Chapter 13
Genetic Approaches to Aging in Budding and Fission Yeasts: New Connections and New Opportunities

Bo-Ruei Chen and Kurt W. Runge

Abstract Yeasts are powerful model systems to examine the evolutionarily conserved aspects of eukaryotic aging because they maintain many of the same core cellular signaling pathways and essential organelles as human cells. We constructed a strain of the budding yeast *Saccharomyces cerevisiae* that could monitor the distribution of proteins involved in heterochromatic silencing and aging, and isolated mutants that alter this distribution. The largest class of such mutants cause defects in mitochondrial function, and appear to cause changes in nuclear silencing separate from the well-known Rtg2p-dependent pathway that alters nuclear transcription in response to the loss of the mitochondrial genome. Mutants that inactivate the *ATP2* gene, which encodes the ATPase subunit of the mitochondrial F_1F_0-ATPase, were isolated twice in our screen and identify a lifespan extending pathway in a gene that is conserved in both prokaryotes and eukaryotes. The budding yeast *S. cerevisiae* has been used with great success to identify other lifespan-extending pathways in screens using surrogate phenotypes such as stress resistance or silencing to identify random mutants, or in high throughput screens that utilize the deletion strain set resource. However, the direct selection of long-lived mutants from a pool of random mutants is more challenging. We have established a new chronological aging assay for the evolutionarily distant fission yeast *Schizosaccharomyces pombe* that recapitulates aspects of aging conserved in all eukaryotes. We have constructed a novel *S. pombe* DNA insertion mutant bank, and used it to show that we can directly select for a long-lived mutant. The use of both the budding and fission yeast systems should continue to facilitate the identification and validation of lifespan extending pathways that are conserved in humans.

Keywords *Schizosaccharomyces pombe* · *Saccharomyces cerevisiae* · Silencing · Chromatin · Respiration

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Abbreviations

CLS Chronological LifeSpan, the length of time a cell or organism can survive. In yeast, this corresponds to the length of time cells survive in stationary phase in nutrient depleted medium.

RLS Replicative LifeSpan, the number of times a cell can divide prior to senescence.

Isolation of Mutants that Redistribute Silencing Function

In *S. cerevisiae*, the transcription of genes inserted in the yeast silent mating type cassettes, next to telomeres or within the array of ribosomal RNA genes (the rDNA array) are silenced via a heterochromatin-mediated mechanism that involves the genes Sir2p, Sir3p and Sir4p (for review see Gasser and Cockell (2001), Rusche et al. (2003)). Early work from the Guarente lab showed that a truncated Sir4p could extend Replicative LifeSpan (RLS, the number of times an individual cell can divide prior to senescence), and that Sir proteins in old cells left telomeres and the silent mating type cassettes and relocalized to the nucleolus where the rDNA locus is transcribed (Kennedy et al. 1995, 1997). We and others found that the phosphorylation state of the silencing protein Sir3p correlated with the level of telomeric silencing and length of lifespan (Roy and Runge 2000; Stone and Pillus 1996). To identify the Sir3p kinase, we constructed a yeast "triple silencer strain" where transcriptional silencing at a silent mating type cassette, a telomere and the rDNA array could be monitored (Roy and Runge 2000) (Fig. 13.1). Each reporter gene is only partially silenced, so both increases and decreases in silencing can be monitored.

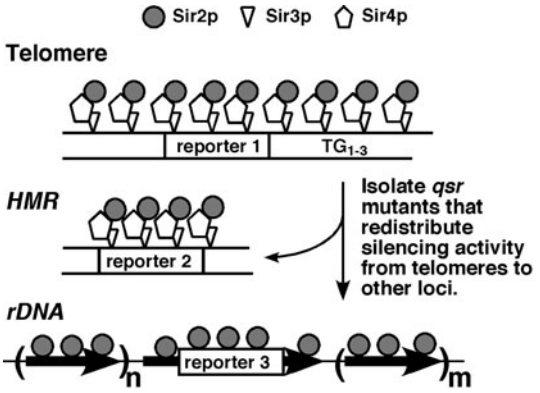

Fig. 13.1 The triple silencer strain concept and selection for *quirky redistribution of silencing* (*qsr*) mutants. The triple silencer strain has three different reporter genes at three different loci whose expression is partially silenced; allowing both increases and decreases in silencing to be monitored. Transcription at telomeres and the *HMR* silent mating type cassette is silenced in part by the actions of Sir2p, Sir3p and Sir4p, and Sir2p also acts at the array of ribosomal RNA genes (the rDNA). The *qsr* mutants were selected by screening for mutants that had the phenotype of relocalizing silencing function from telomeres to other loci

The triple silencer strain was used as the starting point in a transposon mutagenesis to identify mutants with the lower telomere silencing and higher silencing at the mutant *hmr* silent mating type cassette seen in long-lived strains (Roy and Runge 2000) which we call *qsr* mutants for their ***q***uirky ***s***ilencing ***r***edistribution phenotype. The transposon mutagenesis used the library created by Burns et al. that essentially allows one to analyze random transposon insertions throughout the yeast genome (Burns et al. 1994). We generated 33,000 insertion mutants and screened them for increased *hmr* silencing and decreased telomere silencing, and the only kinase we identified was the MAP kinase Slt2p (Ray et al. 2003). We went on to show that Slt2p is an in vivo Sir3p kinase (Ray et al. 2003), which was also shown independently by others (Ai et al. 2002). One interesting aspect of this finding is that Slt2p is activated by rapid growth, which would increase Sir3p phosphorylation and decrease lifespan, while slower growth would leave Sir3p unphosphorylated and extend lifespan. In many organisms, a nutrient-rich medium correlates with more rapid growth and shorter lifespan while a nutrient-poor medium correlates with slower growth and longer lifespan (Metcalfe and Monaghan 2003; Piper and Partridge 2007; Sinclair 2005; Sohal and Weindruch 1996). Thus, the Slt2p-Sir3p circuit appears to be one of the control pathways activated in yeast that regulates lifespan in response to nutrient levels.

