransferase (HAT) catalytic subunit of the SAS complex (Sas2p-Sas4p-Sas5p), which acetylates free histones and nucleosomes and regulates transcriptional silencing; member of the MYSTacetyltransferase family
sch9	CR	Protein kinase involved in transcriptional activation of osmostress-responsive genes; regulates G1 progression, cAPK activity, nitrogen activation of the FGM pathway; involved in life span regulation; homologous to mammalian Akt/PKB
sip2	-	One of three beta subunits of the Snf1 serine/threonine protein kinase complex involved in the response to glucose starvation; null mutants exhibit accelerated aging; N-myristoylprotein localized to the cytoplasm and the
sps1	-	Putative protein serine/threonine kinase expressed at the end of meiosis and localized to the prospore membrane, required for correct localization of enzymes involved in spore wall synthesis
tif2	-	Translation initiation factor eIF4A, identical to Tif1p; DEA(D/H)-box RNA helicase that couples ATPase activity to RNA binding and unwinding; forms a dumbbell structure of two compact domains connected by a linker; interacts
tis11	-	mRNA-binding protein expressed during iron starvation; binds to a sequence element in the 3'-untranslated regions of specific mRNAs to mediate their degradation; involved in iron homeostasis
tma19	-	Protein that associates with ribosomes; homolog of translationally controlled tumor protein; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm and relocates to the mitochondrial outer surface upon oxidative
tor1	CR	PIK-related protein kinase and rapamycin target; subunit of TORC1, a complex that controls growth in response to nutrients by regulating translation, transcription, ribosome biogenesis, nutrient transport and autophagy; involved

Table 4.4 (continued).

Mutant	Likely Pathway	Gene Description
ure2	-	Nitrogen catabolite repression transcriptional regulator that acts by inhibition of GLN3 transcription in good nitrogen source; has glutathione peroxidase activity and can mutate to acquire GST activity; altered form creates [URE3]
ybr238c	-	Mitochondrial membrane protein with similarity to Rmd9p; not required for respiratory growth but causes a synthetic respiratory defect in combination with rmd9 mutations; transcriptionally up-regulated by TOR; deletion increases

Worm Life Span Analysis

Life span experiments were conducted as previously described (Sutphin and Kaeberlein, 2009). Animals were maintained on solid nematode growth media (NGM) agar plates using standard techniques. Experiments were performed on NGM plates supplemented with 25 mg/mL ampicillin to prevent contamination and FUdR during adulthood to prevent reproduction. All experiments were conducted at 15°C except where otherwise noted. Statistical significance was determined using the Wilcoxon Rank Sum test.

Yeast Replicative Life Span Analysis

Replicative life span assays were performed as previously described (Kaeberlein et al., 2004; Steffen et al., 2009). YEP agar plates (1% yeast extract, 2% Bacto-peptone, 2% agar) containing 2% glucose were, except as otherwise indicated in the dietary restriction experiments where 0.05% glucose or alternative carbon sources were utilized. Statistical significance was determined using the Wilcoxon Rank Sum test.

rDNA Recombination and Silencing Assays

For the both assays, the W303AR strain background was used (Kaeberlein et al., 1999).W303AR has an *ADE2* marker integrated into the rDNA locus. Reduced activity of Ade2 results in the formation of a red pigment. For the recombination assay, cultures were inoculated into YPD and allowed to grow overnight at 30°C. Cells were then serially diluted to approximately 1000-3000 colonies per 15 cm YPD plate and allowed to grow for 2-3 days at 30°C before being placed in a 4°C for 2 days to allow red pigment to accumulate and for full color intensity to be observed. The recombination rate at the rDNA site containing *ADE2* is estimated by counting the frequency of half-red half-white colonies and comparing to the number of total colonies. For the silencing assay, the strains were streaked onto SC-Ade plates, incubated for 4 days at 30°C, followed by 2 days at 4°C. Pictures were then taken to assay for small pink colonies, which indicates silencing at the rDNA locus.

Table 4.5. Yeast strains used in this study.

Strain Name	Full Genotype
LF960 (BY4742)	his3 leu2 ura3 MET15 lys2 MATalpha
JD100	sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
KK102	sir2::URA3 his3 leu2 ura3 MET15 lys2 MATalpha
GS426	afg3::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
GS428	alg12::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
GS618	dbp3::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
JD56	elp4::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
JD104	fob1::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
KK144	fob1::LEU2 sir2::HIS3 his3 leu2 ura3 MET15 lys2 MATalpha
SK1095	fob1::HIS3 sir2::URA3 his3 leu2 ura3 MET15 lys2 MATalpha
KK201	gpa2::KanMX sir2::URA3 his3 leu2 ura3 MET15 lys2 MATalpha
KK75	hxk2::KanMX sir2:URA3 his3 leu2 ura3 MET15 lys2 MATalpha
GS629	idh1::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
KS186	idh2::KanMX sir2::URA3 his3 leu2 ura3 MET15 lys2 MATalpha
GS433	inp51::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
JD11	msw1::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
JD35	mtc4::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
KS290	rei1::LEU2 sir2::URA3 his3 leu2 ura3 MET15 lys2 MATalpha
JD15	rim1::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
JD23	rpl7a::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
JD19	rpl13a::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
JD20	rpl21b::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
JD145	rpl22a::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
JD55	rpl23a::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
JD22	rpl37b::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
JD94	rpl43b::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
JD96	rpp2b::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
GS591	sam1::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
KS68	sch9::HIS3 his3 leu2 ura3 MET15 lys2 MATalpha
KS283	sch9::HIS3 sir2::URA3 his3 leu2 ura3 MET15 lys2 MATalpha
JD29	sip2::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
JD97	sps1::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
GS624	tif2::KanMX sir2::LEU2 his3 leu2 ura3 met15 lys2 MATalpha
JD31	tma19::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
KS184	tor1::KanMX sir2::URA3 his3 leu2 ura3 MET15 lys2 MATalpha
JD32	ure2::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
JD146	ybr238c::KanMX sir2::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
GS2391	sir2::LEU2 fob1::URA3 dbp3::KanMX his3 leu2 ura3 MET15 lys2 MATalpha

Table 4.5 (continued).

Strain Name	Full Genotype
KK214	gpa2::KanMX sir2::HIS3 fob1::LEU2 MET15 lys2 MATalpha
KK156	hxk2::KanMX sir2::URA3 fob1::KanMX MET15 lys2 MATalpha
KS292	rei1::URA3 sir2::HIS3 fob1::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
GS2313	rpl6b::KanMX sir2::LEU2 fob1::URA3 his3 leu2 ura3 met15 lys2 MATalpha
GS406	rpl19a::KanMX sir2::LEU2 fob1::URA3 his3 leu2 ura3 met15 lys2 MATalpha
JO202	rpl31a::URA3 sir2::HIS3 fob1::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
KS189	rpl31a::KanMX sir2::URA3 fob1::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
KK206	sch9::URA3 sir2::HIS3 fob1::LEU2 his3 leu2 ura3 MET15 lys2 MATalpha
JO204	fob1::URA3 his3 leu2 ura3 MET15 lys2 MATalpha
KK163	gpa2::kanMX fob1::LEU2 his3 leu2 ura3 met15 lys2 MATalpha
KS5	fob1::LEU2 hxk2::URA3 his3 leu2 ura3 met15 lys2 MATalpha
KS125	rpl31a::KanMX fob1::LEU2 his3 leu2 ura3 met15 lys2 MATalpha
JO206	sch9::HIS3 fob1::URA3 his3 leu2 ura3 met15 lys2 MATalpha
JO211	RDN::ADE2 ade2 can1 his3 leu2 trp1 ura3 MATa
JD107	sir2::LEU2 RDN::ADE2 ade2 can1 his3 leu2 trp1 ura3 MATa
LF802	fob1::URA3 RDN::ADE2 ade2 can1 his3 leu2 trp1 ura3 MATa
GS1742	dbp3::KanMX RDN::ADE2 ade2 can1 his3 leu2 trp1 ura3 LYS2 MATa
GS1246	inp51::HIS3 RDN::ADE2 ade2 can1 his3 leu2 trp1 ura3 MATa
GS701	sam1::KanMX RDN::ADE2 ade2 can1 his3 leu2 trp1 ura3 MATa
DH485	tor1::URA3 RDN::ADE2 ade2 can1 his3 leu2 trp1 ura3 MATa
JO295	sir2::HIS3 tor1::URA3 RDN::ADE2 ade2 can1 his3 leu2 trp1 ura3 MATa
GS1245	ypt6::KanMX RDN::ADE2 ade2 can1 his3 leu2 trp1 ura3 MATa

Table 4.6. Worm strains used in this study.

Strain	Outcrossed	Genotype
N2		wild type
CL2099		dvIs22[rol-6(su1006)]
LG100		geIn3[sir-2.1 rol-6(su1006)
MK287	6x	geIn3[sir-2.1 rol-6(su1006)
MK288	6x	geIn3[sir-2.1 rol-6(su1006)
MK289	6x	geIn3[sir-2.1 rol-6(su1006)
MV391	8x	geIn3[sir-2.1(+),rol-6]
MV392	8x	geIn3[sir-2.1(+),rol-6]
MV393	8x	geIn3[sir-2.1(+),rol-6]
MV394	8x	geIn3[sir-2.1(+),rol-6]
MV395	8x	geIs101[rol-6]
MV396	8x	geIs101[rol-6]
MV397	8x	geIs101[rol-6]
MV398	8x	geIs101[rol-6]

Chapter 5: The Genetics of Life Span Extension using Caffeine in *C. elegans*

CHAPTER SUMMARY

The longevity of an organism is influenced by both genetic and environmental factors. With respect to internal factors, a significant effort is being made to identify novel pharmacological agents that extend life span by targeting genetic pathways with a defined role in the aging process. On the external side, the molecular mechanisms responsible for the positive influence of environmental interventions, such as dietary restriction and mild stress, are widely being explored. The environment experienced by humans in modern societies already contains countless compounds that may influence longevity. Understanding the role played by the compounds with the most significant effect on the aging process will be critical for predicting and interpreting the outcome of introducing new interventions.

Caffeine is the most widely used psychoactive drug worldwide. Prior studies in flies, worms, and mice indicate that caffeine may positively impact age-associated neurodegenerative pathology, such as that observed in Alzheimer's disease. In the experiments outlined in this chapter, we observe that caffeine is capable of extending life span and improving health span in *Caenorhabditis elegans,* a finding that is in agreement with a recently published screen looking for FDA-approved compounds capable of extending worm life span. We also find that caffeine delays pathology in a nematode model of polyglutamine disease. Life span extension using caffeine shows clear epistatic interaction with two known longevity interventions: dietary restriction and reduced insulin signaling. This chapter details our findings related to the use of caffeine to influence aging and age-related pathology in worms.

INTRODUCTION

Numerous interventions have been identified that extend life span across an evolutionarily diverse range of organisms. These include external (environmental) interventions, such as dietary restriction, heat shock, or treatment with a pharmacological agent, as well as internal (genetic) interventions, such as reduced TOR signaling or reduced IIS. In most cases, studies identifying these interventions are carried out using genetically homogenous population in a controlled, low risk, and pathogen-free environment. Interventions that are successful under laboratory conditions are beginning to be introduced into human clinical trials. Thus, it is important to understand how the artificial nature of the populations and environments in laboratory setting may impact the outcomes of specific interventions in more variable environments. In terms of genetics, efforts are underway to understand the effects of dietary restriction in genetically heterogeneous populations. Early evidence indicates that the benefits observed in lab populations might not be universally realized by all members of genetically diverse populations (Liao et al., 2010; Liao et al., 2011; Rikke et al., 2010; Schleit et al., submitted). From an environmental perspective, human populations are exposed to a wide range of diets, climates, and pharmacological agents that are not present in the laboratory setting. Understanding the impact of these factors on longevity and age-associated disease will be important to predicting unintended effects that might arise from introducing novel interventions.

Caffeine is the most widely used psychoactive substance worldwide. Average consumption in the United States is 168 mg/person/day (equivalent to 1 to 2 cups of Starbucks® coffee), and reaches 414 mg/person/day in the Netherlands (Fredholm et al., 1999). Chronic, moderate consumption of caffeine has been linked with reduced risk of age-associated neurodegenerative disorders in humans, including dementia (Eskelinen and Kivipelto, 2010), Alzheimer's disease (Eskelinen and Kivipelto, 2010; Lindsay et al., 2002; Maia and de Mendonca, 2002), and Parkinson's disease (Ascherio et al., 2001; Benedetti et al., 2000; Costa et al., 2010; Ross et al., 2000). In addition, studies performed on elderly human populations have correlated habitual caffeine consumption with reduced mortality (Fortes et al., 2000; Paganini-Hill et al., 2007) and improvements in various measures of health span, including reduced cognitive decline (Santos et al., 2010b; van Gelder et al., 2007), improved memory (Hameleers et al., 2000; Johnson-Kozlow et al., 2002; Ritchie et al., 2007), and increased motor speed (Hameleers et al., 2000). Other studies failed to find similar correlations, though these differences are attributed to wide variation in methodology (Rosso et al., 2008; Santos et al., 2010a).

The potential for caffeine consumption to reduce neuropathology and impart age-associated neuroprotection has motivated numerous studies using animal models. Generally, research using rodent models of neurodegenerative disease have demonstrated that caffeine administration successfully

alleviates degenerative symptoms and pathology (review by Cunha and Agostinho (2010)). Acute treatment with caffeine prevents avoidance memory impairment in a rat model of Parkinson's disease (Gevaerd et al., 2001), while chronic caffeine treatment prevents cognitive defects in mice expressing toxic forms of amyloid beta, a common rodent model of Alzheimer's disease (Arendash et al., 2006). Furthermore, acute caffeine treatment reduces amyloid beta levels in the plasma and brain interstitial fluid (Cao et al., 2009) and delays memory defects following intracerebral administration of amyloid beta (Canas et al., 2009; Cunha et al., 2008; Dall'Igna et al., 2007). One study using these mice suggests that caffeine treatment may even restore performance in individuals that are already displaying memory deficits (Arendash et al., 2009). Neurodegenerative disorders aside, caffeine treatment also prevents memory impairment in rodent models of various other diseases and conditions, including chronic stress, child convulsions, type 1 and type 2 diabetes, attention deficit and hyperactivity disorder, heavy alcohol consumption, and sleep deprivation (Cunha and Agostinho, 2010). Importantly, caffeine improves age-associated memory impairment in both mice and rats (Costa et al., 2008; Prediger et al., 2005).

Recently, several groups have started investigating the physiological effects of caffeine in non-mammalian models, including worms and yeast. A common strategy to model Alzheimer's disease in *C. elegans* involves transgenic expression of a toxic form of amyloid beta in the body wall muscle, resulting in a time-dependent paralysis phenotype (Link et al., 2003). Dostal et al. (2010) found that both caffeine and non-caffeine components of coffee were capable of delaying paralysis in this model. During the course of the present study, another group identified caffeine in a screen for FDA-approved compounds capable of extending worm life span (Lublin et al., 2011). In yeast, caffeine increases chronological life span, likely through a mechanism related to TOR signaling (Wanke et al., 2008).

Given the high consumption rates of caffeine for people living in the developed world, understanding the impact of caffeine on aging and age-related disease will be important as aging interventions move from the lab into use in clinical trials and the broader population. In this study, we characterize the effect of caffeine on life span and health span in *C. elegans*, and identify clear epistatic interactions between caffeine and both dietary restriction and reduced IIS.

