

Chapter 12

Genome-Wide Analysis of Yeast Aging

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Abstract In the past several decades the budding yeast *Saccharomyces cerevisiae* has emerged as a prominent model for aging research. The creation of a single-gene deletion collection covering the majority of open reading frames in the yeast genome and advances in genomic technologies have opened yeast research to genome-scale screens for a variety of phenotypes. A number of screens have been performed looking for genes that modify secondary age-associated phenotypes such as stress resistance or growth rate. More recently, moderate-throughput methods for measuring replicative life span and high-throughput methods for measuring chronological life span have allowed for the first unbiased screens aimed at directly identifying genes involved in determining yeast longevity. In this chapter we discuss large-scale life span studies performed in yeast and their implications for research related to the basic biology of aging.

Keywords Acetic acid · Apoptosis · Asymmetric segregation · Chronological life span · Counter flow centrifugation elutriation (CCE) · Dietary restriction (DR) · Genome-wide · Genomics · High-throughput · Loss of heterozygosity (LOH) · Metabolomics · Microarrays · Mitochondria · Mitochondrial back-signaling · Mother Enrichment Program (MEP) · Oxidative damage · Proteomics · Replicative life span · Retrograde response · Ribosomal DNA (rDNA) · Sirtuins · Target of rapamycin (TOR) signaling · Translation · Worms · Yeast Outgrowth Data Analysis (YODA)

Abbreviations and Acronyms

CCE	counter flow centrifugation elutriation
DR	dietary restriction
ERC	extrachromosomal rDNA circles
FNR	false negative rate
FPR	false positive rate
LL	long-lived
LLM	long-lived mutant

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MEP	mother enrichment program
NLL	not long-lived
NSE	no significant extension
OD	optical density
ORF	open reading frame
PKA	protein kinase A
ROS	reactive oxygen species
SL	short-lived
SIR	silent information regulator
TOR	target of rapamycin
uORF	upstream open reading frame

Introduction

The budding yeast *Saccharomyces cerevisiae* has been used as a model of cellular aging for more than 6 decades (Fabrizio and Longo 2007; Jazwinski 2005; Kaerberlein et al. 2007; Steinkraus et al. 2008). *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.

Two distinct aging paradigms have been described in yeast: replicative and chronological (Kaerberlein 2006). Replicative aging is the better characterized of the two and refers to the progressive loss of replicative capacity of a cell during vegetative growth (Steinkraus et al. 2008). Replicative life span is typically measured by microdissection of daughter cells away from mother cells and counting the number of daughter cells produced by each mother cell prior to senescence (Mortimer and Johnston 1959; Steffen et al. 2009). Chronological aging, in contrast, refers to the decreased ability of non-dividing cells to re-enter the cell cycle over time. Several methods for measuring chronological life span have been described. The most common variant is to culture cells into proliferative arrest in synthetic defined (SD) media while monitoring survival by periodically plating serial dilutions onto rich media and quantifying colony forming units per unit volume of aging culture (Fabrizio and Longo 2003; Murakami and Kaerberlein 2009). The ability to measure both replicative and chronological life span in yeast provides the opportunity to independently study the aging process for both dividing and non-dividing cell types in the same organismal system.

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 yeast open reading frames (ORFs). The yeast ORF deletion collection contains more than 20,000 unique strains (6061 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. This chapter 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.

Yeast Replicative Life Span

A Brief History of Yeast Replicative Aging

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 (discussed below), 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).

Since these early morphology-based studies, yeast replicative aging has become 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 (DR), sirtuins, TOR signaling, and mitochondrial metabolism (Table 12.1).

