

# A Method for High-Throughput Quantitative Analysis of Yeast Chronological Life Span

Christopher J. Murakami,<sup>1</sup> Christopher R. Burtner,<sup>2</sup> Brian K. Kennedy,<sup>2</sup>  
and Matt Kaeberlein<sup>1</sup>

Departments of <sup>1</sup>Pathology and <sup>2</sup>Biochemistry, University of Washington, Seattle.

Chronological aging in yeast has been studied by maintaining cells in a quiescent-like stationary phase culture and monitoring cell survival over time. The composition of the growth medium can have a profound influence on chronological aging. For example, dietary restriction accomplished by lowering the glucose concentration of the medium significantly increases life span. Here we report a novel high-throughput method for measuring yeast chronological life span by monitoring outgrowth of aging cells using a Bioscreen C MBR machine. We show that this method provides survival data comparable to traditional methods, but with decreased variability. In addition to reducing the glucose concentration, we find that elevated amino acid levels or increased osmolarity of the growth medium is sufficient to increase chronological life span. We also report that life-span extension from dietary restriction does not require any of the five yeast sirtuins (Sir2, Hst1, Hst2, Hst3, or Hst4) either alone or in combination.

**Key Words:** Longevity—Dietary restriction—Glucose—Amino acids—Osmolarity—Sir2.

**E**NVIRONMENTAL nutrients have been shown to influence aging in a variety of organisms. For example, dietary restriction (DR), defined as a reduction in nutrient availability without malnutrition, increases life span and delays the onset of age-associated disease in yeast, worms, flies, and rodents (1,2). The mechanisms by which nutrient availability modulates longevity remain unclear, and a comprehensive analysis of how different dietary compositions affect aging has yet to be performed in any organism.

The budding yeast *Saccharomyces cerevisiae* has served as a model of organismal and cellular aging for more than 50 years (3). Two different types of aging have been described in yeast: replicative and chronological aging (4). Replicative life span (RLS) refers to the mitotic capacity of a yeast cell, as defined by the number of daughter cells produced by a mother cell prior to senescence. In contrast to RLS, chronological life span (CLS) refers to the length of time a nondividing cell can maintain viability, as defined by its ability to re-enter the cell cycle after a prolonged period of quiescence. Yeast CLS has therefore been adopted as a model of the viability of postmitotic cells.

Although both yeast aging paradigms have been generally accepted as valid organismal aging models, replicative aging has been more widely used and better characterized than chronological aging. In the replicative aging paradigm, life-span extension by DR has been described by either reducing the glucose concentration of the growth medium (5) or by reducing the total amino acid concentration (6). It was initially proposed that the increased RLS associated with DR was mediated by activation of the Sir2 histone deacetylase (5), which has been shown to promote longevity in yeast (7), worms (8), and flies (9). This model has since been challenged, however, by a series of studies reporting that RLS

extension from DR occurs in cells lacking Sir2 alone (6,10) or in cells lacking multiple Sir2-family proteins (sirtuins) (11,12). An alternative model has been proposed suggesting that DR increases RLS by decreasing the activity of the nutrient-responsive target of rapamycin (TOR) kinase along with Sch9 and protein kinase A (13). A recent report has suggested that decreased TOR signaling leads to activation of Sir2 via the stress-responsive transcription factors Msn2 and Msn4 (14); however, this model is difficult to reconcile with the observation that RLS extension from deletion of *TOR1* or chemical inhibition of TOR does not require Sir2 (13). Furthermore, genetic epistasis experiments definitively place TOR in a genetic pathway with DR that is distinct from the longevity-promoting activity of Sir2 (13).

Life-span extension from DR has also been described in the yeast chronological aging paradigm by transferring cells from spent culture medium to water (15) or by reducing the glucose concentration of the growth medium (16). Similar to the case for RLS, decreased activity of TOR, Sch9, or protein kinase A is sufficient to increase CLS (17,18), supporting the idea that these nutrient-responsive kinases may mediate the beneficial longevity effects of DR in both dividing and nondividing yeast cells. The downstream effectors of these kinases that are important for increased CLS in response to DR have yet to be determined; however, increased respiration, stress response, and autophagy have all been proposed to play a role (17,19,20).

CLS has traditionally been assayed by culturing cells into stationary phase in liquid culture and measuring the cell survival as a function of time by dilution and plating onto a nutrient-rich, agar-based medium (4). Viability is then calculated based on the number of colonies arising (colony forming units; CFUs) on the nutrient agar. However, this

Table 1. Yeast Strains Used in This Study

Strain	Genotype
BY4742	MAT $\alpha$ <i>his3</i> $\Delta$ 1 <i>leu2</i> $\Delta$ 0 <i>lys2</i> $\Delta$ 0 <i>ura3</i> $\Delta$ 0
KK102	BY4742 <i>sir2</i> $\Delta$ :: <i>kanMX</i>
KK76	BY4742 <i>hst1</i> $\Delta$ :: <i>kanMX</i>
KK77	BY4742 <i>hst2</i> $\Delta$ :: <i>kanMX</i>
KK78	BY4742 <i>hst3</i> $\Delta$ :: <i>kanMX</i>
KK79	BY4742 <i>hst4</i> $\Delta$ :: <i>kanMX</i>
MT043	BY4742 <i>sir2</i> :: <i>LEU2</i> <i>job1</i> :: <i>HIS3</i> <i>hst1</i> :: <i>URA3</i> <i>hst2</i> :: <i>kanMX</i> <i>hst3</i> :: <i>kanMX</i> <i>hst4</i> :: <i>kanMX</i> <i>lys2</i> $\Delta$ 0:: <i>LYS2</i>
PSY316AR	MAT $\alpha$ <i>ura3-52</i> <i>leu2-3,112</i> <i>his3-200</i> <i>ade2-101</i> <i>lys2-801</i> <i>rDN1</i> :: <i>ADE2</i>
KK245	PSY316AR <i>sir2</i> $\Delta$ :: <i>HIS3</i>
W303AR5	MAT $\alpha$ <i>ura3-1</i> <i>leu2-3,112</i> <i>trp1-1</i> <i>his3-11,15</i> <i>can1-100</i> <i>rad5-535</i> :: <i>RAD5</i> <i>rDN1</i> :: <i>ADE2</i>
KK244	W303AR5 <i>sir2</i> $\Delta$ :: <i>TRP1</i>

methodology requires a relatively large investment of investigator time and resources, and is not suited for high-throughput studies. Recently a high-throughput method for qualitatively measuring CLS was described in which cells are aged in 96-well microtiter plates (18). Rather than monitoring survival of individual cells based on CFUs, relative cell viability of the population was determined by diluting the aging culture into rich liquid medium and measuring the optical density (OD) at 600 nm following an 18-hour outgrowth at 30°C. All cell and liquid transfers are automated using a high-density replica pinning robot. Although less quantitative than the traditional methodologies, this drawback is offset by the ability to monitor survival for several thousand strains simultaneously.