RESULTS

Caffeine Extends Worm Life Span in a Temperature-Dependent Manner

In order to determine whether caffeine impacts longevity, life span was measured for worms maintained throughout their adult life on NGM plates containing caffeine. In previous work on the *C. elegans* hypoxia pathway, we found temperature-dependent effects on life span resulting from reduced expression of the hypoxia inducible factor, *hif-1* (Leiser et al., 2011). Specifically, *hif-1* knockdown extended life span at 20°C and 25°C, but not at 15°C. To examine the possibility that caffeine might display a similar dependence on temperature, we measured life span for worms at 15°C, 20°C, and 25°C in the presence of 0 mM, 5 mM, or 7.5 mM caffeine. Caffeine concentrations were selected based on previous studies in yeast and worms (Dostal et al., 2010; Kuranda et al., 2006; Sun et al., 1994; Wanke et al., 2008). In contrast to *hif-1* knockdown, caffeine extended life span at 15°C and 20°C, and slightly shortened life span at 25°C (Figure 5.1; Table 5.1). These data are in agreement with the recently published FDA-approved drug screen that reported 29.4% median life span extension resulting from 0.1% (5.15 mM) caffeine at 20°C (Lublin et al., 2011).

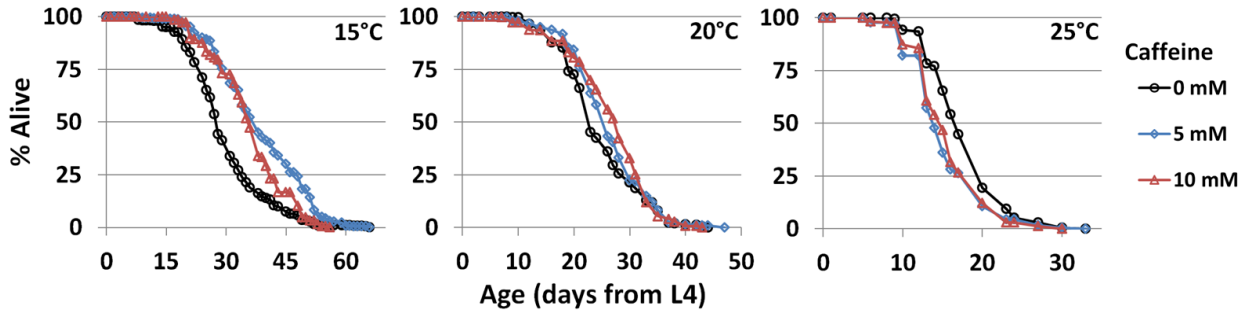


Figure 5.1. Caffeine treatment results in a temperature-dependent life span extension in worms. Maintenance of worms in the presence of 5 mM or 10 mM caffeine throughout adult life increases life span at 15°C and 20°C, but not at 25°C.

Table 5.1. Summary of life span data presented in this chapter.

Intervention					Control							
Name	Temp.	Median Life Span	Mean Life Span	N	Name	Temp.	Median Life Span	Mean Life Span	N	Rank Sum P-Value	Change in Mean Life	Effect on Life Span
5 mM caffeine	15°C	38.0	38.3	695	0 mM caffeine	15°C	28.0	29.6	765	3.45E-53	29.2%	increased
7.5 mM caffeine	15°C	34.0	34.2	186	0 mM caffeine	15°C	28.0	29.6	765	8.36E-10	15.2%	increased
10 mM caffeine	15°C	42.0	40.8	392	0 mM caffeine	15°C	28.0	29.6	765	2.00E-61	37.7%	increased
20 mM caffeine	15°C	34.0	34.7	227	0 mM caffeine	15°C	28.0	29.6	765	5.26E-14	17.2%	increased
50 mM caffeine	15°C	17.0	16.9	188	0 mM caffeine	15°C	28.0	29.6	765	5.88E-54	-43.0%	decreased
70 mM caffeine	15°C	3.0	4.9	57	0 mM caffeine	15°C	28.0	29.6	765	3.72E-36	-83.4%	decreased
100 mM caffeine	15°C	3.0	3.0	83	0 mM caffeine	15°C	28.0	29.6	765	0.00E+00	-89.9%	decreased
0.5 mM caffeine	20°C	26.0	26.8	85	0 mM caffeine	20°C	23.0	24.8	468	0.0017	8.1%	increased
2.5 mM caffeine	20°C	28.0	28.0	96	0 mM caffeine	20°C	23.0	24.8	468	3.28E-07	12.9%	increased
5 mM caffeine	20°C	26.0	26.3	414	0 mM caffeine	20°C	23.0	24.8	468	0.0001	6.0%	increased
7.5 mM caffeine	20°C	28.0	26.7	243	0 mM caffeine	20°C	23.0	24.8	468	5.64E-06	7.7%	increased
10 mM caffeine	20°C	26.0	25.4	131	0 mM caffeine	20°C	23.0	24.8	468	0.1010	2.4%	no effect
30 mM caffeine	20°C	19.0	18.6	92	0 mM caffeine	20°C	23.0	24.8	468	2.18E-17	-25.0%	decreased
5 mM caffeine	25°C	14.0	15.4	178	0 mM caffeine	25°C	17.0	17.8	170	2.00E-07	-13.5%	decreased
7.5 mM caffeine	25°C	15.0	15.8	165	0 mM caffeine	25°C	17.0	17.8	170	2.50E-05	-11.2%	decreased
0 mM caffeine BD	15°C	45.0	41.3	83	0 mM caffeine	15°C	28.0	29.6	765	4.08E-15	39.3%	increased
5 mM caffeine BD	15°C	42.0	41.3	81	5 mM caffeine	15°C	38.0	38.3	695	0.0281	8.0%	increased
10 mM caffeine BD	15°C	42.0	40.0	73	10 mM caffeine	15°C	42.0	40.8	392	0.7847	-2.0%	no effect
20 mM caffeine BD	15°C	38.0	37.9	58	20 mM caffeine	15°C	34.0	34.7	227	0.0208	9.2%	increased
50 mM caffeine BD	15°C	14.0	14.7	95	50 mM caffeine	15°C	17.0	16.9	188	0.0085	-13.0%	decreased
5 mM caffeine BD	15°C	42.0	41.3	81	0 mM caffeine BD	15°C	45.0	41.3	83	5.77E-01	0.1%	no effect
10 mM caffeine BD	15°C	42.0	40.0	73	0 mM caffeine BD	15°C	45.0	41.3	83	0.4409	-3.2%	no effect
20 mM caffeine BD	15°C	38.0	37.9	58	0 mM caffeine BD	15°C	45.0	41.3	83	0.0207	-8.2%	decreased
50 mM caffeine BD	15°C	14.0	14.7	95	0 mM caffeine BD	15°C	45.0	41.3	83	8.59E-27	-64.4%	decreased
0 mM caffeine BD	20°C	23.0	24.4	61	0 mM caffeine	20°C	21.0	21.3	70	0.0001	14.6%	increased
5 mM caffeine BD	20°C	26.0	25.6	73	5 mM caffeine	20°C	26.0	25.3	92	0.5700	1.2%	no effect
10 mM caffeine BD	20°C	23.0	24.2	66	10 mM caffeine	20°C	23.0	23.5	72	0.1700	3.0%	no effect
30 mM caffeine BD	20°C	16.0	16.0	85	30 mM caffeine	20°C	19.0	18.6	92	1.20E-05	-14.0%	decreased
5 mM caffeine BD	20°C	26.0	25.6	73	0 mM caffeine BD	20°C	23.0	24.4	61	0.1010	4.9%	no effect
10 mM caffeine BD	20°C	23.0	24.2	66	0 mM caffeine BD	20°C	23.0	24.4	61	0.9980	-0.8%	no effect
30 mM caffeine BD	20°C	16.0	16.0	85	0 mM caffeine BD	20°C	23.0	24.4	61	7.40E-19	-34.4%	decreased
EV(RNAi) 5 mM caffeine	15°C	35.0	37.8	113	*EV(RNAi)* 0 mM caffeine	15°C	31.0	31.5	98	9.83E-10	20.0%	increased
EV(RNAi) 10 mM caffeine	15°C	38.0	42.0	117	*EV(RNAi)* 0 mM caffeine	15°C	31.0	31.5	98	6.32E-17	33.3%	increased
daf-2(RNAi) 0 mM caffeine	15°C	52.0	53.0	95	*EV(RNAi)* 0 mM caffeine	15°C	31.0	31.5	98	4.67E-26	68.3%	increased
daf-2(RNAi) 5 mM caffeine	15°C	52.0	52.8	123	*EV(RNAi)* 5 mM caffeine	15°C	35.0	37.8	113	5.55E-21	39.7%	increased
daf-2(RNAi) 10 mM caffeine	15°C	52.0	53.8	125	*EV(RNAi)* 10 mM caffeine	15°C	38.0	42.0	117	7.67E-15	28.1%	increased
daf-2(RNAi) 5 mM caffeine	15°C	52.0	52.8	123	*daf-2(RNAi)* 0 mM caffeine	15°C	52.0	53.0	95	0.7550	-0.4%	no effect
daf-2(RNAi) 10 mM caffeine	15°C	52.0	53.8	125	*daf-2(RNAi)* 0 mM caffeine	15°C	52.0	53.0	95	0.7760	1.5%	no effect
daf-16(RNAi) 0 mM caffeine	15°C	28.0	29.0	55	*EV(RNAi)* 0 mM caffeine	15°C	31.0	31.5	98	0.0040	-7.9%	decreased
daf-16(RNAi) 5 mM caffeine	15°C	24.0	26.9	120	*EV(RNAi)* 5 mM caffeine	15°C	35.0	37.8	113	9.73E-24	-28.8%	decreased
daf-16(RNAi) 10 mM caffeine	15°C	28.0	30.3	110	*EV(RNAi)* 10 mM caffeine	15°C	38.0	42.0	117	2.43E-20	-27.9%	decreased
daf-16(RNAi) 5 mM caffeine	15°C	24.0	26.9	120	*daf-16(RNAi)* 0 mM caffeine	15°C	28.0	29.0	55	0.0128	-7.2%	decreased
daf-16(RNAi) 10 mM caffeine	15°C	28.0	30.3	110	*daf-16(RNAi)* 0 mM caffeine	15°C	28.0	29.0	55	0.7530	4.5%	no effect
5 mM caffeine	15°C	42.0	43.4	220	0 mM caffeine	15°C	29.0	32.8	231	1.78E-29	32.4%	increased
10 mM caffeine	15°C	43.0	43.0	232	0 mM caffeine	15°C	29.0	32.8	231	7.03E-28	31.0%	increased
daf-16 0 mM caffeine	15°C	32.0	34.6	141	0 mM caffeine	15°C	29.0	32.8	231	0.0002	5.4%	increased
daf-16 5 mM caffeine	15°C	36.0	36.7	117	5 mM caffeine	15°C	42.0	43.4	220	9.64E-10	-15.5%	decreased
daf-16 10 mM caffeine	15°C	32.0	33.0	149	10 mM caffeine	15°C	43.0	43.0	232	1.61E-25	-23.1%	decreased
daf-16 5 mM caffeine	15°C	36.0	36.7	117	*daf-16* 0 mM caffeine	15°C	32.0	34.6	141	2.50E-05	6.1%	increased
daf-16 10 mM caffeine	15°C	32.0	33.0	149	*daf-16* 0 mM caffeine	15°C	32.0	34.6	141	0.7879	-4.4%	no effect
cep-1 0 mM caffeine	15°C	46.0	45.6	103	0 mM caffeine	15°C	29.0	32.8	231	8.74E-27	39.0%	increased
cep-1 5 mM caffeine	15°C	50.0	53.4	95	5 mM caffeine	15°C	42.0	43.4	220	4.46E-16	22.8%	increased
cep-1 10 mM caffeine	15°C	46.0	48.8	91	10 mM caffeine	15°C	43.0	43.0	232	3.88E-07	13.5%	increased
cep-1 5 mM caffeine	15°C	50.0	53.4	95	*cep-1* 0 mM caffeine	15°C	46.0	45.6	103	2.83E-10	17.0%	increased
cep-1 10 mM caffeine	15°C	46.0	48.8	91	*cep-1* 0 mM caffeine	15°C	46.0	45.6	103	0.0178	7.0%	increased
sir-2.1 0 mM caffeine	15°C	43.0	41.5	95	0 mM caffeine	15°C	29.0	32.8	231	2.55E-16	26.3%	increased
sir-2.1 5 mM caffeine	15°C	46.0	46.0	82	5 mM caffeine	15°C	42.0	43.4	220	0.0082	5.9%	increased
sir-2.1 10 mM caffeine	15°C	43.0	44.6	71	10 mM caffeine	15°C	43.0	43.0	232	0.1435	3.8%	no effect
sir-2.1 5 mM caffeine	15°C	46.0	46.0	82	*sir-2.1* 0 mM caffeine	15°C	43.0	41.5	95	0.0003	10.9%	increased
sir-2.1 10 mM caffeine	15°C	43.0	44.6	71	*sir-2.1* 0 mM caffeine	15°C	43.0	41.5	95	0.0120	7.6%	increased
hif-1 0 mM caffeine	15°C	31.0	31.1	190	0 mM caffeine	15°C	29.0	32.8	231	0.2228	-5.4%	no effect
hif-1 5 mM caffeine	15°C	38.0	37.0	154	5 mM caffeine	15°C	42.0	43.4	220	1.03E-09	-14.9%	decreased
hif-1 10 mM caffeine	15°C	36.0	37.0	128	10 mM caffeine	15°C	43.0	43.0	232	7.48E-09	-13.9%	decreased
hif-1 5 mM caffeine	15°C	38.0	37.0	154	*hif-1* 0 mM caffeine	15°C	31.0	31.1	190	9.41E-16	19.0%	increased
hif-1 10 mM caffeine	15°C	36.0	37.0	128	*hif-1* 0 mM caffeine	15°C	31.0	31.1	190	1.04E-14	19.3%	increased
vhl-1 0 mM caffeine	15°C	42.0	41.8	88	0 mM caffeine	15°C	29.0	32.8	231	2.12E-18	27.3%	increased
vhl-1 5 mM caffeine	15°C	47.0	42.9	48	5 mM caffeine	15°C	42.0	43.4	220	0.7791	-1.2%	no effect
vhl-1 10 mM caffeine	15°C	42.0	40.9	68	10 mM caffeine	15°C	43.0	43.0	232	0.0946	-4.9%	no effect
vhl-1 5 mM caffeine	15°C	47.0	42.9	48	*vhl-1* 0 mM caffeine	15°C	42.0	41.8	88	0.1288	2.8%	no effect
vhl-1 10 mM caffeine	15°C	42.0	40.9	68	*vhl-1* 0 mM caffeine	15°C	42.0	41.8	88	0.4881	-2.1%	no effect

Next, we conducted a caffeine dose response with respect to life span in order to determine the optimal temperature and caffeine concentration for increasing longevity. Life span extension was observed for caffeine concentrations ranging from 0.5 mM to 7.5 mM at 20°C, and from 5 mM to 20 mM at 15°C (Figure 5.2; Table 5.1). The highest mean life span extension (37.7%) was achieved at 15°C use 10 mM caffeine (Figure 5.1A,C; Table 5.1). Interestingly, the maximal life span extension (12.9%) occurred at a lower caffeine concentration (2.5 mM) at 20°C (Figure 5.2B,C; Table 5.1), indicating a shift in caffeine sensitivity with temperature. Caffeine reduced life span at concentrations of 30 mM or greater (Figure 5.2; Table 5.1).