Table 12.1 Genetic interactions of major yeast aging pathways with respect to replicative life span

Pathway	Intervention	Genetic background			
		<i>sir2</i>	<i>fob1</i>	<i>sir2 fob1</i>	<i>tor1</i>
DR	0.05% glucose	N.E.	↑	↑	N.E.
TOR Signaling	<i>TOR1</i> deletion	N.E.	↑	↑	N.E.
Sir2 Fob1	<i>SIR2</i> activation	N.E.	N.E.	N.E.	↑
Sir2 Fob1	<i>FOB1</i> deletion	↑	N.E.	N.E.	↑

The data in this table was compiled from numerous sources (Kaeberlein et al. 1999, 2004, 2005b, c; Lamming et al. 2005; Lin et al. 2000; Tsuchiya et al. 2006)

Table entries indicate effect of each intervention on replicative life span in each genetic background (N.E. = 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 extrachromosomal ribosomal DNA (rDNA) circles, or 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, both phenotypes that correlate with longevity (Kennedy et al. 1995). The SIR complex includes Sir2, Sir3, and Sir4 and acts to repress transcription at telomeres, 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 SIR complex from the telomeres and silent mating-type locus to rDNA (Kennedy et al. 1995, 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 12.2) (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. 1999, 2004; 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;

Table 12.2 Effects of yeast aging pathways on life span and secondary, age-associated phenotypes

Pathway	Gene or intervention	Phenotype						
		Replicative life span	Chronological life span	Oxidative stress resistance	Thermo-tolerance	Telomere silencing	rDNA recombination	rDNA silencing
DR	0.05% glucose	↑	↑	↑	↑	No effect	No effect	No effect
TOR Signaling	tor1	↑	↑	↑	↑	No effect	↓	No effect
Sir2/Fob1	sir2	↓	No effect	↓	?	↓	↑	↓
Sir2/Fob1	fob1	↑	?	?	?	?	↓	↓
Sir2/Fob1	sir2 fob1	No effect	?	?	?	?	↓	?

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. 1999, 2004 2005a, b; Kaeberlein and Powers 2007; Lin et al. 2000, 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, 2009)

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 12.2), 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 12.2) (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 12.2). 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 subtelomeric 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 H_2O_2 (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). One dilemma arising from a DNA damage model is that aging wild-type yeast do not appear to pass heritable mutations to daughter cells, which is based on the observation that daughter cells from aged mothers, although short-lived themselves, eventually produce progeny with full life span potential (Kennedy et al. 1994).

Although *SIR2* orthologs play a role in life span in both *Caenorhabditis elegans* and *Drosophila melanogaster*, there is no evidence that accumulation of ERCs or other non-chromosomal self-replicating DNA elements contribute to aging in these species, suggesting that an ERC-independent mechanism of longevity extension by Sir2 may be responsible for the apparently conserved action of Sir2 on longevity across eukaryotic species. Sirtuins remain a hot topic in aging research and further clarification of their complex role in controlling life span is anticipated (Finkel et al. 2009).

Dietary Restriction

DR, which refers to a reduction in nutrient intake without malnutrition, is the most universally effective intervention to extend life span across a wide range of eukaryotic species. Yeast replicative aging has been used extensively to study the molecular and genetic factors involved in the life span extension resulting from DR. In yeast, DR 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). A less commonly used form of DR involving restriction of amino acids has also been shown to extend life span (Jiang et al. 2000). Genetic models of DR are also available, including deletion of *HXX2*, which encodes a hexokinase responsible for converting glucose into glucose-6-phosphate for entry into the glycolytic pathway (Walsh et al. 1983). Deletion of *HXX2* 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 DR acts to extend life span in yeast are not yet known; however, it is commonly thought that DR manipulates these mechanisms, at least in part, by influencing several partially redundant nutrient-responsive signaling kinases, including target of rapamycin (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 DR (Fabrizio et al. 2004; Kaeberlein et al. 2005c; Lin et al. 2000). TOR signaling is a nitrogen sensitive pathway regulated by glutamine levels and carbohydrate levels that controls a variety of cellular processes, including mRNA translation. TOR is discussed in greater detail later in the chapter. 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 insulin/IGF-1-like signaling (Burgering and Coffey 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 insulin/IGF-1-like signaling pathway, Sch9 may fulfill an equivalent role in yeast to both Akt and S6 kinases in multicellular eukaryotes.