This high-throughput CLS method was used to screen the yeast homozygous diploid open reading frame (ORF) deletion collection for long-lived mutants (18). The entire set of ~5000 deletion mutants was ranked based on relative survival, and among the 90 highest ranked strains, several contained deletions in genes implicated in signaling through the nutrient-responsive TOR signaling pathway (18). The finding that decreased TOR activity increases yeast CLS was a significant discovery from this study (18), and further strengthened the role of TOR as an evolutionarily conserved mediator of longevity (13,21–23). Only five deletion mutants (*gln3* $\Delta$ , *lys12* $\Delta$ , *mep3* $\Delta$ , *mep2* $\Delta$ , and *agp1* $\Delta$ ) were confirmed to have increased CLS from this genome-wide screen (18), however, and subsequent attempts to identify long-lived deletion mutants from among the highest-ranked strains from this screen have proven less successful (our unpublished data). Thus, we have concluded that, although useful for qualitatively identifying long-lived mutants from among a large collection of strains, the previously described high-throughput method (18) is not quantitatively rigorous.

Here we describe a novel method for determining CLS with improved quantitative resolution relative to the previously described assay (18), while also maintaining a capacity for higher-throughput studies than is possible with CFU-based methods. This method provides comparable precision with reduced variability and is easily adaptable to a variety of environmental and genetic perturbations. As proof of

Table 2. Synthetic Defined Medium Used for Chronological Aging Studies

Component	Concentration (g/L)
D-glucose	20
Yeast nitrogen base (–AA/AS)	1.7
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	5.0
Adenine	0.04
L-Arginine	0.02
L-Aspartic acid	0.1
L-Glutamic acid	0.1
L-Histidine	0.1
L-Leucine	0.3
L-Lysine	0.03
L-Methionine	0.02
L-Phenylalanine	0.05
L-Serine	0.375
L-Threonine	0.2
L-Tryptophan	0.04
L-Tyrosine	0.03
L-Valine	0.15
Uracil	0.1

*Note:* The standard recipe is shown. For some experiments, the concentration of D-glucose was varied as indicated. For experiments in which amino acid levels were varied, the concentration of all L-amino acids was varied proportionately, as indicated. Yeast nitrogen base did not contain amino acids (AA) or ammonium sulfate (AS). For experiments with BY4742 and PSY316AT, the concentration of lysine was increased to 0.15 g/L. For experiments with W303AR5, the concentration of tryptophan was increased to 0.2 g/L.

principle, we have used this method to explore the effects of medium composition on CLS and to comprehensively examine the importance of Sir2-family proteins in yeast chronological aging. We found that, between a range of 0.05% and 20% glucose, CLS correlates inversely with glucose concentration. Surprisingly, a direct correlation between total amino acid concentration and CLS was observed. We also found that CLS extension from DR by glucose depletion is not only independent of Sir2 as previously reported (24), but is also capable of increasing life span in cells simultaneously lacking all five yeast sirtuins.

## METHODS

### Strains and Media

Strains were derived from BY4742 (Open Biosystems, Huntsville, AL), W303AR5 (7) or PSY316AR (25). All mutant strains used in this study were either derived from the MAT $\alpha$  yeast ORF deletion collection (26) or were generated by transforming yeast with polymerase chain reaction (PCR)-amplified deletion constructs containing 45 nucleotides of homology to regions flanking the ORF to be deleted and either *HIS3*, *LEU2*, or *URA3* amplified from pRS403, pRS405, or pRS406 (27), respectively. In each case, the entire ORF of the deleted gene was removed. All gene disruptions were verified by PCR. Strains used in this study are listed in Table 1.

YPD medium contained 2% bacto peptone and 1% yeast extract supplemented with filter-sterilized glucose at 2%. The composition of the standard synthetic defined media used in this study is provided in Table 2. This medium

contains excess concentrations of leucine, histidine, and uracil to compensate for auxotrophies present in the laboratory strains used in this study. Additional auxotrophies were compensated for on a strain-by-strain basis as follows: For experiments with BY4742- and PSY316AR-derived strains, the concentration of lysine was increased to 0.15 g/L; for experiments with W303AR5-derived strains, the concentration of tryptophan was increased to 0.2 g/L. Cultures for chronological aging experiments were prepared by inoculating 50  $\mu$ L from a YPD overnight culture into 5 mL of the appropriate aging medium in culture tubes. Tubes were rotated continuously in a roller drum and maintained at 30°C in a water-jacketed incubator.

### *Bioscreen C MBR Outgrowth*

A Bioscreen C MBR (Growth Curves USA, Piscataway, NJ) machine was used for all outgrowth assays. For outgrowth of aged cells, 5  $\mu$ L of the aging culture was inoculated into 145  $\mu$ L of rich YPD (2% glucose, 2% bacto peptone, 1% yeast extract) medium in a Bioscreen Honeycomb 100-well plate (cat no. 9502550). Incubation of the plate is kept constant at 30°C, with the shaking module set to high continuous shaking. Absorbance readings at 600 nm (wideband range) are taken every 30 minutes for 24 hours. OD data were normalized for background prior to presentation by subtracting the initial OD value at  $t = 0$  from each subsequent OD reading.

*Calculation of viability from CFUs.*—To determine viability using the CFU method, stationary cultures were serially diluted in YPD to achieve a cell density of roughly  $1 \times 10^3$  to  $5 \times 10^3$  cells/mL. One hundred microliters of the dilution was spread onto YPD agar plates, incubated at 30°C for 2 days, and manually counted. All cultures were presumed to be 100% viable at day 2, with subsequent CFU measurements normalized to day 2 CFUs. Averages and standard deviations for at least three biological replicates were calculated for each experiment.

*Calculation of viability from Bioscreen data.*—The doubling time for each well in a Bioscreen assay,  $\delta_w$ , was determined by the maximal slope of the semilog plot of OD as a function of time. This value was defined as the median of the three lowest  $\delta$  values obtained for every consecutive pair of OD measurements for that well in that experiment.