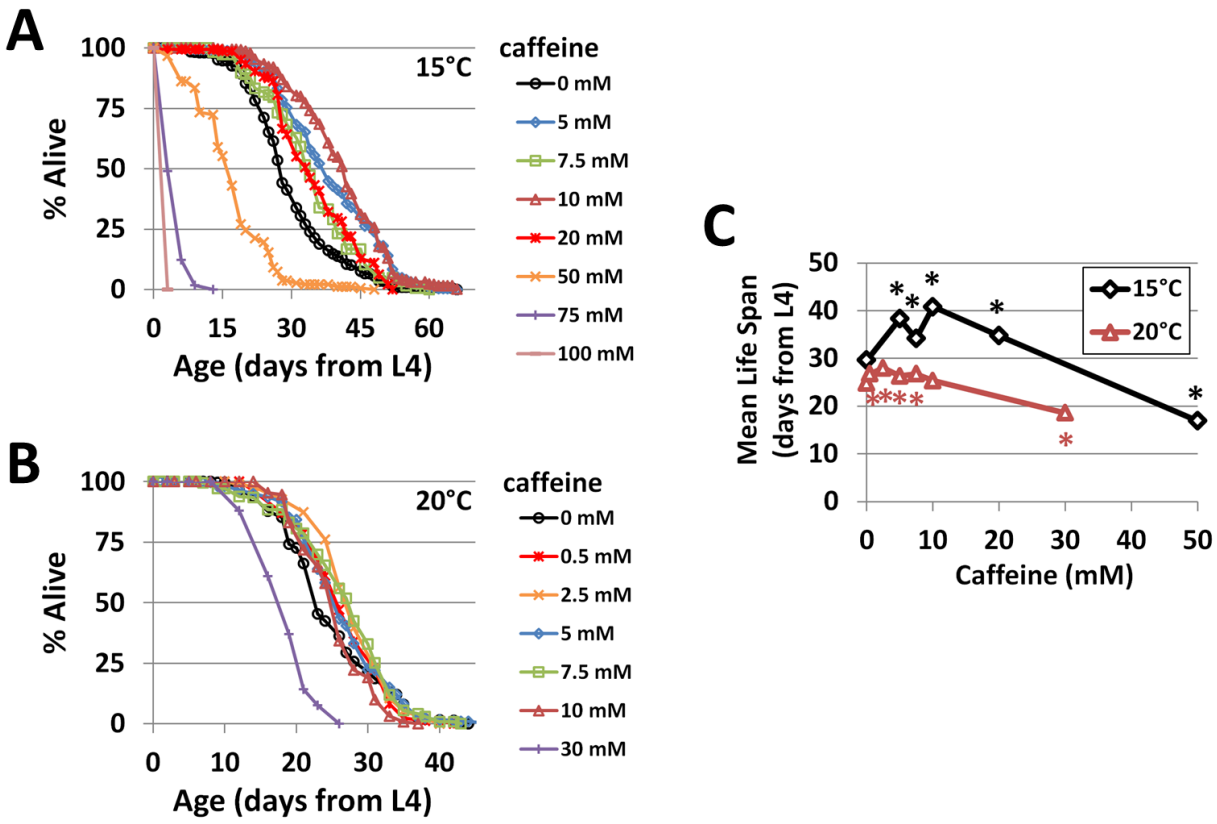


Figure 5.2.Caffeine extends worm life span at 15°C and 20°C. (A) Caffeine concentrations in the 5 to 20 mM range increase life span at 15°C. (B) Caffeine concentrations in the 0.5 to 7.5 mM range increase life span at 20°C. (C) Caffeine dose response curves reveal optimal concentrations for increased life span. * $P < 0.05$ vs. 0 mM at the same temperature.

As detailed in Chapter 1, a number of interventions have been identified that increase life span in multiple evolutionarily divergent species. One previous report found that caffeine extends chronological life span in yeast (Wanke et al., 2008). In order to determine whether caffeine is generally beneficial in this organism, we examined the effect of caffeine on replicative life span. In contrast to both worm life span and yeast chronological life span, yeast replicative life span was either unaffected or decreased by all concentrations of caffeine tested (Figure 5.3). Replicative life span was measured in both wild type yeast and a *sir2*Δ *fob1*Δ strain, which has a replicative life span that is not significantly different from wild type under standard conditions but displays a greater increase in life span in response to dietary restriction (Kaeberlein et al., 2004) (Figure 5.3).

Caffeine Prolongs Mobility and Delays Polyglutamine-Associated Pathology

An intervention that increases longevity does not necessarily extend health span, the time period over which that organism remains healthy. To assess the effect of caffeine on *C. elegans* health span, we examined two types of movement throughout the life span of worms exposed to either 0 mM or 5 mM caffeine. Caffeine delayed age-associated decline in both the thrashing rate in liquid and the rate of travel on solid media in the presence of a bacterial food source (Figure 5.4).

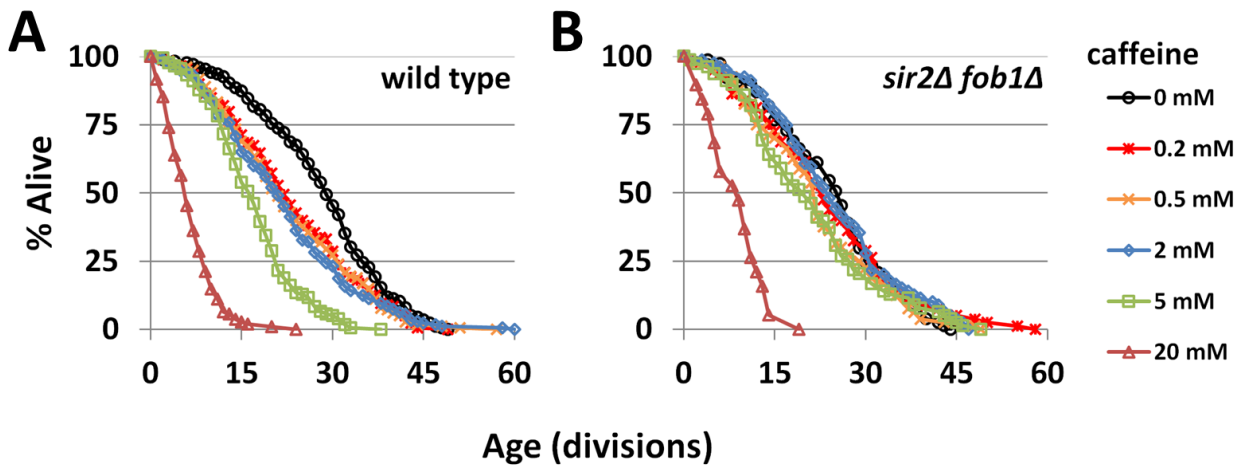


Figure 5.3. Caffeine does not extend replicative life span in yeast. Caffeine concentrations ranging from 0.2 mM to 20 mM failed to extend replicative life span in (A) wild type or (B) *sir2*Δ *fob1*Δ yeast.

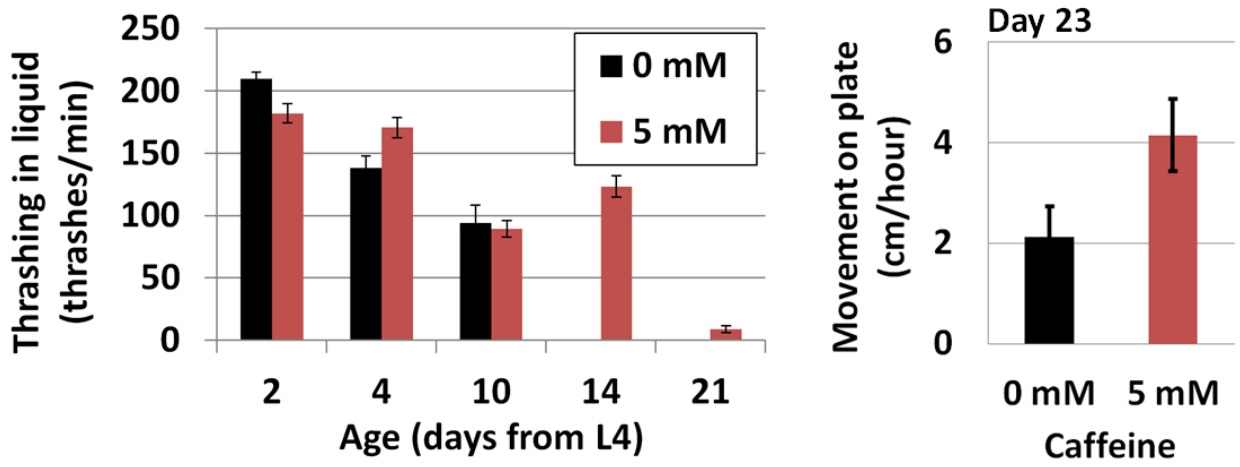


Figure 5.4. Caffeine delays age-associated decline in mobility at 15°C. Worms in the presence of 5 mM caffeine displayed increased thrashing (A) and travel rate (B) compared to untreated control worms.

Two previous studies found that caffeine can prolong survival (Lublin et al., 2011) and delay the onset of paralysis (Dostal et al., 2010) in a worm model of Alzheimer's disease in which amyloid beta is express in the body wall muscles (Link, 1995). Dostal et al. (2010) used an inducible amyloid beta construct that resulted in a severe proteotoxic pathology causing untreated worms to become paralyzed over the course of 20 to 30 hours at 20°C. In order to examine the effect of caffeine in a similar model of age-associated proteotoxicity, we examined paralysis in worms expressing an aggregate-prone, YFP-tagged polyglutamine chain (Q35::YFP) in the body wall muscles. Expanded polyglutamine tracts are a known causative factor in Huntington's disease and related neurodegenerative disorders in humans, though the underlying disease mechanism is not known (Bonini and La Spada, 2005). Q35::YFP worms display a similar paralysis phenotype to the amyloid beta worms, but pathology occurs more slowly, with the majority of worms becoming paralyzed over the course of approximately 3 weeks. Similar to the results of the amyloid beta study, we found that caffeine delayed the onset of paralysis in Q35 worms (Figure 5.5).

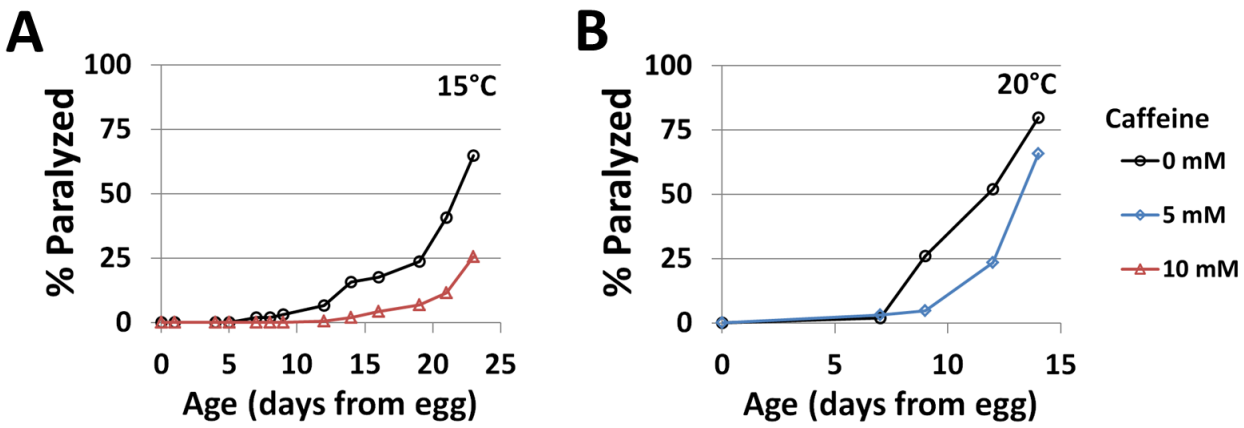


Figure 5.5. Caffeine delays age-associated paralysis in worm polyglutamine toxicity model. Caffeine treatment delayed the onset of paralysis in worms expressing Q35::GFP in their body wall muscles at (A) 15°C and (B) 20°C.

Life Span Extension by Bacterial Deprivation and Exposure to Caffeine are Non-Additive

Dietary restriction is the most widely studied intervention capable of increasing life span. A hallmark goal of dietary restriction research is to discover dietary restriction mimetics, pharmacological compounds capable of reproducing the beneficial effects of dietary restriction without a reduction in food intake. A dietary restriction mimetic should activate the same mechanism of action as dietary restriction, and thus life span extension should be non-additive when the mimetic is applied to dietary restricted animals.

In order to investigate the potential for caffeine to act as a dietary restriction mimetic, we measured life span for worms with combined exposure to both caffeine and bacterial deprivation, a form of dietary restriction in which the bacterial food source is completely removed after the worms have reached early adulthood (Kaeberlein et al., 2006b). Caffeine extended life span of *ad libitum* fed worms, but not worms subjected to bacterial deprivation, at both 15°C and 20°C (Figure 5.6A,B; Table 1). Conversely, bacterial deprivation slightly increased life span of worms treated with either 5 mM or 20 mM caffeine, but to a far smaller extent than untreated worms (Table 1). When worms were subjected to caffeine concentrations of 30 mM or greater, bacterial deprivation had a detrimental effect on life span (Figure 5.6A,B; Table 1). The abrogation of life span extension resulting from bacterial deprivation in the presence of caffeine is consistent with the idea that caffeine and dietary restriction influence aging via similar downstream mechanisms. Importantly, caffeine does not mimic dietary restriction by limiting food intake via reduced pharyngeal pumping (Figure 5.6C), as is the case with long-lived *eat-2* mutants (Avery, 1993; Lakowski and Hekimi, 1998).

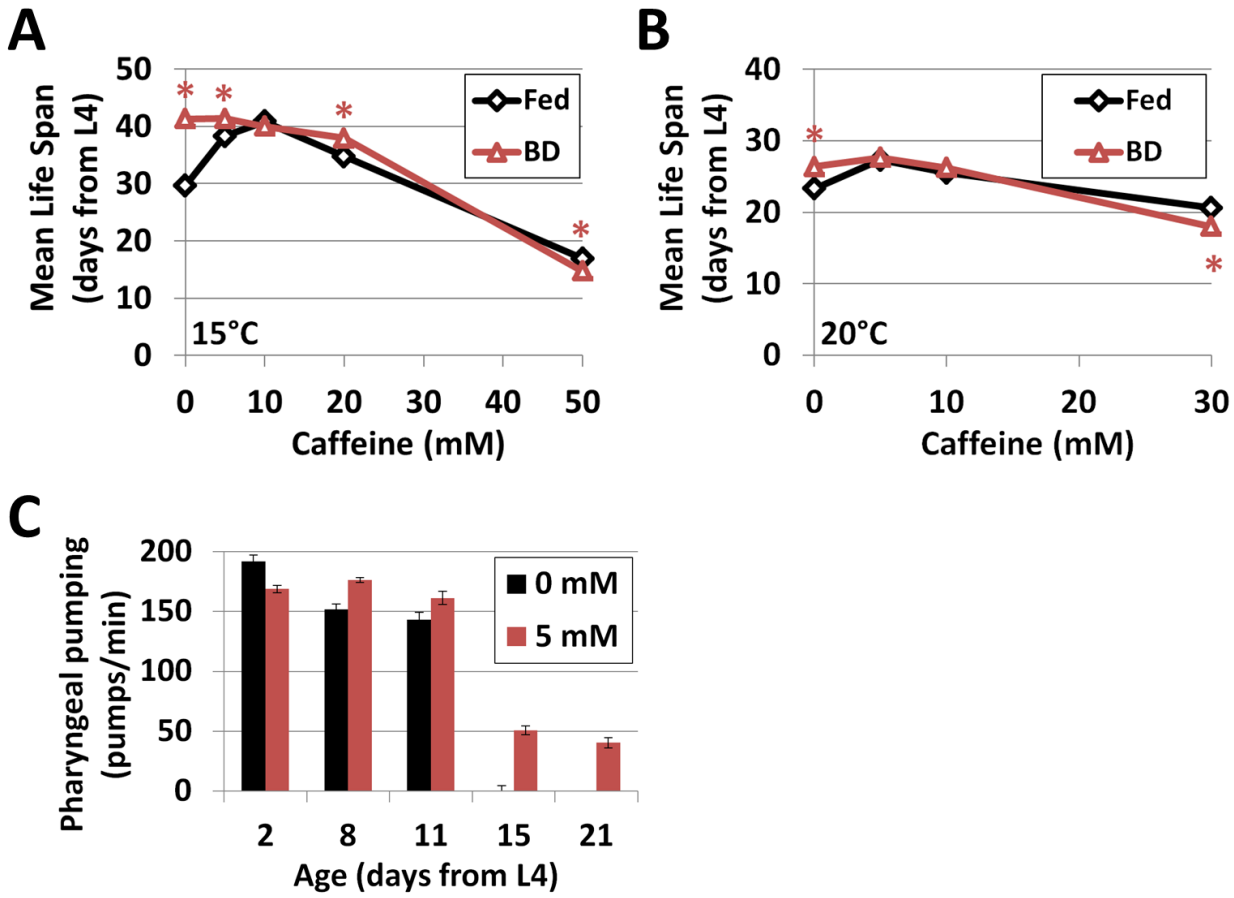


Figure 5.6. Caffeine increases life span in a manner that is non-additive with dietary restriction. Life span extension from caffeine and bacterial deprivation are non-additive at (A) 15°C and (B) 20°C. (C) 5 mM caffeine does not reduce pharyngeal pumping at 15°C. * $P < 0.05$ vs. *ad libitum* fed worms at the same caffeine concentration.