A conclusive answer has not yet been reached to the question of what downstream mechanisms mediate replicative life span extension by DR. Two non-mutually exclusive models have been proposed: increased sirtuin activity and altered mRNA translation (Kaeberlein et al. 2005c; Medvedik et al. 2007; Steffen et al. 2008). DR may activate Sir2 by either elevating NAD levels through increased respiration (Lin et al. 2002) or by increasing transcription of *PCN1* in an Msn2/4 dependent manner. *PNC1* is necessary for the full life span extension from DR (Anderson et al. 2003; Lin et al. 2004) and encodes an enzyme that deaminates nicotinamide, which otherwise inhibits Sir2. Contrary to the idea of sirtuins as mediators of DR, *SIR2* is not required for the replicative life span extension caused by DR (Table 12.1). Specifically, DR does not increase replicative life span in the short-lived *sir2* Δ background (Kaeberlein et al. 2004; Lin et al. 2000), but when the short life span of *sir2* Δ is repressed by deletion of *FOB1*, DR robustly extends replicative life span (Kaeberlein et al. 2004; Lamming et al. 2005). One proposed explanation is that in the absence of Fob1, other sirtuins (such as Hst2) are activated by DR to repress ERC formation (Lamming et al. 2005), though this result has not yet been

independently verifiable for unknown reasons (Kaeberlein et al. 2004; Tsuchiya et al. 2006). Two recent studies found that DR does not alter transcriptional silencing at the rDNA (Riesen and Morgan 2009; Smith Jr et al. 2009), indicating that DR does not extend life span by increasing rDNA silencing via increased Sir2 activity. Interestingly, rDNA recombination was decreased by DR despite the lack of change in rDNA silencing. DR reduced rDNA recombination to a similar degree in both wild type yeast and strains lacking *SIR2*.

An alternate model places DR and Sir2 in separate pathways with respect to replicative life span. ERC levels may limit replicative life span in yeast lacking *SIR2*, such that all cells die from ERC toxicity before the beneficial effects of DR can be realized (Kaeberlein et al. 2004). Removing ERCs as a limiting factor by deleting *FOB1* or overexpressing *SIR2* thus allows the typical extension of replicative life span in response to DR (Kaeberlein et al. 2004). The debate as to whether sirtuins act downstream of DR is ongoing and interested readers are referred to several reviews that discuss the topic in detail (Chen and Guarente 2007; Kaeberlein and Powers 2007; Kennedy et al. 2005; Longo and Kennedy 2006; Sinclair 2005).

Mediation of the beneficial effects of DR via altered translation through reduced TOR signaling is more straightforward. Reduced TOR signaling is known to decrease ribosome levels (Jorgensen et al. 2004; Powers et al. 2004), and DR does not further extend long-lived mutants lacking either *TOR1* or a ribosomal large subunit gene (Steffen et al. 2008). The mechanism by which reduced translation might extend replicative life span is not known. One possibility is that an overall reduction in translation may slow accumulation of damaged or misfolded proteins. An alternative but not mutually exclusive explanation is that reducing overall translation differentially alters translation of specific mRNAs involved in life span determination (see discussion of *GCN4* below) (Steffen et al. 2008).

Asymmetric Segregation, Oxidative Damage, and Mitochondria

An aspect of yeast replicative aging that has generated much interest 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). This change is not caused by late-life heritable mutations, as subsequent asymmetric division results in progeny with renewed replicative potential (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).

Reactive oxygen species (ROS) have long been at the center of the debate on causes of aging and 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 orthologues 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 (Buttner 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).

Genome-Wide Analysis of Replicative Life Span

True high-throughput methods for quantitatively measuring replicative life span in yeast have yet to be described. Replicative life span determination currently requires 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 *MAT α* deletion set contains ~4800 strains with an average life span for the parental strain (BY4742) of approximately 26 generations (daughter cells produced) (Kaeberlein et al. 2005b). In order to screen the entire *MAT α* 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.