For each age-point, a  $\Delta t$  value was calculated as the shift in the Bioscreen growth curve relative to the initial age-point for that strain (day 2). The  $\Delta t$  value was calculated by first determining the linear regression equation of the natural logarithm of  $OD_{600}$  as a function of time for each well ( $0.05 \leq OD_{600} \leq 0.3$ ). Based on the linear regression equation for each well, the time,  $t_{OD}$ , at which  $OD_{600} = 0.3$ , was estimated. This  $OD_{600}$  value was chosen because it is near the middle of the linear range on a plot of  $\ln(OD_{600})$  versus time. The  $t_{OD}$  value was calculated for each age-point and the time shift,  $\Delta t_n$ , was calculated as the difference of the  $t_{OD}$  for each age-point and the  $t_{OD}$  for the first age-point (day 2 of culture).

Relative survival for each strain at each age-point was calculated by the formula:

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

where  $v_n$  = viability at age-point  $n$ ,  $\Delta t_n$  equals the time shift between the outgrowth curves at the initial age-point and age-point  $n$  at  $OD = 0.2$ , and  $\delta$  equals the median of the  $\delta_w$  values calculated for that strain at each individual age-point. The Bioscreen method, as described here, was used to measure CLS in all of the experiments, except for the data shown in Figure 2 that were obtained by counting CFUs.

## RESULTS

### *Relative Cell Viability Quantified by a Shift in the Growth Curve*

To improve the quantitative capacity and increase the throughput of the previously described method for high-throughput CLS screening (18), we explored incorporation of a Bioscreen C MBR machine (Growth Curves) into yeast life-span analysis. The Bioscreen C MBR machine is a computer-controlled shaker/incubator/reader equipped with eight filters from 405 nm to 600 nm and a temperature control system that maintains the set temperature in each well with a 0.1°C accuracy. Plates specifically designed for use with the Bioscreen C MBR avoid condensation of liquid on the inside of the microplate lid and prevent volume loss from outer wells. Our group and others have used the Bioscreen C MBR to determine growth kinetics of yeast strains under a variety of conditions (12,28). We speculated that use of a Bioscreen C MBR machine to obtain OD readings every 30 minutes during outgrowth after dilution of the aging culture, rather than after a single fixed incubation period as had been previously done, would greatly improve the quantitative capacity for measuring relative cell survival over time.

To determine whether the Bioscreen C MBR machine could be used to accurately measure CLS, we carried out a proof-of-principle experiment with the haploid ORF deletion collection wild-type strain (BY4742) (26). BY4742 cells were aged in 5 mL of synthetic defined (SD) medium on a rotating drum, and viability was determined at each age-point by outgrowth in the Bioscreen C MBR machine. To monitor viability at each age-point, 5  $\mu$ L of the aging culture was inoculated into 145  $\mu$ L of YPD in one well of a Bioscreen Honeycomb plate. Outgrowth of the inoculated cells took place in the Bioscreen C machine at 30°C with continuous shaking. OD at the Bioscreen C wideband wavelength ( $\sim 600$  nm) was determined every 30 minutes for 24 hours (Figure 1).

The growth curves of BY4742 cells showed a distinct rightward shift with age, such that for a given OD value, the length of time required to achieve that value increased with age (Figure 2A). A survival curve was generated from the Bioscreen growth data, based on the estimated fraction of cells retaining viability at each age-point (Figure 2B). The viable fraction was calculated relative to the initial age-point (viability at day 2 is defined as 100%) based on the

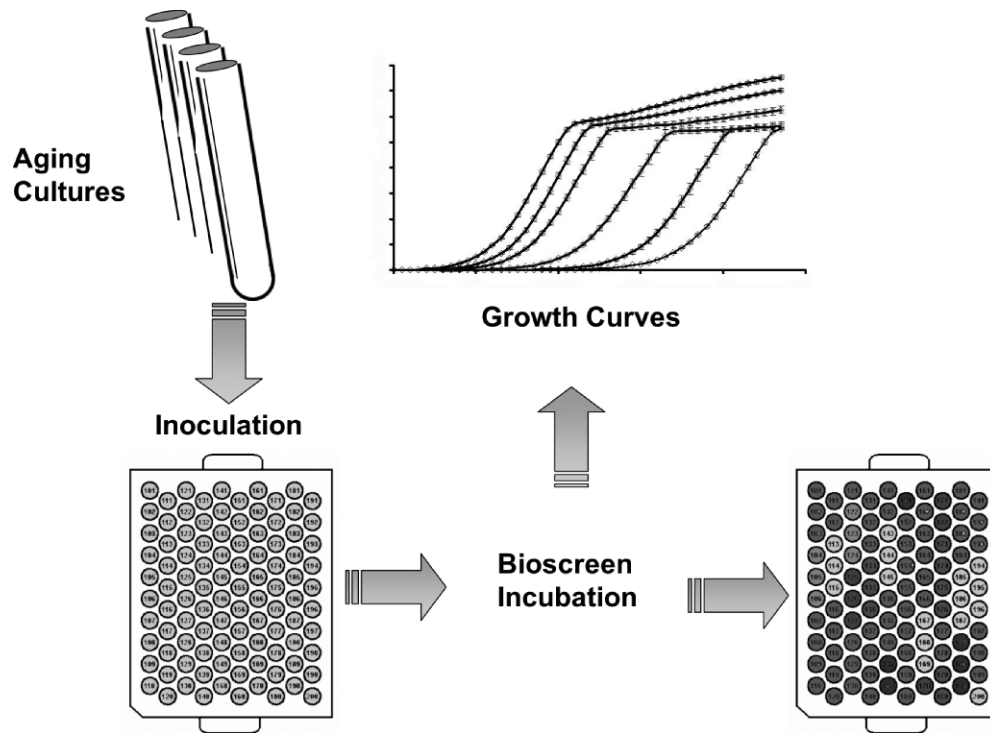


Figure 1. A new method for measuring yeast chronological life span. Aging cultures are kept at 30°C on a roller drum. At each age-point, 5  $\mu$ L from each aging culture is inoculated into 145  $\mu$ L of rich growth medium in an individual well of the Bioscreen Honeycomb plate. The Bioscreen machine incubates the plates at 30°C under constant agitation, while the optical density (OD) of each well is measured in intervals of 30 minutes. At the end of a specified incubation time (24 hours for our experiments), outgrowth curves for multiple strains can be plotted from the OD measurements as a function of time.

rightward time shift required for outgrowth to reach an OD value of 0.2 using the formula:

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

where  $v_n$  = viability at age-point  $n$ ,  $\Delta t_n$  equals the time shift between the outgrowth curves at the initial age-point and age-point  $n$  at OD = 0.2, and  $\delta$  equals the doubling time of the strain (determined by the maximal slope of the semilog plot of OD as a function of time).