Caffeine Extends Life Span by a Mechanism Related to IIS

Several genetic pathways are known to influence longevity and may mediate the life span-extending effects of caffeine. In order to identify interactions between caffeine and canonical aging pathways, we measured the effect of caffeine on life span for strains with loss of function mutations in (1) *daf-16*, which encodes the FOXO transcription factor that acts downstream of IIS; (2) *sir-2.1*, which encodes the worm ortholog of Sir2, a histone deacetylase linked to aging in many species; (3) *hif-1*, which encodes the hypoxia inducible factor; and (4) *cep-1*, the worm ortholog of the tumor suppressor p53.

Neither 5 mM nor 10 mM concentrations of caffeine extended life span of *daf-16* mutant worms (Figure 5.7A,B; Table 1). This result is in agreement with similar findings by Lublin et al. (2011) at 20°C,

and suggests a functional link between caffeine and IIS. Mutation or knockdown of *daf-2*, which encodes the *C. elegans* insulin/IGF-1 receptor, robustly increases worm life span (Kenyon et al., 1993; Kimura et al., 1997). To further explore the link between caffeine and IIS, we investigated whether caffeine is capable of extending life span of worms with *daf-16* or *daf-2* knocked down using RNAi. As with mutation of *daf-16*, 5 mM or 10 mM caffeine failed to increase life span in worms subjected to *daf-16(RNAi)* (Figure 5.7C,D; Table 1). In addition, caffeine did not further increase the long-life span of worms subjected to *daf-2(RNAi)* (Figure 5.7C,E; Table 1).

Activity of DAF-16 is post-translationally regulated through subcellular localization. Reduction of IIS causes dephosphorylation of the DAF-16 protein, allowing it to enter the nucleus and thereby activate transcription of target genes. In order to determine whether caffeine activates DAF-16 in a similar manner, we examined transgenic worms expressing a GFP tagged DAF-16 protein (DAF-16::GFP). Worms exposed to caffeine for 2-3 hours displayed an increase in DAF-16::GFP nuclear localization compared to untreated controls (Figure 5.8). These data are consistent with a model where caffeine impacts life span, at least in part, by reducing insulin signaling and activating DAF-16.

In contrast to *daf-16* and *daf-2*, caffeine did extend life span of worms with mutations in *sir-2.1*, *hif-1*, and *cep-1* (Figure 5.9; Table 1), indicating that the action of caffeine on life span is at least partially independent of these genes. Notably, effect of caffeine on life span in each of these mutant backgrounds was smaller than that observed in wild type worms. While these data are not as strongly indicative of an overlapping mechanism as the clear epistatic interaction between caffeine and IIS, the reduced life span extension in each of these backgrounds nevertheless suggest that caffeine may be interacting with multiple aging pathways.

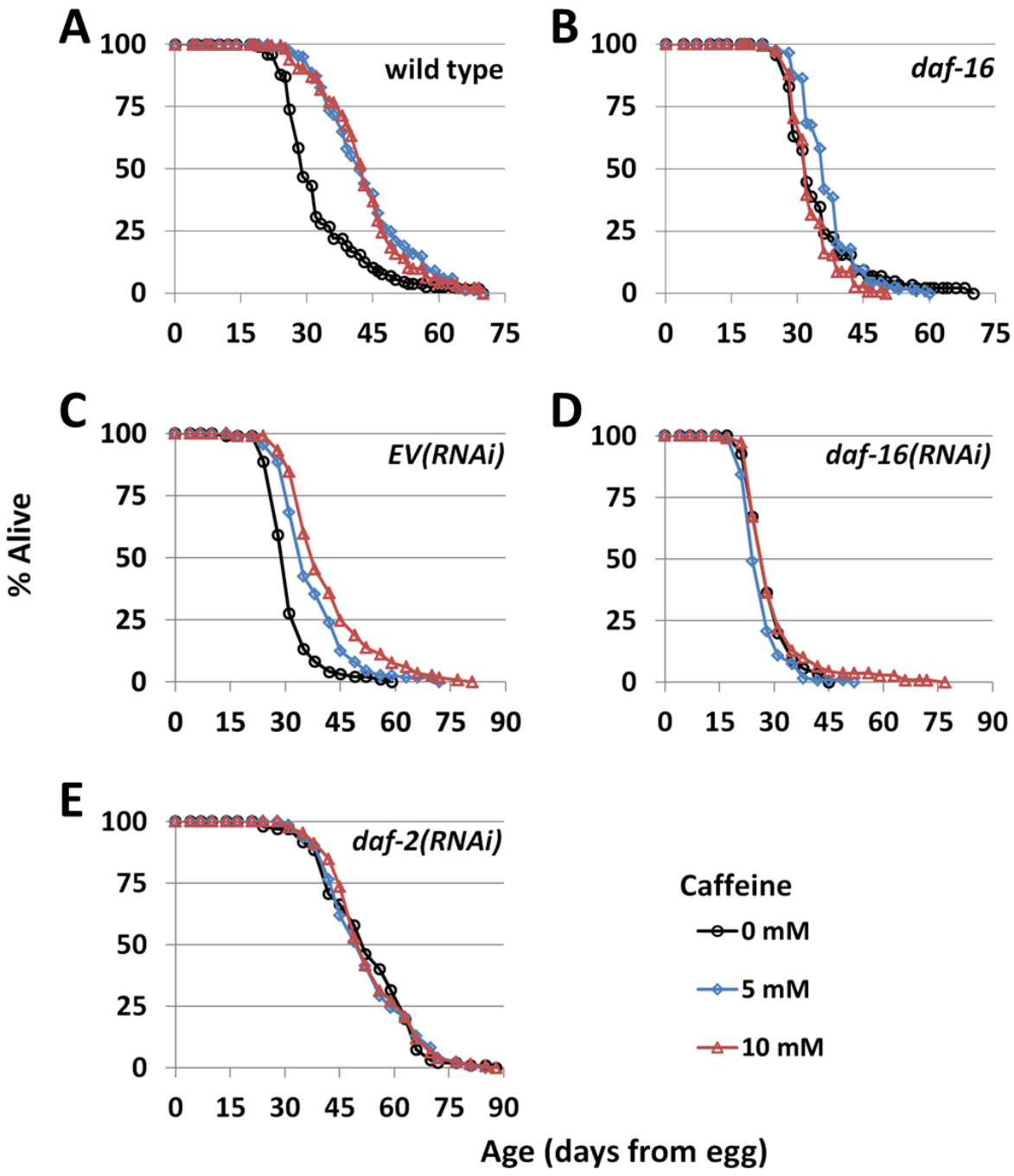


Figure 5.7. Caffeine displays epistatic interaction with IIS components. 5 mM or 10 mM caffeine extends life span in wild type (A), but not *daf-16(mu86)* (B), worms, and in wild type worms fed *EV(RNAi)* (C), but not worms fed *daf-16(RNAi)* (D) or *daf-2(RNAi)* (E).

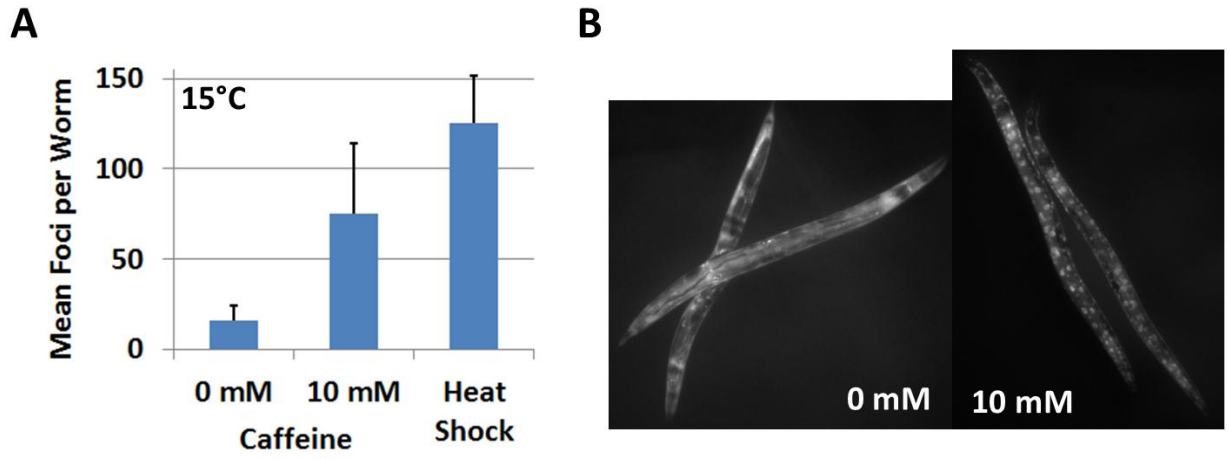


Figure 5.8. Caffeine causes DAF-16 nuclear localization. (A) Treatment with 5 mM caffeine causes nuclear localization of transgenically expressed DAF-16::GFP. A 2 hour heat shock at 37°C robustly activates DAF-16 and was used as a positive control. (B) Representative image showing DAF-16::GFP nuclear localization in response to caffeine.

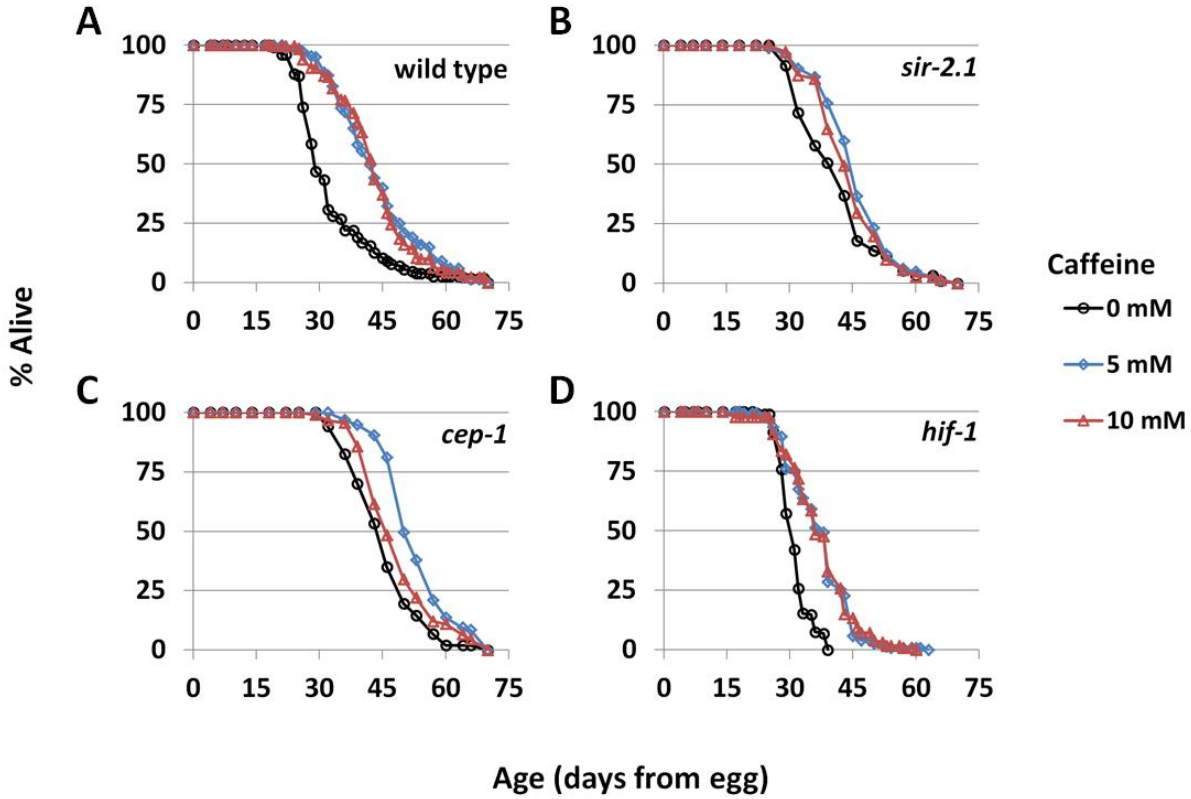


Figure 5.9. Caffeine extends life span of non-IIS longevity mutants. Treatment with 5 mM or 10 mM caffeine extends life span of (A) wild type, (B) *sir-2.1(ok434)*, (C) *cep-1(gk138)*, and (D) *hif-1(ia4)* worms.

DISCUSSION

In this study we demonstrate that chronic caffeine exposure during adulthood extends life span and health span of *C. elegans* in a temperature-dependent manner. Life span extension from caffeine is non-additive with life span extension by bacterial deprivation, and independent of the hypoxia inducible factor, HIF-1, the *C. elegans* p53 ortholog, CEP-1, and the *C. elegans* Sir2 ortholog, SIR-2.1. Caffeine appears to act, at least in part, by activating the FOXO transcription factor DAF-16 in a manner related to reduced IIS. Chronic caffeine exposure also delays paralysis in a *C. elegans* model of polyglutamine disease.

Perhaps the most intriguing possibility for caffeine is its potential to reduce the risk and delay the onset of age-associated neurodegenerative disease. As described in the introduction, studies in humans and rodents indicate that caffeine treatment reduces risk of disease onset and improves cognitive decline in models of Alzheimer's and Parkinson's disease. Previous work has also shown that caffeine is capable of delaying pathology in worm models of Alzheimer's disease (Dostal et al., 2010; Lublin et al., 2011). In this study we expand upon these findings to show that caffeine is capable of delaying pathology is a worm model of polyglutamine disease. Although mammalian studies investigating the use of caffeine in neurodegenerative disorders have focused primarily on Alzheimer's and Parkinson's disease, published research that examines the effects of caffeine consumption on Huntington's disease in human populations are currently underway. Our finding suggests that a more detailed examination of the influence of caffeine on the progression of Huntington's disease in mammalian models may be warranted.

This study and that by Lublin et al. (2011) both identify an epistatic connection between caffeine and IIS. A related link has been made in mammals. While acute treatment with caffeine has been shown to increase blood pressure (Riksen et al., 2009) and reduce insulin sensitivity (Keijzers et al., 2002; Moisey et al., 2008), long-term coffee consumption shows a strong correlation with reduced risk of type 2 diabetes in humans (van Dam and Hu, 2005) and chronic caffeine exposure prevents diet-induced insulin resistance and hypertension in rats (Conde et al., 2012). Chronic caffeine consumption may prove useful in mimicking reduced insulin signaling and improving diet induced insulin resistance.