In 2005, 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. 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 *MAT α* 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 (Fig. 12.1) (Kaeberlein et al. 2005c). Based on the average replicative life span of these 5 cells relative to the wild type parental strain, each mutant is given a putative longevity classification. Additional cells are assayed for strains that show potential for long life span until a definitive classification can be made. Once a deletion mutant has been definitively classified as long-lived in the *MAT α* background, the corresponding deletion from the *MAT α* 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 (*hvk2 Δ* , *gpa2 Δ* , *gpr1 Δ* , and *fob1 Δ*). 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.

The replicative life span data for wild-type mother cells (N > 500) were pooled into one set (WT) and replicative life span data for the mother cells of long-lived mutants (LLMs) *hvk2 Δ* , *gpa2 Δ* , *gpr1 Δ* , and *fob1 Δ* (N > 500) were pooled into a second set. Probability distributions were then generated for mean life span as

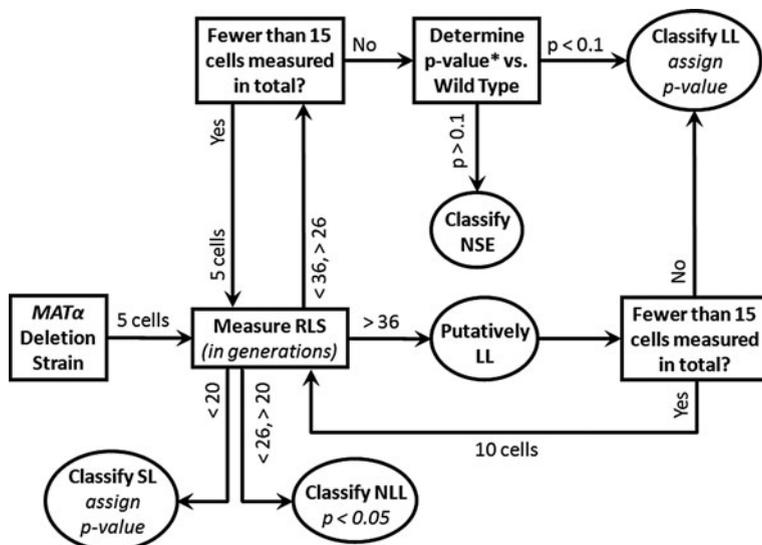


Fig. 12.1 Flow diagram illustrating the iterative approach for identifying long-lived strains from the yeast *MATα* deletion collection. LL – Long-Lived; NSE – No Significant Extension; NLL – Not Long-Lived; SL – Short-Lived. Mutations classified as LL by this process are subsequently verified as replicatively long-lived in the *MATa* background. P-values are for comparison of mean replicative life span for the deletion strain in question to the cumulative probability distribution for BY4742 wild type 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

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, we were able to establish that an iterative strategy initially analyzing the replicative life span of 5 cells per deletion strain should allow us to identify a large fraction of strains with mean life span greater than 30% longer than wild type.

In the final iterative method (Fig. 12.1), 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 *MATa* background by determining the replicative life span for the corresponding deletion strain contained in the *MATa* deletion collection.

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, each plate 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”, with each strain coded in a manner such that the researcher performing the microdissection has no knowledge of the identity of any strain until after the experiment is completed.

The iterative strategy described substantially reduces the manual labor necessary to identify gene deletions that confer increased replicative life span. Even with this advantage, screening the entire *MATa* deletion collection requires a significant investment of time and effort and is still in progress. An initial screen was performed to characterize the replicative aging properties for 564 single-gene deletion strains (Kaeberlein et al. 2005c). Of the 564 deletion mutants, 13 were verified to have extended replicative life span relative to the parental strain (Kaeberlein et al. 2005c). The expected false positive rate is low due to validation of long-lived strains by testing of an independently derived deletion allele from the *MATa* deletion collection. The false negative rate is not known. For comparison, it is worth noting that the percent of deletions conferring increased replicative life span for the initial 564 strains examined (~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 screen) (Smith et al. 2007). 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.