#### Reduced Variability with the Bioscreen CLS Method

We next examined the relative variability of cell survival measurements using the Bioscreen C MBR machine versus serial dilution and plating for CFUs. A dilution series ranging from 1.25-fold to 1000-fold was generated from a 2-day-old culture of BY4742, and the relative survival was measured both by plating for CFUs and by using the Bioscreen C MBR machine. The fraction of viable cells inoculated, relative to the initial dilution, was calculated using the formula described above and was compared to the value based on known dilution. The coefficient of variation was calculated and compared between both methods (Table 3). Relative to CFUs, the Bioscreen CLS method provided reduced variance over the entire range of dilutions.

A similar trend was observed in the context of a chronological aging experiment involving two genetic backgrounds commonly used in aging experiments (W303AR5

and PSY316). At each age-point, the Bioscreen CLS method and the CFU method gave comparable estimations of survival (Figure 3). The Bioscreen method did, however, produce less variable measurements than the CFU method, as determined by the standard deviation of five biological replicates (error bars in Figure 3). This trend is most likely due to variation introduced during serial dilution and plating of cells, and has held up in multiple different experiments (our unpublished data).

In addition to survival, the Bioscreen CLS method provides information about the aging cells (such as doubling time and final density) that is not available using other methods. For example, we observed that the growth rate following dilution does not change substantially with chronological age, because normalizing for the  $\Delta t$  time shift associated with each age-point causes the growth curves to overlay (data not shown, but can be visualized in Figure 2A). From this, we conclude that chronological age does not lead to substantial genetic or epigenetic changes at the population level in wild-type cells sufficient to alter growth rate. It will be of interest to determine whether this trend is also observed in a variety of mutant backgrounds.

#### Effect of Glucose on CLS

We have used the Bioscreen CLS method experimentally to explore the relationship between CLS and DR. The largest increase in RLS from DR has been previously reported to occur at either 0.5% or 0.05% glucose, depending on the genetic background (11,12,29,30). We began our

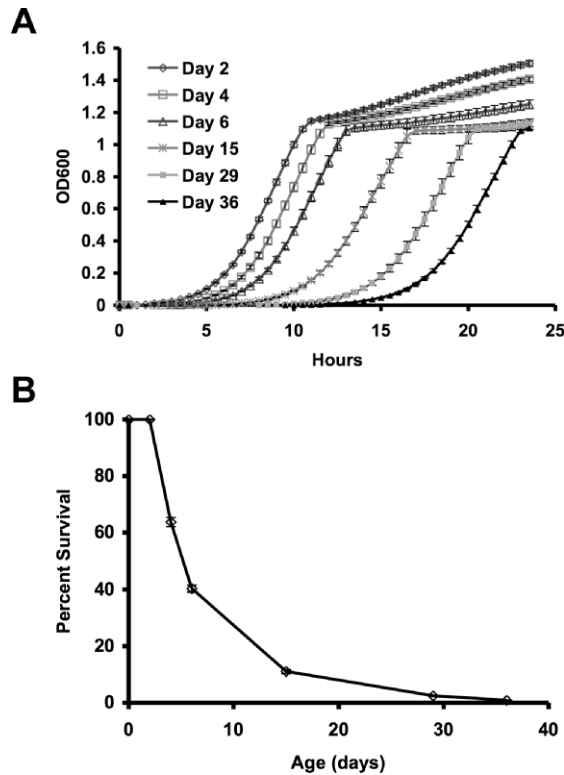


Figure 2. Determination of chronological life span using a Bioscreen C MBR machine. **A**, Outgrowth curves for following dilution of 5  $\mu$ L from a culture of aging yeast cells into 145  $\mu$ L of YPD measured using a Bioscreen machine. The rightward shift in curves with age is due to decreased viability in the population of aging cells. OD = optical density. **B**, Survival of BY4742 calculated from the shift in outgrowth curve at each age-point relative to the initial age-point.

analysis by measuring the CLS of cells grown in SD medium containing 0.05%, 0.5%, 1%, 2%, 10%, or 20% glucose. As can be observed from the survival curves at each glucose level, an inverse relationship between glucose and CLS was observed across the entire range of glucose concentrations tested (Figure 4). The observation that glucose levels higher than 2% reduce CLS in a dose-dependent fashion contrasts dramatically with RLS where glucose in excess of 2% has been shown to extend life span in the PSY316 background (29).

#### Effect of Amino Acid Concentration on CLS

Because decreasing availability of amino acids in yeast media has been reported to extend RLS (6), the relationship between amino acid concentration of the growth medium and CLS was also examined. In contrast to glucose, reduced amino acid levels (0.5X or 0.1X) did not increase CLS. To our surprise, 10-fold higher concentrations of amino acids substantially increased CLS (Figure 5A). This result is interesting given that CLS can be extended by growth in medium lacking a single high- or intermediate-nitrogen quality amino acid, such as asparagine or glutamate (18), and suggests that total amino acid abundance as well as the relative amounts of individual amino acids can have differential effects on longevity.

Table 3. The Bioscreen Method Provides Improved Precision for Estimating Relative Viability in a Population of Yeast Cells

Dilution Factor (% Viability)	Bioscreen	CFU
1 (100%)	0	16.4
1.25 (80%)	2.1	11.7
1.67 (60%)	4.1	12.3
2.5 (40%)	2.2	8.2
5 (20%)	3.2	19.2
10 (10%)	2.7	21.8
100 (1%)	4.3	11.3
1000 (0.1%)	7.8	11.9

Note: A dilution series was prepared from a yeast culture. The relative fraction of viable cells was measured for each sample using the Bioscreen method and by plating for colony forming units (CFUs). Based on six technical replicates for each method, the coefficient of variation for the Bioscreen method was less than for the CFU method at each dilution.