The interaction observed between bacterial deprivation and caffeine suggests that caffeine may emulate aspects of dietary restriction, which is particularly interesting given that caffeine is already in common used in human society. The observation that caffeine interacts with both bacterial deprivation and insulin/IGF-1-signaling is complicated by the fact that bacterial deprivation extends life span independently of both DAF-2 and DAF-16 (Kaeberlein et al., 2006b), suggesting that caffeine may activate overlapping downstream targets in both pathways. The additional observation that life span extension from caffeine is reduced in three other strain backgrounds with mutations in genes linked to

aging suggests that caffeine may be activating a common set of cellular processes important for increased longevity via a range of interventions. Alternatively, it is possible that caffeine alters the molecular or physiological state of the organisms in such a way that renders the organism unable to respond normally to signals that result in increased life span in untreated animals. As discussed in detail in Chapter 3, epistatic interactions provide limited information regarding the potential mechanism of life span extension, and further study will be required to unravel the complexities of caffeine's impact on the aging process.

This study identifies interactions between caffeine and both the IIS and the dietary restriction pathways. The direct molecular targets and downstream mechanisms by which caffeine influences longevity remain to be investigated. Mammalian research suggests that caffeine primarily impacts cognitive phenotypes by antagonizing adenosine receptors A_1 and A_{2A} (Cunha and Agostinho, 2010). At sub-toxic levels of caffeine, adenosine receptors are the only clear molecular targets and many of the beneficial effects of caffeine are mimicked by specific agonists of adenosine receptor A_{2A} (Cunha and Agostinho, 2010). Mild inhibition of phosphodiesterase activity has only been observed at higher concentrations of caffeine (Spinetta et al., 2008). Functionally, caffeine prevents memory impairment induced by heavy alcohol consumption in rats, an effect which can be mimicked by simultaneous treatment with inhibitors of phophodiesterase 5 and adenosine receptor A_{2A} (but not by either inhibitor alone) (Spinetta et al., 2008). This suggests that specific subclasses of phophodiesterases may be important for the action of sub-toxic doses of caffeine in some circumstances.

Clear orthologs of mammalian adenosine receptors have not yet been identified in *C. elegans*, though there are several candidate genes based on sequence homology. The *C. elegans* genome contains six phosphodiesterases that fall into two functional classes: one class that specifically targets cAMP (PDE-4,6), and another that is thought to target cGMP (PDE-1,2,3,5) (Liu et al., 2010; Omori and Kotera, 2007). High-doses of caffeine have been shown to inhibit mammalian cAMP phosphodiesterases (Butcher and Potter, 1972; Tsuzuki and Newburgh, 1975), indicating that the former class is of greater interest. Further investigation will determine whether adenosine receptors, phosphodiesterases, or other targets are involved in caffeine's effect on worm life span.

Two factors have complicated mammalian research with caffeine. In human populations, most studies determine caffeine intake by consumption of caffeine-containing foods and beverages, such as coffee, tea, soft drinks, and chocolate, all of which contain other compounds that have the potential to affect the diseases under investigation. For example, one study found that long-term coffee consumption shows a strong correlation with reduced risk of type 2 diabetes (van Dam and Hu, 2005), while a later study found a similar correlation for both caffeinated and decaffeinated coffee consumption (van Dam et al., 2006). The collective findings of these two studies suggest that some of the insulin-related benefit

from coffee consumption may result from sources other than caffeine. Interpretations are further complicated by seemingly contradictory effects resulting from acute and chronic caffeine treatment in some circumstances, as discussed previously with respect to insulin sensitivity and hypertension. *C. elegans* may be a useful model decoupling these types of complications. For example, worm studies completed to date have already begun to separate influences from caffeine and non-caffeine sources with respect to coffee. Dostal et al. (2010) identified *skn-1* as a primary downstream factor in the caffeine-independent delay in amyloid beta toxicity using coffee extract, while this study and that by Lublin et al. (Lublin et al., 2011) identify IIS as an important player in life span extension by caffeine.

A growing accumulation of evidence in humans, rodents, and worms suggests that chronic caffeine use may yield significant health benefits by delaying aging and preventing specific age-associated pathologies. Recent studies indicate that *C. elegans* will be a useful system for dismantling the molecular events that underlie the beneficial effects of caffeine. *C. elegans* research may also help unravel the complications associated with acute versus chronic caffeine treatment, and identify other compounds in coffee and tea with the potential to promote longer life span. Overall, based on the observations that caffeine is capable of increasing life span in worms and has been correlated with decreased mortality in humans, we anticipate the expansion of future studies examining the influence of caffeine on longevity to mammalian systems.

EXPERIMENTAL PROCEDURES

Strains and Media

Strains used in this study are listed in Table 5.2 and were obtained from the *Caenorhabditis* Genetics Center, the laboratory of Dr. Chris Link (University of Colorado, Boulder, CO, USA), or the laboratory of Dr. Jim Thomas (University of Washington, Seattle, WA, USA). Animals were maintained on solid nematode growth media (NGM) agar plates using standard techniques. Experiments were performed on NGM plates supplemented with 25 mg/mL ampicillin to prevent contamination. Adult worms were placed on NGM plates containing FUdR to prevent reproduction. With the exception of RNAi bacteria, bacterial food was killed by exposure to UV. Solid anhydrous caffeine (MP Biomedicals, Solon, OH, USA) was added directly to the NGM solution prior to autoclaving. Neither autoclaving nor UV treatment during plate preparation influenced life span in the presence of caffeine (data not shown). All experiments were conducted at 15°C except where otherwise noted.

Table 5.2. Strains used in this study.

Strain	Genotype
N2	wild type
CF1038	*daf-16(mu86) I*
VC199	*sir-2.1(ok434) IV*
TJ1	*cep-1(gk138) I*
ZG31	*hif-1(ia4) V*
TJ356	zIs356[*pdaf-16::daf-16-gfp; rol-6*]
MQ35	*Q35::YFP*

RNAi

RNAi experiments were conducted using feeding protocols according to standard procedures. The RNAi feeding strains targeting *daf-16* and *daf-2* were obtained from the Vidal RNAi library (Rual et al., 2004) and J. McElwee, respectively. RNAi plasmids were sequenced to verify target sequence. RNAi plates consisted of NGM supplemented with 1 mM β-D-isothiogalactopyranoside (IPTG) and 25 μg/mL carbenicillin. Worms were raised on RNAi bacteria from egg to the L4 stage of development, and then transferred to plates containing freshly seeded RNAi bacteria plus 50 μM FUdR to prevent production.

Life Span Analysis

Life span experiments were conducted as previously described (Sutphin and Kaeberlein, 2009). Bacterial deprivation was conducted by maintaining animals on UV killed OP50 bacteria food until day 4 of adulthood. Worms were then transferred to plates without a bacterial food source.

Paralysis Analysis

Paralysis of worms was assessed visually. Worms were scored as paralyzed if they were unable to make forward progress on the NGM surface in response to plate-tapping or tail-prodding.

Movement Assays

Thrashing was quantified visually by suspending individual animals in a droplet of M9 buffer on the surface of an NGM plate and counting the number of body bends in 60 seconds. Movement rate was quantified by placing an individual worm onto a fresh OP50-seeded NGM plate. After 60 minutes, pictures were taken of tracks left in the bacterial lawn using a standard SLR camera with a microscope eyepiece adapter. Track length was measured using ImageJ software. Pharyngeal pumping was quantified visually by taking video recordings of the head region of individual animals and counting the number of pumps in 30 seconds.

DAF-16::GFP Nuclear Localization

Transgenic worms expressing DAF-16::GFP were transferred to plates containing 0 mM or 5 mM caffeine at the L4 stage of development. A third population of worms was exposed to 30°C for 2 hours immediately prior to analysis. Worms were immobilized by treatment with 25 nM NaN_3 and still images captured using a mounted digital camera and the GFP fluorescence channel of a Zeiss SteREO Lumar V.12 microscope. Captured images were used to quantify visible GFP foci.

Replicative Life Span Analysis

Replicative life span assays were performed as previously described (Kaeberlein et al., 2004; Steffen et al., 2009). Strains were maintained and all experiments carried out on YEPD agar plates (1% yeast extract, 2% Bacto-Peptone, 2% agar, 2% glucose). Solid anhydrous caffeine (MP Biomedicals, Solon, OH, USA) was added directly to the NGM solution prior to autoclaving.

Statistical Analysis

Statistical significance was determined for worm life span and yeast replicative life span using the Wilcoxon Rank Sum test and all other comparisons were conducted using an unpaired two-tailed Student's T-test assuming unequal variance.

Chapter 6: Uncovering the Role of Manganese Homeostasis in the Response to Dietary Restriction in *S. cerevisiae*

CHAPTER SUMMARY

Dietary restriction extends life span and improves numerous age-associated health parameters in evolutionarily divergent species ranging from the budding yeast to the rhesus monkey. The majority of dietary restriction studies have been carried out in a controlled laboratory environment on populations of genetically homogenous individuals. In genetically heterogeneous populations such as humans, it remains unknown whether dietary restriction will have a generally positive effect on survival and health, or whether different genotypes within the population will display differential responses. In the budding yeast, we have identified single gene deletion mutants that show a wide range of responses to dietary restriction, from severely shortened to greatly extended replicative life span. Two of the most severely negative responses to dietary restriction were observed in strains lacking either *PMR1*or *SOD2*. *PMR1* encodes a Golgi apparatus calcium/manganese ATPase responsible for transporting calcium and manganese from the cytosol into the Golgi lumen. *SOD2* encodes the mitochondrial manganese superoxide dismutase. The poor growth and reduced replicative life span of *pmr1*Δ cells subject to dietary restriction are rescued by point mutations in *SMF2*, an Nramp family manganese transporter involved in maintaining manganese homeostasis. Together, these observations indicate that proper control of intracellular manganese is important for an appropriate response to dietary restriction in yeast. Dietary restriction by reducing available glucose or replacing glucose with a nonfermentable carbon source causes yeast to switch from fermentive to respiratory metabolism. We propose a model in which cells with reduced Sod2 activity have decreased replicative life span in response to dietary restriction due to an inability handle the increased reactive oxygen species produced in the mitochondria in conditions that increase respiration. Cells lacking *PMR1* have reduced Sod2 activity due to reduced mitochondrial manganese concentrations resulting from improper control of manganese homeostasis. This chapter describes genetic and molecular evidence supporting this model, and discusses ongoing research to further test its predictions

INTRODUCTION

Dietary restriction is generally considered a widely applicable intervention to extend life span and improve health. When looking across evolutionarily diverse species, this appears to be the case. Reduced food intake without malnutrition increases life span in virtually all examined species including yeast, worms, flies, spiders, fish, mice, rats, dogs, and monkeys. A primary goal of studying longevity interventions is to develop treatments to extend human longevity, improve general human health, and fight age-associated human disease. The possibility of applying dietary restriction or drugs mimicking the effects of dietary restriction to human populations leads to a key question: are the benefits truly universal, or will dietary restriction have differential effects when applied to different individuals within genetically diverse populations living in non-standardized environments? Dietary restriction studies using model organisms to date do not provide a clear answer to this question, as most have been carried out using inbred, genetically homogenous populations of animals maintained for generations under laboratory conditions (i.e. abundant food, no predation, limited exposure to pathogens).

Two sets of mouse studies suggest that genetic diversity may have a significant impact on the individual response to dietary restriction. Harper et al. (2006) found that subjecting the non-inbred second generation offspring of wild-captured mice to dietary restriction did not extend the mean or median life span of the population. Instead, the population suffered increased mortality early in life and decreased mortality late in life. This survival response indicates that certain genotypes within the population promoted a positive response to dietary restriction, while other genotypes promoted a negative response. Liao and colleagues (Liao et al., 2010; Liao et al., 2011; Rikke et al., 2010) examined the effect of dietary restriction on life span in 42 recombinant inbred mouse strains. The response ranged from a 40% reduction in life span to a 98.5% increase in life span. Two additional studies noted different life span characteristics in response to dietary restriction in different inbred strain backgrounds (Fernandes et al., 1976; Forster et al., 2003). A limitation inherent to all of these studies was the use of a single level of dietary restriction, leaving open the possibility that an optimal restriction regimen may need to be adjusted for each genotype within a population. Technical considerations aside, the degree of variation observed in response to dietary restriction strongly suggests that the genotype of each individual in a population will determine whether dietary restriction will have a beneficial, neutral, or deleterious effect on life span and health span.

Recently, we completed a study examining the effect of dietary restriction on replicative life span in 165 single gene deletion yeast strains by reducing the glucose in the growth media from 2% to 0.05% (Schleit et al., submitted). Similar to the case for mice, we observe a response ranging from an 82% reduction in life span to a 103% increase in replicative life span (Schleit et al., submitted). We are now in

the process of examining the strains with the most divergent response to dietary restriction compared to wild type yeast in order to understand the underlying molecular events that are important for survival in nutrient-limiting conditions.

Two of the strains that displayed the greatest negative response to dietary restriction lack *PMR1* and *SOD2*. *PMR1* encodes a calcium/manganese ATPase in the Golgi apparatus. Pmr1 is the primary protein responsible for providing the Golgi apparatus with calcium and manganese, and for removing excess manganese from the cytoplasm. Strains lacking *PMR1* display distinct phenotypes associated with abnormal localization of each cation. *PMR1* mutants with calcium transport defects have increased cytosolic calcium, display protein processing and sorting defects, and are sensitive to low-calcium media or to compounds that result in increased protein misfolding (e.g. tunicamycin or DTT) (Antebi and Fink, 1992; Chen et al., 2005b; Durr et al., 1998; Mandal et al., 2003). *PMR1* mutants with manganese transport defects have increased cytosolic manganese, display defects in glycosylation, and are sensitive to high-manganese media (Bolton et al., 2002; Durr et al., 1998; Mandal et al., 2003; Mandal et al., 2000).

SOD2 encodes one of two highly conserved superoxide dismutases in yeast. Sod2 localizes to the mitochondrial matrix and acts to detoxify ROS produced as a byproduct of respiratory metabolism by converting superoxide to hydrogen peroxide (Luk et al., 2005). The activity of Sod2 requires manganese, which is provided the by manganese chaperone Mtm1 (Luk et al., 2003), and delivered to the mitochondria through a poorly understood mechanism involving the Nramp manganese transporter Smf2 (Luk and Culotta, 2001).

A central role for manganese in the function of two of the genes with the greatest impact on the response to dietary restriction implicates manganese homeostasis as a potentially important system for a general response to environmental nutrients. This chapter explores the role of manganese in the altered response of strains lacking *PMR1* and *SOD2* to dietary restriction.

RESULTS

Deletion of PMR1 *or* SOD2 *results in severe negative response to dietary restriction*

In a recent study examining the response of single deletion strains to dietary restriction, replicative life span of *pmr1*Δ and *sod2*Δ strains was found to be dramatically shortened on yeast extract peptone (YEP) media when glucose was reduced from 2% to 0.05% (Schleit et al., submitted). We confirmed this result and examined the replicative life span of both genotypes in response to an alternative form of dietary restriction in which the 2% glucose in the media is replaced by 3% glycerol, a

nonfermentable carbon source (Delaney et al., 2011; Kirchman and Botta, 2007). Replicative life span of *pmr1*Δ and *sod2*Δ cells was greatly reduced by both forms of dietary restriction (Figure 6.1A). Growth rate was severely reduced for *pmr1*Δ cells on YEP 3% glycerol, but only slightly reduced for *sod2*Δ cells (Figure 6.1B,C).