Since this initial report, 5 cell replicative life span data has been obtained for the entire haploid MAT α ORF deletion collection (our unpublished data). Iterative validation and verification is ongoing. To date, 87 single-gene deletions have been identified from this screen as having increased replicative life span in both haploid mating types of which 51 have been published and are listed in Table 12.3 (Kaeberlein et al. 2005b, c; Managbanag et al. 2008; Smith et al. 2008; Steffen et al. 2008). The largest functional group represented in this list is genes involved in mRNA translation, including ribosomal proteins, translation initiation factors, *TOR1*, and *SCH9*. Also represented are genes involved in transcription, post-translational protein modification and processing, metabolism, and cell wall integrity.

Table 12.3 Genes for which deletion results in increased replicative life span identified from an ongoing screen of the haploid yeast ORF deletion collections. The genes with nematode orthologs indicated were identified as part of the worm to yeast ortholog screen for conserved longevity determinants (Smith et al. 2008)

Yeast ORF	Yeast gene	Nematode ORF	Nematode gene	Function
YNR051C	BRE5			Molecular function unknown
YBL087C	RPL23A			Structural constituent of ribosome
YBRO84C-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

Table 12.3 (continued)

Yeast ORF	Yeast gene	Nematode ORF	Nematode gene	Function
YFR032C-A	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
YJR094W-A	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 (NAB) activity
YNL229C	URE2			Transcription co-repressor activity

Table 12.3 (continued)

Yeast ORF	Yeast gene	Nematode ORF	Nematode gene	Function
YNR030W	ALG12	T27F7.3		Alpha-1,6-mannosyltransferase activity
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 ^a		C01G8.5	erm-1	Molecular function unknown
YHR205W ^a	SCH9	Y47D3A.16	rsk-1	Protein serine/threonine kinase activity

^a Indicates genes published a part of the worm to yeast ortholog study but not verified in the *MATa* background.

The data in this table was compiled from numerous sources (Kaeberlein et al. 2005b, c; Managbanag et al. 2008; Smith et al. 2008; Steffen et al. 2008)

Insights into Mechanisms of Replicative Aging from Genome-Wide Screens

TOR Signaling Links Nutrient Availability and Replicative Life Span

The 51 long-lived gene deletion strains reported to date 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, initial analysis of 564 randomly selected deletion strains led to the hypothesis that DR is mediated primary via reduced signaling through the target of rapamycin (TOR) kinase (Kaeberlein et al. 2005c). 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. 2005c). TOR kinases are evolutionarily conserved proteins that function to mediate mRNA translation, cell growth, metabolism, degradation, and stress resistance (among other processes) in response to nutrient and growth factor cues (Table 12.2) (Arsham and Neufeld 2006; Martin and Hall 2005). Mutations that decrease TOR activity have also been reported to increase life span in both *C. elegans* (Jia et al. 2004; Vellai et al. 2003) and *D. melanogaster* (Kapahi et al. 2004), suggesting an evolutionarily conserved link between TOR signaling and aging.

Unlike most multicellular eukaryotes, yeast has two TOR paralogs: *TOR1* and *TOR2*. Tor1 is believed to function specifically in the rapamycin-sensitive TOR complex 1 (TORC1), while Tor2 functions in both TORC1 and TOR complex 2 (TORC2). While Tor2 is essential, strains lacking *TOR1* are viable and have increased replicative life span (Table 12.1), suggesting that TORC1 is an important player in replicative aging. Epistasis analysis with respect to replicative life span places *TOR1* in a genetic pathway that includes DR, but is independent of *SIR2* and *FOBI* (Table 12.1) (Kaeberlein et al. 2005c). This relationship between DR and TOR is supported by studies carried out in both worms and flies (Hansen et al. 2008; Kapahi et al. 2004), indicating that TOR signaling may represent an evolutionarily conserved nutrient response pathway important for mediating the longevity effects of DR.

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). 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 insulin/IGF-1-like signaling (Melendez et al. 2003). Several recent studies have also uncovered an important role for autophagy in the response to DR. DR 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 DR 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, *tor1* Δ 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. 2005c). Thus, like autophagy, the importance of mitochondrial metabolism in TOR-mediated control of replicative life span remains unclear.