#### High Osmolarity Increases CLS

Given that elevated glucose levels shortened CLS but elevated amino acid levels increased CLS, we considered the possibility that osmotic stress could influence chronological aging. To determine whether this was the case, we measured CLS when cells were grown in the presence of standard CLS medium (Table 2) supplemented with either 18% sorbitol (an acyclic polyol that cannot be metabolized as a carbon source) or 300 mM NaCl. Addition of either of these osmolytes increased CLS (Figure 5B). This trend is consistent with data demonstrating that high osmolarity increases RLS (29). Thus, the short CLS associated with high glucose appears to be glucose-specific, whereas the

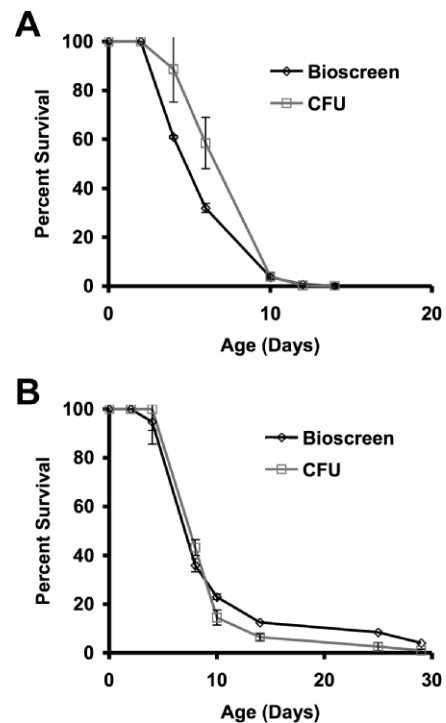


Figure 3. Comparison of chronological viability estimations obtained from the Bioscreen method versus the traditional colony forming unit (CFU) method. Chronological survival of W303AR (A) and PSY316AR (B) cells. Error bars show standard deviation of five biological replicates.

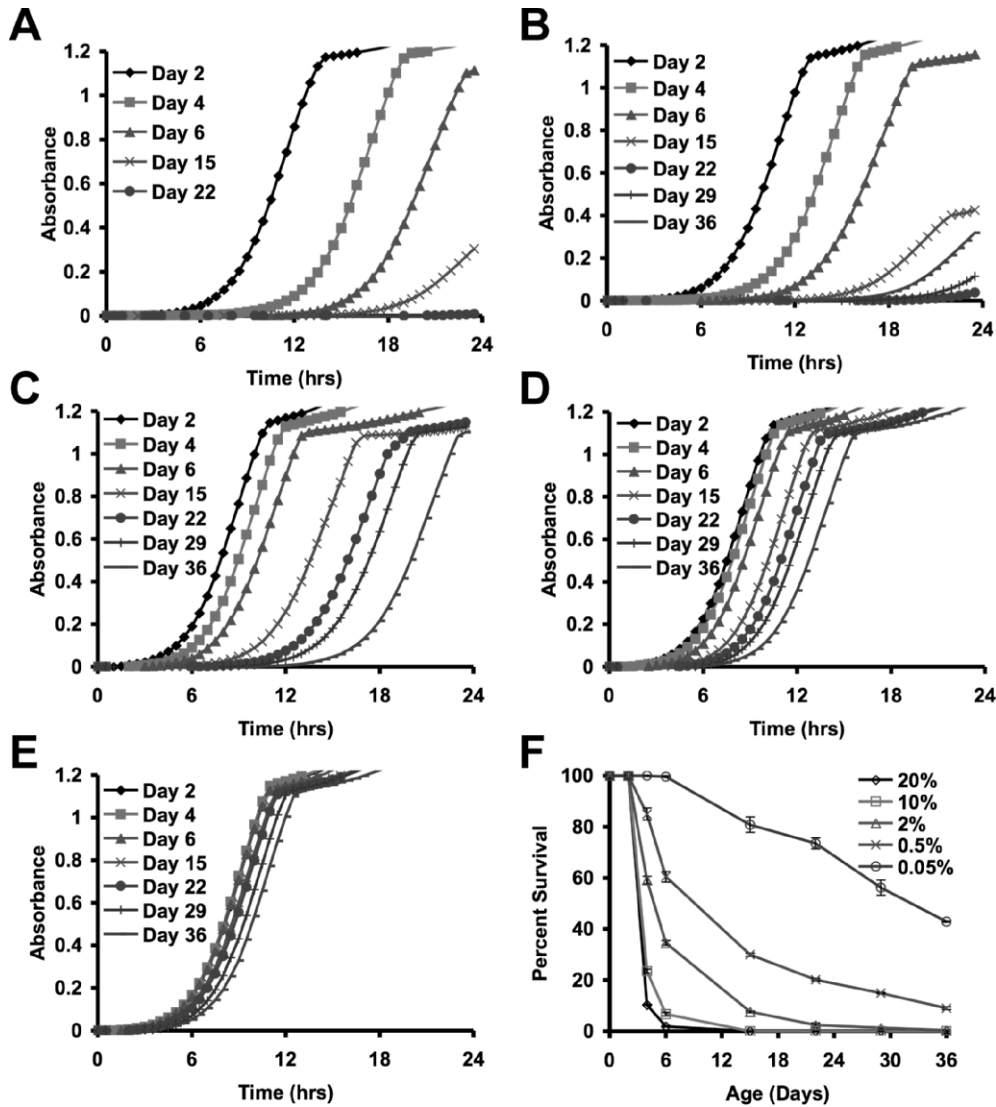


Figure 4. Glucose reduced the chronological life span of BY4742 cells. Bioscreen outgrowth curves obtained from aging cell growth in medium with 20% (A), 10% (B), 2% (C), 0.5% (D), or 0.05% glucose (E). F, Relative survival of cell growth in medium containing different glucose concentrations. Error bars show standard deviation of five biological replicates.

increased CLS associated with a 10-fold increase in amino acids may reflect activation of an osmotic stress response pathway that promotes chronological longevity.

#### *CLS Extension from DR is Independent of Sirtuins*

The involvement of Sir2 and Sir2 homologs in DR-mediated life-span extension has been controversial (31). To determine if CLS extension by DR requires Sir2 function, Sir2 was deleted in three different strain backgrounds, BY4742, W303, and PSY316, and subjected to DR by reduction of glucose. In all three strains, low glucose at both 0.5% and 0.05% increased CLS extension independently of Sir2 (Figure 6). This observation is consistent with a prior report that Sir2 is not required for CLS extension using an alternate DR method, i.e., aging postmitotic cells in water (24) and with a more recent report using 0.5% glucose for DR in BY4742 (16).

To determine if other yeast sirtuins might contribute to CLS extension from DR, single gene deletions in Hst1, Hst2, Hst3, and Hst4 in the BY4742 background were assayed for life span in low-glucose medium. Similar to the case for RLS (12), DR was sufficient to increase CLS in cells individually lacking any of the four Sir2 homologs (Figure 7). Additionally, DR increased the CLS of cells simultaneously lacking all five yeast sirtuins (Figure 8), ruling out the possibility that CLS extension in a strain lacking a single sirtuin is due to a functionally redundant homolog. Thus, we conclude that sirtuins do not mediate the CLS extension associated with DR by growth in reduced glucose medium.