Point mutations in SMF2 *suppress poor response of* pmr1Δ *cells to dietary restriction*

Pmr1 is involved in both calcium and manganese transport, and impacts multiple processes downstream of each function including protein sorting and secretion, glycosylation, and cellular calcium and manganese homeostasis. In order to gain unbiased insight into which of these processes are relevant to the response of *pmr1Δ* cells to dietary restriction, we examined *pmr1*Δ cells that spontaneously regained the ability to grow in the presence of glycerol.

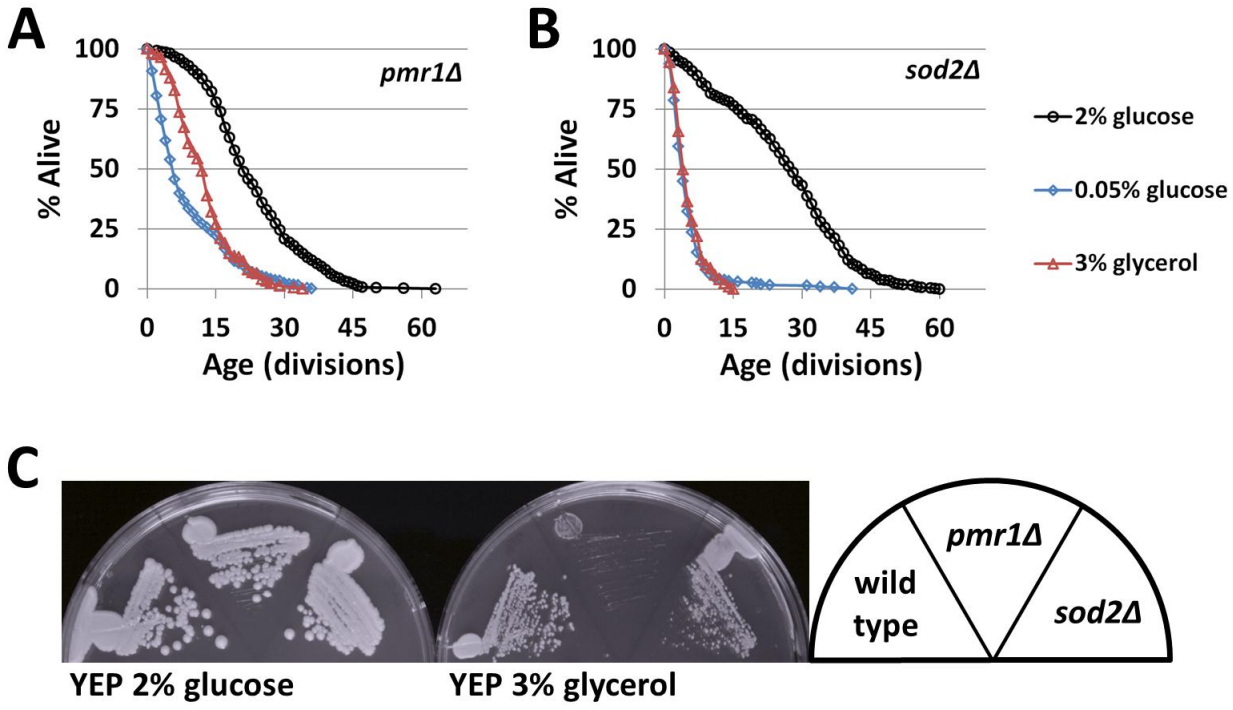


Figure 6.1. Dietary restriction shortens replicative life span and reduces growth rate of *pmr1*Δ and *sod2*Δ cells. Reducing media glucose or replacing glucose with glycerol severely shortens replicative life span of strains lacking either *PMR1* (A) or *SOD2* (B). Growth rate of *pmr1*Δ cells is severely reduced and the growth rate of *sod2*Δ cells slightly reduced on media containing glycerol in place of glucose (C).

A large number of wild type and *pmr1Δ* cells were plated on YEP 3% glycerol plates in order to isolate spontaneous suppressors of the *pmr1*Δ growth defect. After three days of growth, *pmr1Δ* colonies that had reached a similar size to wild type colonies were selected and backcrossed in order to isolate strains in which growth on glycerol was restored by a mutation at a single locus. Four strains were isolated that retained their ability to growth on glycerol following backcross (Figure 6.2).

Strains containing *pmr1Δ* and the *PMR1* glycerol growth suppressor (*PSG)* mutations were intercrossed to look for complementation and determine whether the four *PSG* mutations represented a single gene or multiple genes. The complementation experiment was performed by mating haploid strains with known glycerol growth characteristics and examining the ability of the resulting diploid strain to grow on glycerol. Importantly, wild type alleles were dominant for both the *PMR1* and *PSG* loci in the diploid context (i.e. heterozygous diploid strains with one wild type and one mutant allele at a given locus behaved in the same manner as a strain with two wild type alleles) and homozygous mutants behaved in the same manner as the equivalent haploid strain (e.g. a *pmr1*Δ*;pmr1*Δ *psge;psge* diploid and a *pmr1*Δ *psge* haploid both grow on glycerol) (Table 6.1A-K). In all cases, the *psg* mutations failed to complement, indicating that all four mutations occurred in the same gene (Table 6.1L-Q).

The identity of each suppressing mutation was determined by whole genome sequencing. All four suppressor strains contained one of two point mutations in the *SMF2* gene (Table 6.2). A mentioned in the introduction, *SMF2* encodes an Nramp family manganese transporter that is thought involved in supplying manganese to Sod2 in the mitochondria (Luk and Culotta, 2001). Smf2 has been linked to Pmr1 in the context of managing cytosolic manganese levels, and appears to reside in intracellular vesicles, though the precise subcellular localization is not known (Luk and Culotta, 2001).

Replicative life span was determined for *pmr1Δ* cells containing the *smf2(L149P)* mutation. In both cases, *smf2(L149P)* suppressed the short replicative life span of *pmr1Δ* cells in response to dietary restriction (Figure 6.3). The identification of *SMF2* as the carrier of the suppressing mutation strongly implicated manganese homeostasis as the relevant process for the short replicative life span of *pmr1Δ* cells in response to dietary restriction.

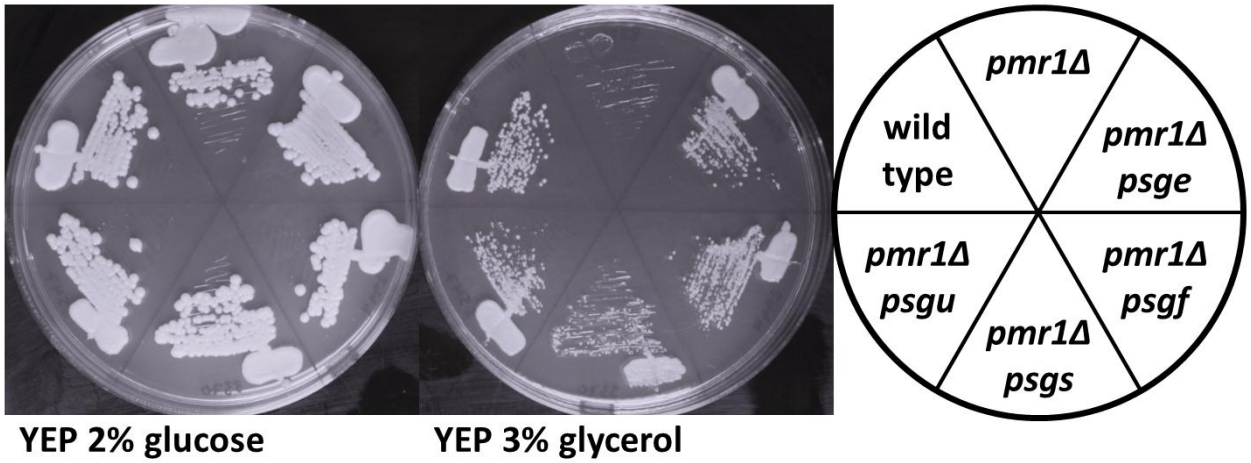


Figure 6.2. Spontaneous *PSG* mutants rescue growth of *pmr1*Δ cells on glycerol.

Table 6.1. Failure of *pmr1*Δ *psg* strains to complement with respect to growth in the presence of glycerol indicates that all mutations affect the same gene.

	Genotype			Growth on YEP 3% Glycerol		
Cross	MATα	MATa	Diploid	MATα	MATa	Diploid
(A)	wild type	wild type	wild type	normal	normal	normal
(B)	wild type	*pmr1 Δ*	*PMR1;pmr1 Δ*	normal	very slow	normal
(C)	*pmr1 Δ*	*pmr1Δ*	*pmr1 Δ;pmr1Δ*	very slow	very slow	very slow
(D)	*pmr1 Δ*	*pmr1 Δ psge*	*pmr1 Δ;pmr1Δ psge;psge*	very slow	normal	very slow
(E)	*pmr1 Δ*	*pmr1Δ psgf*	*pmr1Δ;pmr1Δ psgf;psgf*	very slow	normal	very slow
(F)	*pmr1 Δ*	*pmr1Δ psgs*	*pmr1Δ;pmr1Δ psgs;psgs*	very slow	normal	very slow
(G)	*pmr1 Δ*	*pmr1Δ psgu*	*pmr1Δ;pmr1Δ psgu;psgu*	very slow	normal	very slow
(H)	*pmr1 Δ psge*	*pmr1 Δ psge*	*pmr1 Δ;pmr1Δ psge;psge*	normal	normal	normal
(I)	*pmr1Δ psgf*	*pmr1Δ psgf*	*pmr1Δ;pmr1Δ psgf;psgf*	normal	normal	normal
(J)	*pmr1Δ psgs*	*pmr1Δ psgs*	*pmr1Δ;pmr1Δ psgs;psgs*	normal	normal	normal
(K)	*pmr1Δ psgu*	*pmr1Δ psgu*	*pmr1Δ;pmr1Δ psgu;psgu*	normal	normal	normal
(L)	*pmr1 Δ psge*	*pmr1Δ psgf*	*pmr1 Δ;pmr1Δ ?;psge ?;psgf*	normal	normal	normal
(M)	*pmr1 Δ psge*	*pmr1Δ psgs*	*pmr1 Δ;pmr1Δ ?;psge ?;psgs*	normal	normal	normal
(N)	*pmr1 Δ psge*	*pmr1Δ psgu*	*pmr1 Δ;pmr1Δ ?;psge ?;psgu*	normal	normal	normal
(O)	*pmr1 Δ psgf*	*pmr1Δ psgs*	*pmr1 Δ;pmr1Δ ?;psgf ?;psgs*	normal	normal	normal
(P)	*pmr1 Δ psgf*	*pmr1Δ psgu*	*pmr1 Δ;pmr1Δ ?;psgf ?;psgu*	normal	normal	normal
(Q)	*pmr1 Δ psgs*	*pmr1Δ psgu*	*pmr1 Δ;pmr1Δ ?;psgs ?;psgu*	normal	normal	normal

Table 6.2. Genome sequencing of *PSG* mutants identifies two point mutations in the *SMF2* gene conferring single amino acid substitutions in the Smf2 protein.

pmr1 Δ	Supressing	Genomic Mutation			Amino Acid Substitution		
Suppressor	Gene	Position	Wild Type	Mutant	Position	Wild Type	Mutant
psge	*SMF2*	208099	T	C	149	L	P
psgf	*SMF2*	208099	T	C	149	L	P
psgs	*SMF2*	208002	G	T	117	G	C
psgu	*SMF2*	208002	G	T	117	G	C

Deletion of SMF2 *Does Not Rescue* pmr1Δ *Replicative Life Span or Growth in Response to Dietary Restriction*

We next asked whether complete deletion of *SMF2* would replicate the effect of the *SMF2* point mutations on growth and replicative life span in the *pmr1*Δ background. Intriguingly, *pmr1*Δ *smf2*Δ cells behaved similarly to *pmr1*Δ cells and not *pmr1*Δ *smf2(L149P)* or *pmr1*Δ *smf2(G117C)* in both contexts (Figure 6.4). In order to examine the interaction between the deletion and point mutation in *SMF2* in the context of cells lacking *PMR1*, we performed a complementation assay with respect to growth on YEP 3% glycerol media (Table 6.3). The following discussion refers only to *smf2(L149P)*, but the identical phenotypes were observed in interactions with *smf2(G117C)* (data not shown).

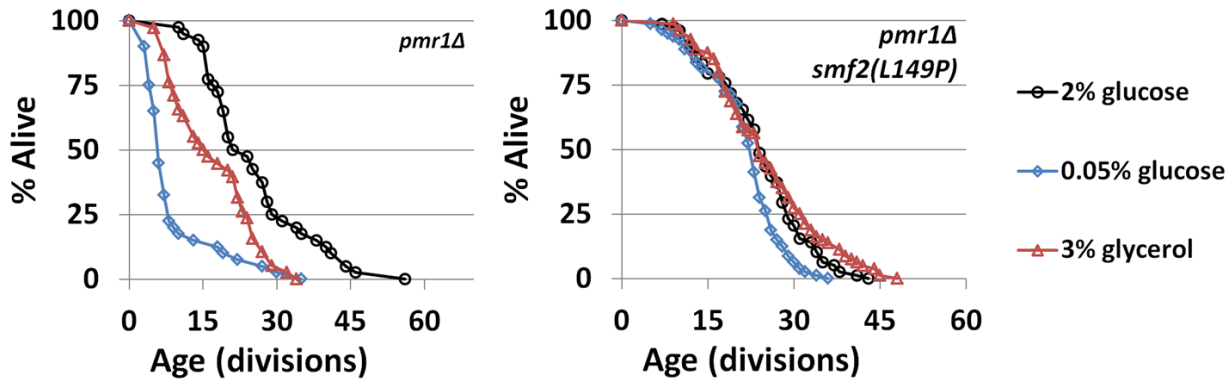


Figure 6.3. The *smf2(L149P)* point mutation rescues the short replicative life span of *pmr1*Δ cells in response to dietary restriction.

As before, wild type alleles were dominant in all cases at the *PMR1* and *SMF2* loci and homozygous diploids behaved similarly to an equivalent haploid strain with the same alleles (Table 6.3A-I). The one piece missing from this pattern was the cross between two *pmr1*Δ *smf2*Δ haploid strains, which failed to produce viable diploid when mated (Table 6.3J). Haploid *pmr1*Δ yeast are inefficient at mating due to an impaired ability to properly process and secrete alpha factor (Antebi and Fink, 1992). While this process is rescued by the addition of calcium to the growth media and has thus been associated with the calcium transport function of Pmr1 (Antebi and Fink, 1992), it has been suggested that manganese can substitute for calcium in some aspects of mating behavior in other contexts (Loukin and Kung, 1995). We suspect that further impairment of manganese homeostasis in *pmr1*Δ cells by deletion of *SMF2* may result in an even lower mating efficiency.