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 also seems 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 DR (Lin et al. 2000).

Regulation of mRNA Translation Modulates Replicative Life Span

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). The yeast S6 kinase, Sch9, is known to also modulate replicative life span and genetically maps to the same epistasis group as DR and TOR, consistent with a role downstream of TOR in modulating aging (Fabrizio et al. 2004b; Kaerberlein et al. 2005c).

In the initial analysis of 564 deletion strains, strains lacking two different genes coding for ribosomal large subunit proteins (*rpl31a* Δ and *rpl6b* Δ) were among the long-lived mutants (Kaerberlein et al. 2005c). 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 (*tif1* Δ , *tif2* Δ , and *tif4631* Δ) have been found to increase replicative life span from the deletion set analysis (Table 12.3) (Steffen et al. 2008).

A recent study identified Gcn4 as a potential mediator of reduced mRNA translation with respect to life span (Steffen et al. 2008). Gcn4 is a transcription factor that 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 expression of a Gcn4-luciferase reporter was upregulated in strains lacking *RPL20B* and *RPL31A*, and that deletion of *GCN4* partially blocked the life span extension resulting from DR, deletion of *TOR1*, deletion of *SCH9*, or deletion of an *RPL*.

Cellular levels of Gcn4 are translationally regulated by four small inhibitory upstream open reading frames (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).

Yeast Chronological Life Span

As briefly described in the [Chapter 1](#), Introduction, chronological life span is typically determined by growing yeast cells into stationary phase and monitoring cell survival over time. Detailed descriptions of prior studies in this area are provided elsewhere in this book, and we refer interested readers to the chapters written by Fabrizio and Longo ([Chapter 5](#)), Werner-Washburne et al. ([Chapter 6](#)), and Piper ([Chapter 7](#)). Unlike replicative aging, which involves maintaining cells on solid media and physically removing daughter cells from individual mother cells, the conditions for chronological aging experiments are much more amenable to high-throughput analysis. In this section, we describe the development of new methods for monitoring yeast chronological survival and their application to identify long-lived mutants from the yeast ORF deletion collection. We also discuss insights gained from these genome-wide studies of chronological aging.

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 and identified five deletion strains with increased chronological life span: *gln3Δ*, *lys12Δ*, *mep3Δ*, *mep2Δ*, and *apg1Δ*.

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 μ L of the aging culture is inoculated into 145 μ L 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 (Fig. 12.2a). A survival curve can be generated from the Bioscreen growth data based on the estimated fraction of cells retaining viability at each time point (Fig. 12.2b). 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 , Δt_n is the time shift between the outgrowth curves at OD = 0.3 for the initial and n th 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).

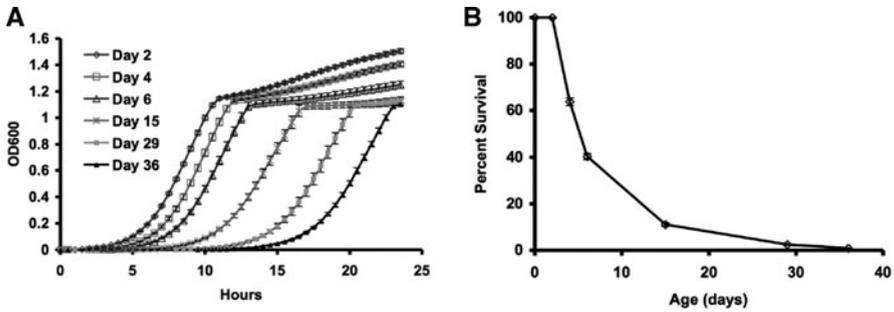


Fig. 12.2 Chronological life span survival curves are calculated based on time delay for strain specific outgrowth. **a** Outgrowth curves shift rightward as stationary phase cells age. **b** Survival curves are calculated from the time shift between outgrowth curves

Insights into the Mechanisms of Chronological Aging from Genome-Wide Studies

TOR Signaling Promotes Chronological Aging

As mentioned previously, the nutrient sensitive TOR pathway appears to play an important role in both replicative and chronological life span. 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 DR (Table 12.2) (Fabrizio et al. 2001; Kaerberlein et al. 2005c; Powers 3rd et al. 2006). DR 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 DR is believed to be independent of Sir2. In contrast to replicative life span, the mechanism by which reduced TOR signaling and DR promote chronological life span appears to be mediated primarily by regulation of carbon metabolism, which is discussed in detail below (Bonawitz et al. 2007).