#### DISCUSSION

The Bioscreen C MBR method for measuring CLS represents a novel high-throughput CLS assay with improved

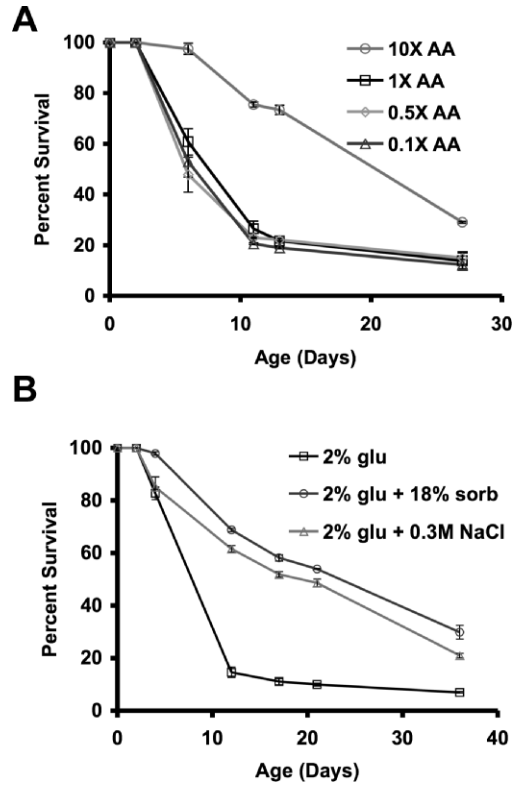


Figure 5. Elevated amino acid (AA) concentrations and increased osmolarity extend chronological life span. **A**, BY4742 cells grown in medium supplemented with 10-fold higher AA levels live longer than cells grown in control medium or medium with reduced AA levels (0.5X or 0.1X). **B**, BY4742 cells grown in medium supplemented with either 18% sorbitol (sorb) or 300 mM sodium chloride live longer than cells grown in control medium. Glu = glucose.

accuracy and precision relative to prior methods. We have used this assay to examine the relationship between nutrient availability and CLS and to examine the importance of sirtuins for mediating effects of DR. Similar to the replicative aging model (5,10), and as others have reported for CLS (16), we find that CLS is extended by a reduction in glucose. Also similar to the replicative aging model, this life-span extension does not require the presence of sirtuins. Although these data do not rule out a role for sirtuins in mediating some aspects of DR, they do further support accumulating evidence in the nematode *Caenorhabditis elegans* (32–34), suggesting that DR acts by a sirtuin-independent mechanism in evolutionarily divergent eukaryotes.

In contrast to the effect of reduced glucose, which increases both RLS and CLS, our studies also identified two cases in which medium composition affects CLS and RLS differently: high glucose and low amino acids. The CLS-shortening effect of high glucose, for example, is opposite to the increase in RLS observed at 20% glucose (29). This difference appears to be specific for glucose, as high osmolarity increases both CLS and RLS when sorbitol or NaCl are used as osmolytes (29). Further studies will be required to characterize the mechanisms by which high osmolarity increases CLS whereas increased glucose shortens CLS.

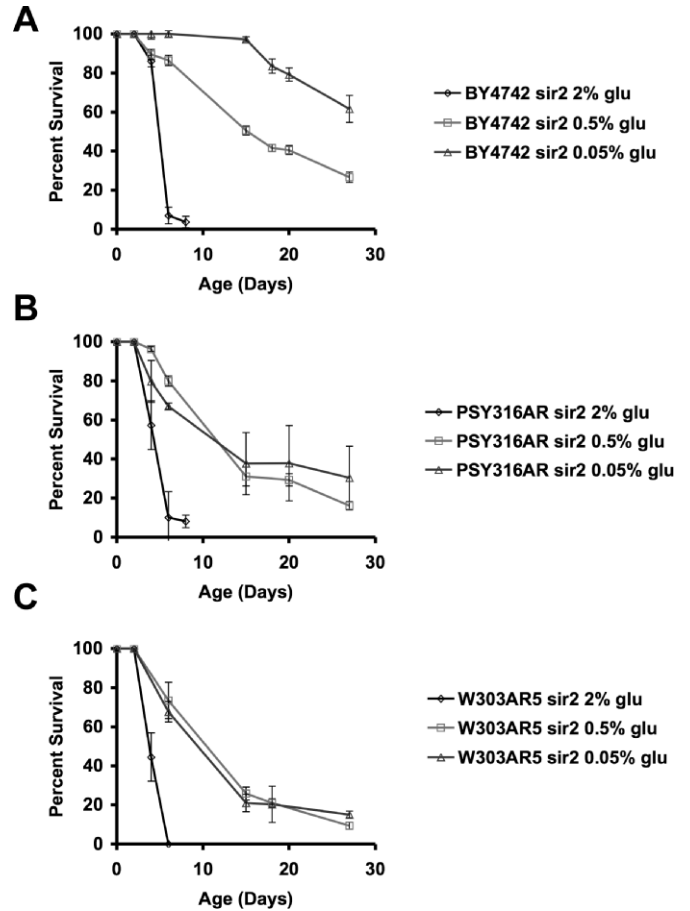


Figure 6. Sir2 is not required for chronological life-span extension from dietary restriction (DR) in multiple genetic backgrounds. Relative to cells grown in control medium, DR by growth at either 0.5% or 0.05% glucose (glu) increased life span in BY4742 *sir2Δ* (**A**), PSY316AR *sir2Δ* (**B**), and W303AR *sir2Δ* (**C**) cells.

The CLS-shortening effect of reducing the total amino acid levels of the medium was unexpected, as it has been previously observed that reducing the concentration of either asparagine or glutamate in the growth medium increases CLS (18). Thus, it may be the case that CLS is affected differently by the relative concentrations of individual amino acids, and it will be of interest to explore this possibility in future studies. The increase in CLS observed when amino acid levels are increased is most likely related to increased osmolarity, as evidenced by the increase in CLS observed at either 1M sorbitol or 300 mM NaCl.