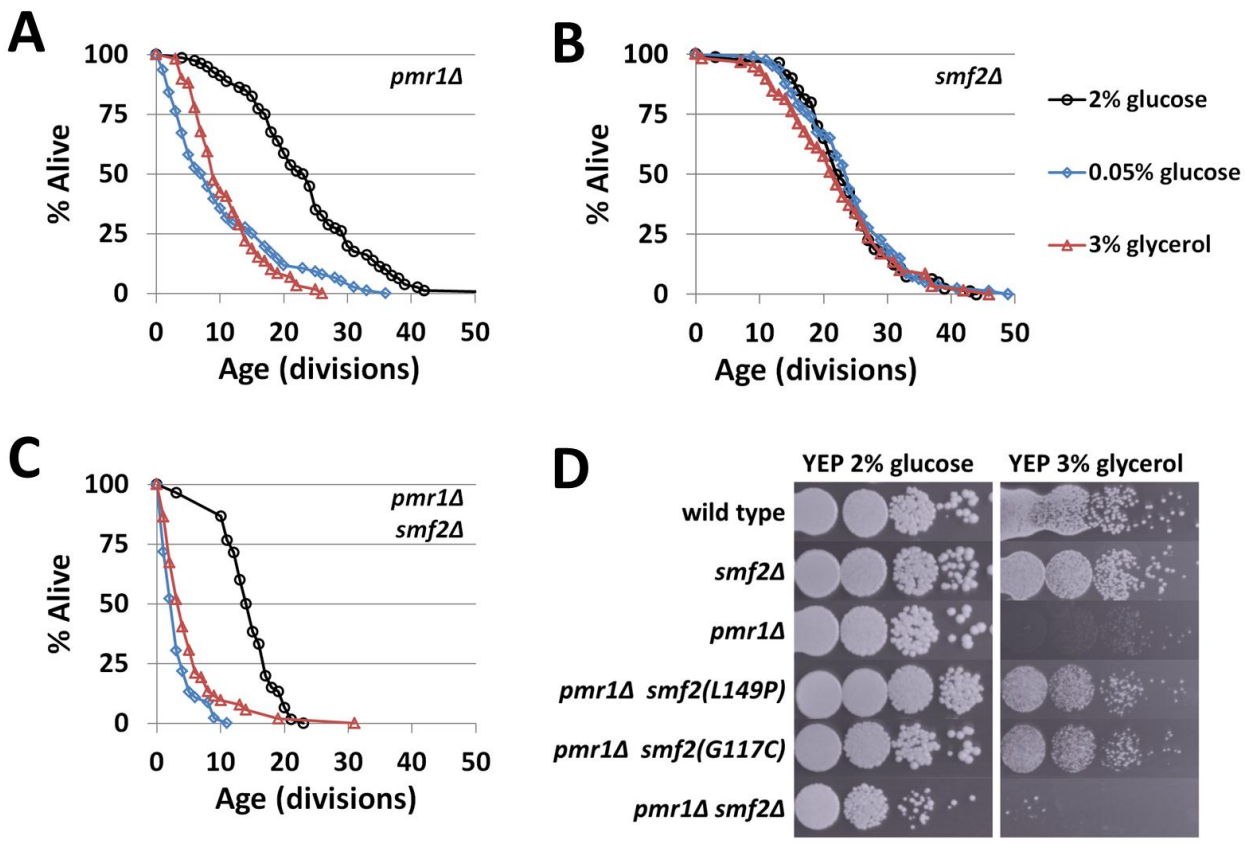


Figure 6.4. Deletion of *SMF2* fails to rescue *pmr1*Δ response to dietary restriction. *pmr1*Δ (A) and *pmr1*Δ *smf2*Δ (C) but not *smf2*Δ (B) cells respond poorly to dietary restriction with respect to replicative life span. (D) Growth of *pmr1*Δ cells on glycerol is rescued by *smf2(L149P)* and *smf2(G117C)* but not *smf2*Δ.

When a *pmr1*Δ single mutant haploid is crossed to a *pmr1*Δ *smf2(L149P)* mutant, the resulting *pmr1*Δ*;pmr1*Δ *SMF2;smf2(L149P)* diploid strain behaves in the same manner as a *pmr1*Δ*;pmr1*Δ *SMF2;SMF2* or *pmr1*Δ*;pmr1*Δ *SMF2;smf2*Δ diploid strain with respect to glycerol growth, indicating that the wild type *SMF2* allele is dominant to *smf2(L149P)* (Table 6.3C,H,K). However, when a *pmr1*Δ *smf2*Δ haploid strain is crossed to a *pmr1*Δ *smf2(L149P)* haploid strain, the resulting heterozygous diploid with a single *smf2(L149P)* version of *SMF2* is able to grow normally on glycerol media, emulating the rescue of the homozygous *PMR1* deletion by the homozygous *smf2(L149P)* mutant (Table 6.3I,L). This pattern of interaction between wild type *SMF2, smf2*Δ, and the identified *SMF2* point mutants suggests that *smf2(L149P)* and *smf2(G117C)* are partial loss of function alleles, and are clearly acting in a manner different from the *smf2*Δ strain, which lacks the entire *SMF2* ORF.

Table 6.3. Complementation between *smf2*Δ and *smf2(L149P)* in the *pmr1*Δ background with respect to growth on YEP 3% glycerol. The *smf2(L149P)* and *smf2(G117C)* alleles displayed identical complementation patterns. Growth was scored qualitatively with "normal" growth being visually similar to that of wild type haploid strains, and "very slow" growth being visually similar to *pmr1*Δ strains when cells are streaked on solid media (see Figure 6.1A).

	Genotype			Growth on YEP 3% Glycerol		
Cross	MATα	MATa	Diploid	MATα	MATa	Diploid
(A)	wild type	wild type	wild type	normal	normal	normal
(B)	wild type	*pmr1 Δ*	*PMR1;pmr1 Δ*	normal	very slow	normal
(C)	*pmr1 Δ*	*pmr1Δ*	*pmr1 Δ;pmr1Δ*	very slow	very slow	very slow
(D)	wild type	*smf2Δ*	*SMF2;smf2Δ*	normal	normal	normal
(E)	*smf2Δ*	*smf2Δ*	*smf2Δ;smf2Δ*	normal	normal	normal
(F)	wild type	*smf2(L149P)*	*SMF2;smf2(L149P)*	normal	normal	normal
(G)	*smf2(L149P)*	*smf2(L149P)*	*smf2(L149P);smf2(L149P)*	normal	normal	normal
(H)	*pmr1 Δ*	*pmr1 Δ smf2Δ*	*pmr1Δ;pmr1 Δ SMF2;smf2Δ*	very slow	very slow	very slow
(I)	*pmr1Δ smf2(L149P)*	*pmr1Δ smf2(L149P)*	*pmr1Δ;pmr1Δ smf2(L149P);smf2(L149P)*	normal	normal	normal
(J)	*pmr1 Δ smf2Δ*	*pmr1 Δ smf2Δ*	*pmr1Δ;pmr1 Δ smf2Δ;smf2Δ*	very slow	very slow	failed mating
(K)	*pmr1 Δ*	*pmr1Δ smf2(L149P)*	*pmr1Δ;pmr1Δ SMF2;smf2(L149P)*	very slow	normal	very slow
(L)	*pmr1 Δ smf2Δ*	*pmr1Δ smf2(L149P)*	*pmr1Δ;pmr1Δ smf2Δ;smf2(L149P)*	very slow	normal	normal

Copper rescues short replicative life span of* pmr1Δ *and* sod2Δ *cells

Growth on media containing either a reduced concentration of glucose or a nonfermentable carbon source, such as glycerol, causes a shift from fermentation to respiration (Pronk et al., 1996). Given the role of Sod2 in detoxifying ROS produced in response to increased respiration, the identification of mutations in *SMF2* as the suppressors the *pmr1*Δ glycerol growth defect led us to hypothesize that deletion of *PMR1* results in reduced Sod2 activity caused by impaired trafficking of manganese to the mitochondria. Thus the poor response of both *pmr1*Δ and *sod2*Δ cells to dietary restriction results from an inability of these cells to eliminate the increased superoxide produced in the mitochondria during respiratory metabolism. This model is further supported by a previous report that *pmr1*Δ cells are sensitive to paraquat, which induces mitochondrial superoxide production (Outten et al., 2005).

If the poor response to dietary restriction in *pmr1*Δ and *sod2*Δ cells results from a decreased ability to handle increased superoxide production, interventions that improve superoxide scavenging independent of Sod2 may rescue the short replicative life span in response to dietary restriction. *SOD1* encodes the copper/zinc superoxide dismutase in yeast. While Sod1 is primarily cytosolic, a portion of the protein localizes to the mitochondria intermembrane space (Sturtz et al., 2001). We hypothesized that adding copper to the media may rescue the short life span of yeast lacking Pmr1 or Sod2 by increasing superoxide scavenging through Sod1. Indeed, the short replicative life span of *pmr1Δ* and *sod2Δ* cells in response to dietary restriction was rescued by the addition of copper to the growth media (Figure 6.5A,B). We also examined the response *sod2Δ* cells to several other types of metals to verify that the observed rescue was unique to copper, and not a general response to increased metal content in the cell. Adding manganese, magnesium, calcium, iron, zinc, or aluminum to the media did not improve the response of *sod2Δ* cells to dietary restriction (Figure 6.5C).

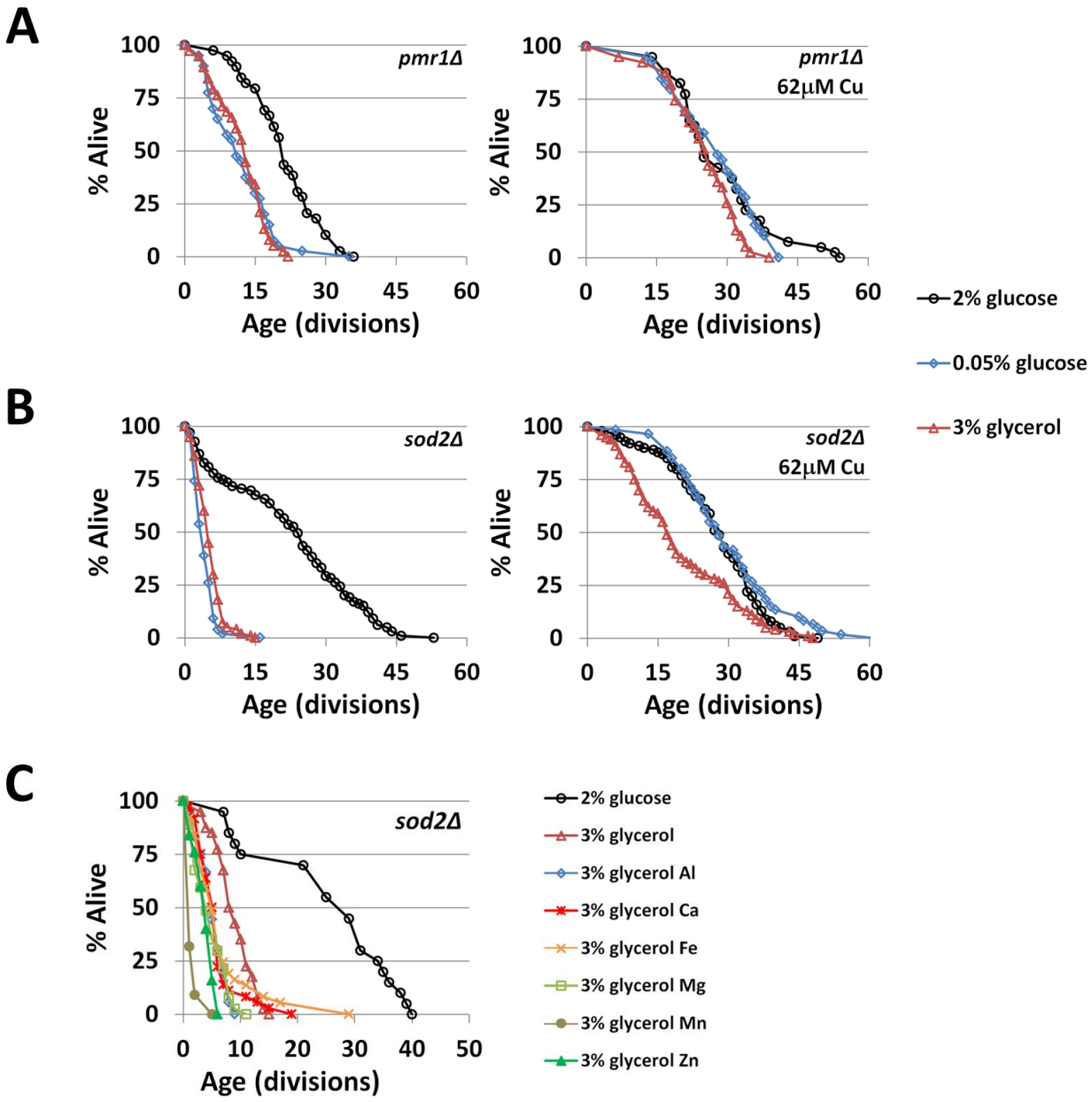


Figure 6.5. Copper rescues short replicative life span of *pmr1*Δ and *sod2*Δ cells in response to dietary restriction. Increasing the media copper content rescued the short replicative life span of strains lacking either (A) *PMR1* or (B) *SOD2* subjected to dietary restriction. (C) Adding aluminum, calcium, iron, magnesium, manganese, or zinc to the media had no effect on the response of *sod2*Δ cells to dietary restriction.

DISCUSSION

This chapter presents an investigation of the molecular mechanisms behind the shortening of replicative life span observed in *pmr1*Δ and *sod2*Δ yeast subjected to dietary restriction. We propose a model in which cells with reduced Sod2 activity are unable to deal with increased superoxide resulting from an increase in respiration during dietary restriction. Delivery of manganese to Sod2 in the mitochondria is disrupted in *pmr1*Δ cells, resulting in a decrease in Sod2 activity and emulation of the *sod2*Δ phenotypes under dietary restriction. Manganese is considered as a potential linking factor between Pmr1 and Sod2 in this model based on three facts: (1) the requirement of manganese for the activity of Sod2, (2) the central role for Pmr1 in cellular manganese homeostasis, and (3) the identification of mutations in the gene encoding the Smf2 manganese transporter as suppressors of *pmr1*Δ growth defects on glycerol. Smf2 further strengthens the link between Pmr1 and Sod2 due to its previously identified role in supplying manganese to Sod2 in the mitochondria and its interaction with Pmr1 with respect to regulating cytosolic manganese concentrations (Luk and Culotta, 2001).

The previous observation that *pmr1*Δ cells are sensitive to paraquat, which induces superoxide production in the mitochondria (Outten et al., 2005), further supports the idea *pmr1*Δ cells have reduced Sod2 activity and therefore a reduced capacity for superoxide detoxification. The ability of copper to rescue the short replicative life span of *pmr1Δ* and *sod2Δ* cells in response to dietary restriction strengthens the argument that both strains are suffering from a similar defect. Since copper is a required for the activity of Sod1, its ability to rescue *pmr1*Δ and *sod2*Δ cells is consistent with a model in which increased superoxide is the mechanism of reduced replicative life span. The role of Sod1 in this model is a topic of current investigation.

The presented evidence is not sufficient to make a definitive statement about the validity of a model linking Pmr1 to Sod2 and dietary restriction via manganese homeostasis. However, the study is still ongoing and current efforts are focused on testing different aspects of this model. One prediction made by the model is that mitochondrial manganese levels should be low in *pmr1*Δ cells relative to wild type cells, and that the *smf2(L149P)* and *smf2(G117C)* point mutations, but not deletion of *SMF2*, should raise these values. This prediction is currently being examined by using mass spectrometry to quantify manganese content in mitochondrial extracts from these strains under both standard and dietary restricted conditions.

A second prediction is that Sod2 activity should be reduced in *pmr1*Δ cells. One previous study reported that *pmr1*Δ cells actually have a similar level of Sod2 activity to wild type cells (Luk and Culotta, 2001); however, in this case superoxide dismutase activity was examined in total cell lysates. Cells lacking *PMR1* are known to have elevated manganese in the cytoplasm (Bolton et al., 2002). If our

model is correct, then reduced Sod2 activity would result from insufficient manganese specifically in the mitochondria. Once mitochondrial and cytoplasmic compartments are mixed in a total cell lysate, any loss in Sod2 activity resulting from insufficient manganese would be recovered when the mitochondrial Sod2 is exposed to the abundant manganese from the cytosolic compartment. To investigate this possibility, Sod2 activity from both total cell lysates and from mitochondrial extracts will be measured independently in both *pmr1*Δ strains with wild type or mutant *SMF2*. MitoSOX Red dye will also be employed to determine whether superoxide levels are increased in *pmr1*Δ and *sod2*Δ cells relative to wild type cells in response to dietary restriction. Related to Sod2 activity assays is the interaction between *SOD2* and the *SMF2* point mutants. If our model is accurate, the point mutants rescue *pmr1*Δ phenotypes by restoring Sod2 activity. The short replicative life span of *sod2*Δ cells should therefore not be rescued by these point mutations. This is currently being tested.