The chronological life span screen of the homozygous diploid ORF deletion collection performed by Powers et al. (2006) identified 16 genes implicated in the TOR pathway. Of these, 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 3rd et al. 2006). Powers et al. (2006) also demonstrate that pharmacological inhibitors of TOR signaling (methionine sulfoximine and rapamycin) increase chronological life span.

Acetic Acid Is a Molecular Cause of Chronological Aging

Ongoing studies are currently aimed at using the Bioscreen method described above 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. 2007), DR 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 DR 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 alkalized 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 12.4). 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* lifespan, (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 lifespan 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

Table 12.4 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
<i>sch9Δ</i>	X	X
<i>ras2Δ</i>		X
<i>tor1Δ</i>	X	
<i>HAP4</i> overexpression	X	
<i>ADH1</i> overexpression	X	
<i>adh2Δ</i>	X	
<i>ycalΔ</i>		X

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.

Recent Genome-Scale Screens Identify New Potential Chronological Aging Pathways

The past 2 years have seen the emergence of a competitive-survival strategy for identifying genes involved in chronological aging. In this strategy, the ~4800 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 Maticic 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; Maticic 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.

Alternative High-Throughput Methods for Studying Aging

Measurement of life span is the only way to directly determine whether an intervention influences the aging process. With that acknowledged, indirect, genome-scale approaches to studying age-related phenomena can provide valuable insight into processes involved in normal aging and the determination of life span. Such approaches include microarrays for studying genome-wide transcriptional changes, proteomic techniques for looking at protein interactions and changes in protein content, and metabolomic approaches that measure the range of small molecule metabolites present in an organism. Such techniques have been useful primarily in cases where a large number of relevant cells can be obtained for analysis, such as studying the background effects of interventions and genes that influence aging and for studying changes that occur with chronological age. Historically, relatively pure populations of replicatively aged cells have been difficult to obtain in large enough numbers to be useful for such large scale approaches. In the past few years techniques have become available that allow enrichment for replicatively aged cells from mixed populations. In addition, new methods are being developed with potential for efficient high-throughput measurement of replicative life span.

Emerging High-Throughput Strategies for Studying Replicative Aging

Biochemical and genomic studies of replicative aging are 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 produced cell populations highly enriched for cells aged up to 20 generations (Egilmez et al. 1990). This technique was used in a transcriptome study to identify the long-lived *LAG1* deletion strain (D’Mello 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 a 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.

Microarrays, Proteomics, and Metabolomics

Genome-scale technologies have become standard throughout the biological sciences and have been applied to study yeast 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. (2001) 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. Specifically, pure populations of chronologically aged cells are easy to obtain in large quantities using the same media conditions used to measure chronological life span.

An alternative microarray approach to comparing yeast of different ages is to compare the expression profiles of different age-matched long-lived mutants and look for pattern similarities that may identify genes that are generally important in the aging process. 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, b). The expression patterns implied an overall reduction in transcription in the three mutant strains as compared to wild type, as well as a downregulation in genes involved in the TCA cycle and oxidative phosphorylation relative to genes involved in glycolysis. The *ras2* Δ strain also showed a reduced expression of genes involved in mitosis, distinguishing it from the other two long-lived mutants.

Microarrays are currently the standard approach used to measuring transcript levels in a cell population or tissue. 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 DR 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 which 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.