As demonstrated by our studies, one advantage of the Bioscreen CLS method is that it is easily adaptable to varying assay conditions. In the experiments reported here, we aged the cells in culture tubes on a rolling drum in a variety of different media compositions. Because viability is determined by outgrowth in the Bioscreen C machine, however, other culture conditions (e.g., aeration, temperature, volume) are equally adaptable to this method. For example, an alternative protocol for chronologically aging yeast cells involves growing cells to stationary phase in rich

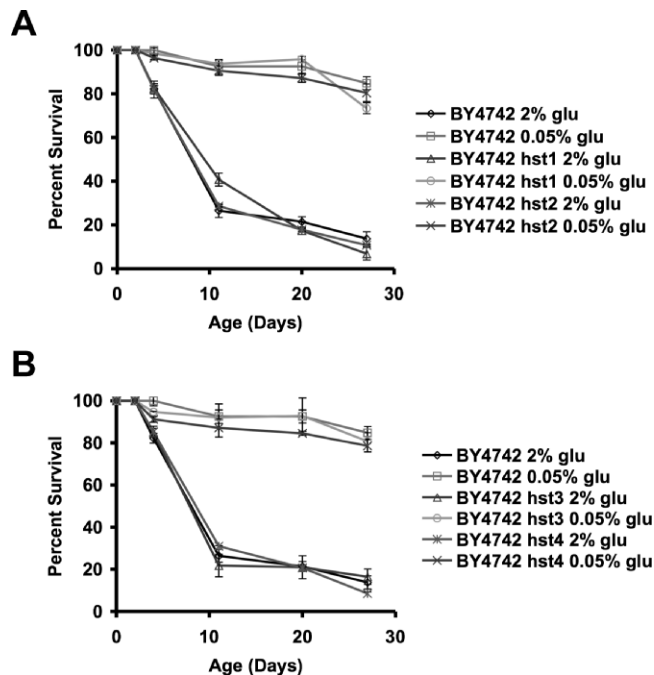


Figure 7. Sir2 homologs are not involved in life-span extension from dietary restriction (DR). Chronological life-span extension from DR was unaffected by individual deletion of any of the four Sir2 homologs: (A) *HST1*, *HST2*, (B) *HST3*, or *HST4*.

medium then transferring them to water. A major difference between cells aged in SD versus those aged in water is the metabolic state of the quiescent cells; cells aged in SD maintain a high metabolic rate, whereas cells transferred to water from rich medium enter a so-called hypometabolic state (35,36). The Bioscreen CLS method is equally adaptable to either method and would be particularly useful for systematically comparing how different mutants age under each condition.

From our experience, aside from the initial cost of purchasing the machine (~\$35,000) the use of a Bioscreen C MBR machine for determining yeast CLS has only two significant limitations. First, the Honeycomb plates used with this machine are 100-well plates, which are not easily adapted for robotic 96- or 384-well assays. There is no reason in principle that the Honeycomb plates cannot be made in a 96-well format, and hopefully the manufacturer will address this limitation in the near future. Second, a maximum of 200 wells can be assayed per machine per overnight incubation. This translates to a maximum throughput of ~600 simultaneous CLS assays per Bioscreen C MBR machine, assuming two age-points per week, or 1400 assays assuming 1 age-point per week. Thus, this method provides much higher throughput capacity than traditional CLS assays involving CFU determination, without loss of accuracy or precision. Application of this method in a genome-wide manner should allow for future comparative analyses of yeast CLS with prior and ongoing genomic studies of longevity in the yeast replicative aging paradigm (13,37) and in *C. elegans* (38–43).

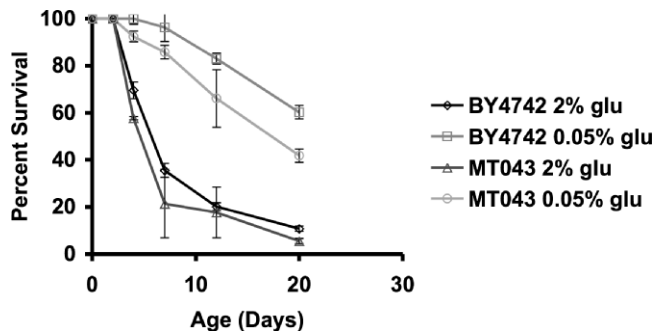


Figure 8. Dietary restriction (DR) increases the chronological life span of sirtuinless yeast. Cells lacking all five yeast sirtuins (MT043) live longer in medium containing 0.05% glucose (glu) compared to control medium (2% glu).

#### ACKNOWLEDGMENTS

This work was supported by a pilot grant to M. K. from the University of Washington Nathan Shock Center for Excellence in the Basic Biology of Aging (National Institutes of Health Grant 5P30 AG013280) and by a grant to B. K. K. and M. K. from the Ellison Medical Foundation. C. R. B. is supported by National Institutes of Health Training Grant 5P30 AG013280. C. J. M. and C. R. B. contributed equally to this work.

#### CORRESPONDENCE

Address correspondence to Matt Kaerberlein, PhD, Department of Pathology, University of Washington, Box 357470, Seattle, WA 98195-7470. E-mail: kaerber@u.washington.edu

#### REFERENCES

- Masoro EJ. Overview of caloric restriction and ageing. *Mech Ageing Dev.* 2005;126:913–922.
- Weindruch RH, Walford RL. *The Retardation of Aging and Disease by Dietary Restriction*. Springfield, IL: Thomas; 1988.
- Kaerberlein M, Burtner CR, Kennedy BK. Recent developments in yeast aging. *PLoS Genet.* 2007;3:e84.
- Kaerberlein M. Longevity and aging in the budding yeast. In: Conn PM, ed. *Handbook of Models for Human Aging*. Boston: Elsevier; 2006:109–120.
- Lin SJ, Defossez PA, Guarente L. Requirement of NAD and SIR2 for life-span extension by calorie restriction in *Saccharomyces cerevisiae*. *Science.* 2000;289:2126–2128.
- Jiang JC, Jaruga E, Repnevskaya MV, Jazwinski SM. An intervention resembling caloric restriction prolongs life span and retards aging in yeast. *FASEB J.* 2000;14:2135–2137.
- Kaerberlein M, McVey M, Guarente L. The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. *Genes Dev.* 1999;13:2570–2580.
- Tissenbaum HA, Guarente L. Increased dosage of a sir-2 gene extends lifespan in *Caenorhabditis elegans*. *Nature.* 2001;410:227–230.
- Rogina B, Helfand SL. Sir2 mediates longevity in the fly through a pathway related to calorie restriction. *Proc Natl Acad Sci U S A.* 2004;101:15998–16003.
- Kaerberlein M, Kirkland KT, Fields S, Kennedy BK. Sir2-independent life span extension by calorie restriction in yeast. *PLoS Biol.* 2004;2:E296.
- Kaerberlein M, Steffen KK, Hu D, et al. Comment on “HST2 mediates SIR2-independent life-span extension by calorie restriction.” *Science.* 2006;312:1312; author reply 1312.
- Tsuchiya M, Dang N, Kerr EO, et al. Sirtuin-independent effects of nicotinamide on lifespan extension from calorie restriction in yeast. *Aging Cell.* 2006;5:505–514.
- Kaerberlein M, Powers RW 3rd, Steffen KK, et al. Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. *Science.* 2005;310:1193–1196.