Finally, the mechanism underlying the ability of copper to rescue the short replicative life span of both *pmr1*Δ and *sod2*Δ cells is being examined. In our model, copper improves the response to dietary restriction by activating Sod1, thus dealing with the increased superoxide resulting from increased respiration in the absence of typical Sod2 activity. Two predictions can be made. First, copper should be able to rescue the poor growth of *pmr1*Δ and *sod2*Δ cells in the presence of paraquat. Second, copper should increase Sod1 activity and decrease the presence of superoxide in *pmr1*Δ and *sod2*Δ cells in response to dietary restriction.

This study provides insight into the mechanisms of decreased replicative life span in two yeast mutants with altered response to dietary restriction. A primary goal in this line of investigation is to identify mechanisms by which genetic variation influence response to dietary restriction. Developing a comprehensive understanding of these mechanisms will be useful in determining what genetic and environmental factors should be taken into consideration when transitioning longevity interventions into clinical applications to slow aging and fight age-related disease. This study also highlights the importance of the manganese superoxide dismutase Sod2 in the response of yeast to dietary restriction, and identifies proper control of cellular manganese homeostasis as a vital component of this response.

EXPERIMENTAL PROCEDURES

Strains and Media

All strains used in this study were derived from the parent strains of the haploid yeast ORF deletion collections (Winzeler et al., 1999), BY4742 (MATα *his3*Δ *leu2*Δ *lys2*Δ *ura3*Δ) and BY4741 (MAT**a** *his3*Δ *leu2*Δ *met15*Δ *ura3*Δ) and are listed in Table 6.4. The MATα and MAT**a** haploid ORF deletion collection and parental strains were obtained from Research Genetics. Single-deletion strains were either taken directly from one of the ORF deletion collection or constructed by replacing the entire ORF of the indicated gene with a selectable marker using standard PCR-base protocols as previously described (Sikorski and Hieter, 1989) and verified by PCR. Double mutant strains were produced by mating haploid mutants with the desired mutations, sporulating the resulting heterozygous diploid strain, selecting haploids with the desired markers via tetrad analysis, and verifying mutations by PCR..

Strains were maintained on YEP (1% yeast extract, 2% Bacto-Peptone) agar plates and experiments carried out on either YEP or synthetic defined (SD) (Sherman et al., 1978) agar plates supplemented with either 2% glucose, 0.05% glucose, or 3% glycerol as indicated. For reduced manganese experiments, the quantity of $MnSO_4*H_2O$ was altered to 0x or 0.1x the standard concentration during plate preparation. For experiments examining the response metal, 62 μM of $CuSO_4*5H_2O$, $MnSO_4*H_2O$, $MgSO_4*7H_2O$, $CaCl_2$, $FeSO_4*7H_2O$, $ZnSO_4*7H_2O$, or $Al_2(SO_4)_3*18H_2O$ was added during media preparation.

Replicative Life Span Analysis

Replicative life span assays were performed as previously described (Kaeberlein et al., 2004; Steffen et al., 2009). All presented data is pooled across experiments and mating types for strains of the same genotype and growth conditions.

Spot Assays

For spot growth assays, yeast cultures were seeded with single colonies and grown overnight at 30°C in liquid YEP or SD media supplemented with 2% glucose. OD_{600} was measured for all cultures, and each culture diluted to the OD_{600} of the most dilute culture to normalize cell number. Each culture was

then serial diluted 8 times in 10-fold dilution steps. 15 μL (SD) or 10 μL (YEP) volumes of each dilution level was spotted on the indicated media type. Spot plates were incubated for 2-4 days at 30°C to allow colony growth.

Complementation

Single colonies of haploid strains containing the mutations of interest were patched close together one YEP 2% glucose plates and incubated for 2 hours at 30°C to induce mating pheromone secretion and cell cycle arrest. Patches were then swirled together and incubated for 4 hours at 30°C to allow mating. Patches were then streaked for single cells and individual zygotes selected by microdissection and allowed to produce diploid colonies. The colonies were then streaked to YEP 3% glycerol and placed at 30°C for 3 days. Growth was assessed relative to wild type ("normal" growth) or *pmr1*Δ ("very slow"). For each complementation test, at least 4 crosses were examined for the desired genotype using different parental strains. For each cross, at least 4 zygotes were selected.

Table 6.4. Strains used in this study. All strains are in the BY4742 (MATα *his3*Δ *leu2*Δ *lys2*Δ *ura3*Δ) and BY4741 (MAT**a** *his3*Δ *leu2*Δ *met15*Δ *ura3*Δ). Strains labeled "DC:XXXXX" are from the yeast ORF deletion collection.

Strain	Mating Type	Genotype	Strain	Mating Type	Genotype	Strain	Mating Type	Genotype
DC:15B2	smf2::KanMX	MAT**a**	GS3003	pmr1::KanMX psgf	MAT**a**	GS3529	pmr1::KanMX psgr	MATα
DC:36C7	sod2::KanMX	MAT**a**	GS3007	pmr1::KanMX psgf	MATα	GS3530	pmr1::KanMX psgr	MATα
DC:114B12	smf2::KanMX	MATα	GS3013	pmr1::KanMX psgj	MAT**a**	GS3532	pmr1::KanMX psgr	MAT**a**
DC:131D6	smf1::KanMX	MATα	GS3014	pmr1::KanMX psgj	MATα	GS3533	pmr1::KanMX psgr	MAT**a**
DC:134G2	sod2::KanMX	MATα	GS3016	pmr1::KanMX psgj	MAT**a**	GS3535	pmr1::KanMX psgs	MATα
GS1222	pmr1::KanMX	MAT**a**	GS3021	pmr1::KanMX psgj	MAT**a**	GS3536	pmr1::KanMX psgs	MATα
GS1228	pmr1::KanMX	MATα	GS3025	pmr1::KanMX psgj	MAT**a**	GS3538	pmr1::KanMX psgs	MAT**a**
GS1231	pmr1::KanMX	MATα	GS3032	pmr1::KanMX psgg	MAT**a**	GS3539	pmr1::KanMX psgs	MAT**a**
GS1528	pmr1::URA3	MATα	GS3033	pmr1::KanMX psgg	MATα	GS3541	pmr1::KanMX psgt	MATα
GS1821	wild type	MAT**a**	GS3036	pmr1::KanMX psga	MAT**a**	GS3542	pmr1::KanMX psgt	MATα
GS1875	wild type	MATα	GS3037	pmr1::KanMX psga	MATα	GS3545	pmr1::KanMX psgt	MAT**a**
GS2421	wild type	MAT**a**	GS3038	pmr1::KanMX psga	MAT**a**	GS3546	pmr1::KanMX psgt	MAT**a**
GS2422	wild type	MATα	GS3043	pmr1::KanMX psgb	MATα	GS3547	pmr1::KanMX psgu	MATα
GS2457	pmr1::KanMX psga	MATα	GS3050	pmr1::KanMX psgb	MAT**a**	GS3548	pmr1::KanMX psgu	MATα
GS2458	pmr1::KanMX psgb	MATα	GS3051	pmr1::KanMX psgb	MAT**a**	GS3551	pmr1::KanMX psgu	MAT**a**
GS2461	pmr1::KanMX psge	MATα	GS3058	pmr1::KanMX psgg	MAT**a**	GS3552	pmr1::KanMX psgu	MAT**a**
GS2462	pmr1::KanMX psgf	MATα	GS3062	pmr1::KanMX psgh	MAT**a**	GS3570	pmr1::KanMX smf2::KanMX	MAT**a**
GS2463	pmr1::KanMX psgg	MATα	GS3066	pmr1::KanMX psgh	MAT**a**	GS3571	pmr1::KanMX smf2::KanMX	MATα
GS2464	pmr1::KanMX psgh	MATα	GS3070	pmr1::KanMX psgh	MATα	GS3578	sod2::KanMX	MATα
GS2465	pmr1::KanMX psgi	MATα	GS3072	pmr1::KanMX psgi	MAT**a**	GS3606	smf2::KanMX	MAT**a**
GS2466	pmr1::KanMX psgj	MATα	GS3073	pmr1::KanMX psgi	MATα	GS3607	smf2::KanMX	MATα
GS2584	pmr1::URA3	MAT**a**	GS3075	pmr1::KanMX psgi	MAT**a**	GS3623	wild type	MATα
GS2968	pmr1::KanMX psge	MATα	GS3077	pmr1::KanMX psgj	MATα	GS3628	wild type	MATα
GS2969	pmr1::KanMX psge	MATα	GS3517	pmr1::KanMX psgp	MATα	GS3657	smf2::KanMX sod2::KanMX	MATα
GS2970	pmr1::KanMX psge	MAT**a**	GS3518	pmr1::KanMX psgp	MATα	GS3658	smf2::KanMX sod2::KanMX	MATα
GS2971	pmr1::KanMX psge	MAT**a**	GS3520	pmr1::KanMX psgp	MAT**a**	GS3668	pmr1::KanMX smf2::KanMX	MAT**a**
GS2979	pmr1::KanMX psge	MATα	GS3522	pmr1::KanMX psgp	MAT**a**	GS3670	pmr1::KanMX	MATα
GS2982	pmr1::KanMX psge	MAT**a**	GS3523	pmr1::KanMX psgq	MATα	GS3674	pmr1::KanMX smf2::KanMX	MATα
GS2994	pmr1::KanMX psgf	MATα	GS3524	pmr1::KanMX psgq	MATα	GS3675	pmr1::KanMX	MATα
GS2995	pmr1::KanMX psgf	MAT**a**	GS3527	pmr1::KanMX psgq	MAT**a**	GS3721	pmr1::KanMX	MAT**a**
GS2999	pmr1::KanMX psgf	MAT**a**	GS3528	pmr1::KanMX psgq	MAT**a**	GS3722	pmr1::KanMX	MAT**a**

Conclusions

The desire to extend healthy human life span and develop treatments for age-associated disease has led to a concerted and growing effort to understand the basic biological mechanisms that underlie the aging process. Mammalian systems are ultimately needed to understand the role of genes and environmental factors in human aging, and murine systems are central to our growing understanding of aging process. A central challenge in studying mammalian aging is the cost in both time and resources of maintaining relatively long-lived animals in a controlled setting over the course of their life span. One answer to this challenge has been the development of several short-lived, easily maintained, genetically tractable, and evolutionarily disparate invertebrate model systems for aging research. These models provide a set of systems in which to develop an understanding of the core network of processes that determine longevity across species. The development of evolutionary theories of aging and the identification of interventions and genetic pathways that similarly impact longevity in multiple species tell us that knowledge gained in these systems is relevant to mammalian aging. This dissertation has broadly discussed advancements made using the three most prominent of these model systems—yeast, nematodes, and fruit flies—and detailed several current lines of study into specific aspects of aging biology.

The past decades have seen the biomedical sciences shift toward systems-level techniques. The range of environmental influences and internal processes that influence longevity, and the apparently high degree of interrelationship between these processes, make aging a prime candidate for this type of approach. The application of genome-wide screens, microarrays, proteomics, and metabolomics to longevity and other age-associated phenotypes is the first phase toward defining the range of genetic and environmental players in longevity determination. Targeted studies allow these players to be grouped into pathways that respond to similar upstream signals and activate common downstream factors. As the number of interactions between different players continues to grow, it has become apparent that traditional linear pathway structures are not sufficient to capture the complexity of longevity determination. The development of more sophisticated tools for analyzing these interactions will allow us examine the aging processes as a network of interrelated processes that integrate environmental and internal signals into a series of biological responses. One ultimate goal of this type of analysis will be to identify key points within the network where pharmacologic or environmental interventions might be applied to positively influence longevity or specific age-associated disease. The formal epistasis system

discussed in Chapter 3 is an early step in the development of such a tool aimed at taking advantage of the growing pool of information regarding specific interactions between aging interventions in the context of life span phenotypes.

A second recent trend in aging research is to question how interventions that robustly increase longevity in the laboratory, such as dietary restriction, will affect genetically distinct individuals exposed to variable environmental conditions. Initial evidence from recent studies in mice and yeast indicate that the influence of such interventions on life span can vary widely across genetically diverse individuals. This line of inquiry naturally ties into the trend toward genomics, as genome-scale techniques are useful in identifying key genetic factors that alter response to a specific intervention within large or diverse populations. The ongoing identification of these factors opens a range of questions regarding the mechanistic underpinning of the altered response. The discussion of the interaction between dietary restriction, manganese transport, Sod2, and Pmr1 outlined in Chapter 6 is one example.

Another side to this trend is to question how longevity altering elements already present in a population might interact with novel interventions applied to that population. The study examining the interaction between caffeine and known aging pathways is an example of one pharmacological agent widely used in human populations. Using worms, we demonstrate that caffeine influences longevity and clearly interacts with at least two commonly studied and evolutionarily conserved longevity interventions: dietary restriction and reduced IIS. As treatments are developed for age-related pathologies based on dietary restriction and IIS and moved to clinical trial, these findings suggest that caffeine intake might be an important factor in predicting treatment outcomes.

Invertebrate models have been invaluable in identifying and exploring novel processes involved in the determination of life span. Many discoveries in these models have already led to new areas of mammalian research, and even to clinical trials in a few cases. As our knowledge of the complexity of the aging process continues to grow, our understanding of the challenges inherent in applying that knowledge to human populations will become more complete. This will ultimately lead to clinical interventions to increase longevity and treat age-associated disease with a more precise understanding of the outcomes based on individual genotype and environmental influences.

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Appendix: Acronyms Used in this Dissertation

AMPK	adenosine monophosphate-activated kinase
CCE	counter flow centrifugation elutriation
CR	caloric restriction (see also DR, sDR)
dilp	*Drosophila* insulin-like peptide
DR	dietary restriction (see also CR, sDR)
Dyf	neuronal dye-filling abnormal phenotype
ERC	extrachromosomal rDNA circle
FNR	false negative rate
FPR	false positive rate
FUdR	floxuridine
GHRKO	growth hormone receptor knockout
GO	gene ontology
HMG	high mobility group
HSF	heat shock factor
IIS	insulin/IGF-1-like signaling
IPTG	β-D-isothiogalactopyranoside
IRS	insulin receptor substrate
LL	long-lived
MEP	mother enrichment program
MNC	median neurosecretory cell
mTOR	mammalian target of rapamycin
mTOR	mammalian target of rapamycin complex
NE	no effect
NGM	nematode growth media
NLL	not long-lived
NSE	no significant extension
OD	optical density at 600 nm
ORF	open reading frame
PI3K	phosphatidylinositol 3-kinase

PKA	AMP-dependent protein kinase
PSG	*pmr1*Δ suppressor of glycerol growth defect
rDNA	ribosomal DNA
RNAi	RNA interference
Rol	roller phenotype
ROS	reactive oxygen species
RPL	ribosomal protein of the large subunit
RPS	ribosomal protein of the small subunit
S6K	ribosomal protein S6 kinase
SD	synthetic defined
sDR	solid dietary restriction (see also CR, DR)
SIR	silent information regulator
SL	short-lived
TIF	translation initiation factor
TOR	target of rapamycin
TOR	target of rapamycin complex
uORF	upstream open reading frame
UPR	unfolded protein response
YEP	yeast extract peptone

VITA

George L. Sutphin was born in Seattle, Washington. At the University of Washington he earned a Bachelor of Science and Master of Science in Aeronautics and Astronautics. Following these degrees he developed an interested in the biology of aging and left the aerospace field to study biology. In 2012 he earned a Doctor of Philosophy from the University of Washington in Molecular and Cellular Biology.