Comparison of Yeast Aging to a Multicellular Eukaryote

The ability to measure life span on a genome-wide scale in yeast has made it possible, for the first time, to compare the degree to which genetic control of aging is shared between yeast and multicellular model organisms. Another invertebrate model organism widely used to aging is the nematode *C. elegans*. Using the same iterative approach described above for identifying long-lived yeast strains, Smith et al. (2008) sought to determine which yeast homologues of known worm aging genes played a similar role in determining yeast replicative life span. Underlying this analysis was the rationale that if genetic control of aging has been evolutionarily conserved, then yeast homologs of worm longevity associated genes should be more likely to influence longevity than randomly selected yeast genes.

Smith et al. (2008) began with a set of 276 *C. elegans* genes reported to increase adult life span when function is decreased. A majority of these genes were identified from large-scale RNAi screens (Curran and Ruvkun 2007; Dillin et al. 2002; Hamilton et al. 2005; Hansen et al. 2005; Lee et al. 2003). Since decreased function led to increased life span in *C. elegans*, strains from the ORF deletion collection lacking genes corresponding to homologs of the worm genes could be examined for replicative life span in yeast. In order to identify ortholog pairs between worms and yeast, a two-tiered approach was taken in which a high-stringency set of ortholog pairs and a lower stringency set of homolog pairs were defined. The high stringency set of ortholog pairs was assembled based on a modified reciprocal BLASTp best match algorithm. The low stringency set of homologs included all cases in which one or more yeast proteins could be identified with at least 20% sequence identity and 10% amino acid alignment to the worm aging protein, with a maximum of 6 yeast homologs selected per worm gene. From 276 worm aging genes, 264 non-essential yeast genes (viable as single-gene deletions) were identified in the low stringency homolog set, of which 78 also met the high-stringency ortholog criterion (Smith et al. 2008). Replicative life span analysis was performed on each of the 264 single-gene deletion strains contained in the low-stringency homolog set. Using the iterative process described above, 25 single-gene deletions from this set were determined to be long-lived, of which 11 were also in the high-stringency ortholog criteria (Table 12.3). In both the high- and low-stringency sets, the frequency of strains with increased replicative life span (14.1 and 9.5% respectively) significantly exceeded the frequency expected based on the analysis of 564 randomly chosen deletion strains (2.3%), as well as the adjusted frequency considering only yeast genes with worm homologs (3.4%) (Kaeberlein et al. 2005c).

The results of Smith et al. (2008) allow for the conclusion that genetic control of longevity has been evolutionarily conserved between yeast and worms (Smith et al. 2008). The fact that this conservation is observed for yeast replicative aging, but not for chronological aging (Burtner et al. 2011), is surprising, since *C. elegans* are primarily post-mitotic as adults and thus intuitively closer to a model of chronological aging. The mechanistic underpinnings of this conservation remain unknown, although the conserved longevity factors identified by Smith et al. (2008)

are substantially enriched for genes that code for proteins involved in regulating mRNA translation. Among the 25 homolog pairs, only two were previously known to modulate aging in both yeast and worms: *TOR1/let-363* and *SCH9/rsks-1*. *SCH9* and *rsks-1* are functional orthologs 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*, and *TIF4631/fig-1*). Given that TOR and S6K are known to negatively regulate both ribosome biogenesis and translation initiation factor activity, it is reasonable to speculate that all of these factors act in a single conserved longevity pathway.

Two yeast homologs of *C. elegans* aging genes, *TOR1* and *SCH9*, are known to modulate both replicative and chronological life span. As mentioned previously, the proteins encoded by these genes are thought to act in a pathway to mediate both replicative and chronological life span extension in response to DR, and parallel studies in *C. elegans* have placed TOR downstream of DR (Hansen et al. 2008). Interestingly, the life span extending effects of DR and TOR signaling in *C. elegans* require autophagy, which has not yet been shown in yeast. This raises the intriguing possibility that TOR signaling and DR modulate longevity by three different mechanisms: altered mRNA translation for yeast replicative aging, altered carbon utilization and acetic acid production for yeast chronological aging, and increased autophagic protein degradation for *C. elegans*. Additional studies are likely to clarify whether the mechanistic details are truly different or whether underlying commonality exists.

Conclusion

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. A subset of homologs of 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.

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