14. Medvedik O, Lamming DW, Kim KD, Sinclair DA. MSN2 and MSN4 link calorie restriction and TOR to siruin-mediated lifespan extension in *Saccharomyces cerevisiae*. *PLoS Biol.* 2007;5(10):e261.
15. Longo VD, Ellerby LM, Bredesen DE, Valentine JS, Gralla EB, Human Bcl-2 reverses survival defects in yeast lacking superoxide dismutase and delays death of wild-type yeast. *J Cell Biol.* 1997;137:1581–1588.
16. Smith DL Jr, McClure JM, Maticic M, Smith JS. Calorie restriction extends the chronological lifespan of *Saccharomyces cerevisiae* independently of the siruins. *Aging Cell.* 2007;6:649–662.
17. Fabrizio P, Pozza F, Pletcher SD, Gendron CM, Longo VD. Regulation of longevity and stress resistance by Sch9 in yeast. *Science.* 2001;292:288–290.
18. Powers RW 3rd, Kaerberlein M, Caldwell SD, Kennedy BK, Fields S. Extension of chronological life span in yeast by decreased TOR pathway signaling. *Genes Dev.* 2006;20:174–184.
19. Piper PW, Harris NL, MacLean M. Preadaptation to efficient respiratory maintenance is essential both for maximal longevity and the retention of replicative potential in chronologically ageing yeast. *Mech Ageing Dev.* 2006;127:733–740.
20. Levine B, Klionsky DJ. Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell.* 2004;6:463–477.
21. Jia K, Chen D, Riddle DL. The TOR pathway interacts with the insulin signaling pathway to regulate *C. elegans* larval development, metabolism and life span. *Development.* 2004;131:3897–3906.
22. Kapahi P, Zid BM, Harper T, Koslover D, Sapin V, Benzer S. Regulation of lifespan in *Drosophila* by modulation of genes in the TOR signaling pathway. *Curr Biol.* 2004;14:885–890.
23. Vellai T, Takacs-Vellai K, Zhang Y, Kovacs AL, Orosz L, Muller F. Genetics: influence of TOR kinase on lifespan in *C. elegans*. *Nature.* 2003;426:620.
24. Fabrizio P, Gattazzo C, Battistella L, et al. Sir2 blocks extreme life-span extension. *Cell.* 2005;123:655–667.
25. Kaerberlein M, McDonagh T, Heltweg B, et al. Substrate-specific activation of siruins by resveratrol. *J Biol Chem.* 2005;280:17038–17045.
26. Winzeler EA, Shoemaker DD, Astromoff A, et al. Functional characterization of the *S. cerevisiae* genome by gene deletion and parallel analysis. *Science.* 1999;285:901–906.
27. Sikorski RS, Hieter P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics.* 1989;122:19–27.
28. Malathi K, Higaki K, Tinkelenberg AH, et al. Mutagenesis of the putative sterol-sensing domain of yeast Niemann Pick C-related protein reveals a primordial role in subcellular sphingolipid distribution. *J Cell Biol.* 2004;164:547–556.
29. Kaerberlein M, Andalis AA, Fink GR, Guarente L. High osmolarity extends life span in *Saccharomyces cerevisiae* by a mechanism related to calorie restriction. *Mol Cell Biol.* 2002;22:8056–8066.
30. Kaerberlein M, Hu D, Kerr EO, et al. Increased life span due to calorie restriction in respiratory-deficient yeast. *PLoS Genet.* 2005;1(5):e69.
31. Kaerberlein M, Powers RW 3<sup>rd</sup>. Sir2 and calorie restriction in yeast: a skeptical perspective. *Ageing Res Rev.* 2007;6:128–140.
32. Hansen M, Taubert S, Crawford D, Libina N, Lee S, Kenyon C. Lifespan extension by conditions that inhibit translation in *C. elegans*. *Aging Cell.* 2007;6:95–110.
33. Kaerberlein TL, Smith ED, Tsuchiya M, et al. Lifespan extension in *Caenorhabditis elegans* by complete removal of food. *Aging Cell.* 2006;5:487–494.
34. Lee GD, Wilson MA, Zhu M, et al. Dietary deprivation extends lifespan in *Caenorhabditis elegans*. *Aging Cell.* 2006;5:515–524.
35. Fabrizio P, Longo VD. The chronological life span of *Saccharomyces cerevisiae*. *Aging Cell.* 2003;2:73–81.
36. Piper PW. Long-lived yeast as a model for ageing research. *Yeast.* 2006;23:215–226.
37. Kaerberlein M, Kennedy BK. Large-scale identification in yeast of conserved ageing genes. *Mech Ageing Dev.* 2005;126:17–21.
38. Chen D, Pan KZ, Palter JE, Kapahi P. Longevity determined by developmental arrest genes in *Caenorhabditis elegans*. *Aging Cell.* 2007;6:525–533.
39. Curran SP, Ruvkun G. Lifespan regulation by evolutionarily conserved genes essential for viability. *PLoS Genet.* 2007;3(4):e56.
40. Dillin A, Hsu AL, Arantes-Oliveira N, et al. Rates of behavior and aging specified by mitochondrial function during development. *Science.* 2002;298:2398–2401.
41. Hamilton B, Dong Y, Shindo M, et al. A systematic RNAi screen for longevity genes in *C. elegans*. *Genes Dev.* 2005;19:1544–1555.
42. Hansen M, Hsu AL, Dillin A, Kenyon C. New genes tied to endocrine, metabolic, and dietary regulation of lifespan from a *Caenorhabditis elegans* genomic RNAi screen. *PLoS Genet.* 2005;1:119–128.
43. Lee SS, Lee RY, Fraser AG, Kamath RS, Ahringer J, Ruvkun G. A systematic RNAi screen identifies a critical role for mitochondria in *C. elegans* longevity. *Nat Genet.* 2003;33:40–48.

Received September 14, 2007

Accepted October 30, 2007

Decision Editor: Huber R. Warner, PhD