The final *qsr* mutant collection contained a total of 18 genes whose deletion caused a transcriptional silencing phenotypes associated with long-lived cells: less silencing at telomeres and more silencing at the silent mating type cassettes (Kennedy et al. 1995; Ray et al. 2003). Each insertion mutant was subsequently verified by deleting the identified gene's ORF and showing that the deletion mutation also had the same silencing phenotype.

An unexpected outcome from this screen was that the largest class of mutants disrupted mitochondrial function. Mutations in *COX4*, encoding a component of the electron transport chain, and *ATP2*, the β-subunit of the F_1F_0-ATP synthase, were isolated twice. In addition, five genes affecting mitochondrial protein synthesis and a chaperone for Cox2p transport into the mitochondrial matrix were also identified (Table 13.1). These data indicated that the transcriptional silencing redistribution phenotype provided an unexpected, novel reporter for mitochondrial function and could monitor mitochondrial-nuclear signaling. All of the strains lacking these genes could not grow on carbon sources that require mitochondrial function, showing that these mutations simultaneously impaired mitochondrial function and changed nuclear silencing. The Nyström lab has also found that nuclear mutations in a subset of gene involved in mitochondrial protein synthesis alter silencing at the rDNA array and silent mating type cassettes (Caballero et al. 2011), further establishing this mitochondrial function-nuclear gene silencing link.

Interestingly, similar transcriptional silencing defects were observed with two proteins from different subcellular compartments: *ATP2*, which encodes the mitochondrial β-subunit of the F_1F_0-ATP synthase, and *SAS4,* which encodes nuclear histone modifying enzyme (Fig. 13.2). We found that the *atp2Δ* strain had a short RLS compared to wild type, in agreement with previous work from the Jazwinski

Table 13.1 The *quirky silencing redistribution* (*qsr*) mutants show that the distribution of silencing responds to mitochondrial function. The largest class of mutants, which alters mitochondrial function, and the two mutants that have established roles in silencing are shown

Gene/protein	Functional class	Systematic name
Mitochondrial components		
ATP2/β-subunit of F_1F_0-ATP synthase	F_1F_0-ATP synthase	YJR121W
COX4/cytochrome c oxidase subunit	Electron transport chain	YGL187C
IFM1/translation initiation factor	Mitochondrial translation	YOL023W
MRLP9/ribosomal protein	Mitochondrial translation	YGR220C
MRSP8/ribsosomal protein	Mitochondrial translation	YMR158W
MSD1/Aspartyl tRNA synthetase	Mitochondrial translation	YPL104W
YER087W/unknown tRNA synthetase	Mitochondrial translation	YER087W
MSS2/required for Cox2p synthesis	Mitochondrial chaperone	YDL107W
Signaling proteins		
SLT2/MAP kinase for cell integrity	Sir3p kinase, links rapid Growth to short lifespan (Ray et al. 2003)	YHR030C
Known silencing regulators		
SAS4/relocalizes Sir proteins	Part of MYST histone Deacetylase complex (Lafon et al. 2007)	YDR181C
8 other genes in other aspects of metabolism		

lab (Lai et al. 2002), while the *sas4Δ* strain had a longer RLS (Fig. 13.3), indicating that the transcriptional silencing phenotype is not a predictor of long lifespan. The short RLS of *atp2Δ* cells contrasts with the extended RLS of some other mitochondrial mutants (Caballero et al. 2011; Kirchman et al. 1999), revealing additional complexities in this lifespan control pathway. The sum of these observations do show that partially dysfunctional mitochondria signal transcriptional changes in the nucleus that alter lifespan and silencing.

The *qsr* Mutant *atp2Δ* Reveals a Novel Mitochondria-to-Nucleus Signaling Pathway

The majority of mitochondrial proteins are encoded in the nucleus, and the functional state of the mitochondria affects the transcriptional regulation of these nuclear genes. Retrograde Signaling or Retrograde Regulation is the general name given to the processes that link nuclear gene expression to the level of mitochondrial

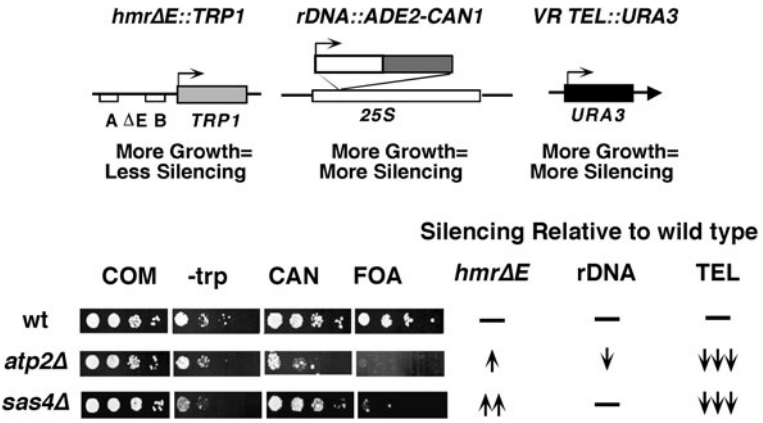

Fig. 13.2 The *qsr* mutants *atp2Δ* and *sas4Δ* have similar silencing phenotypes. A spot test assay of the three reporter genes, using the amount of growth as a measure of the level of gene expression. Ten-fold serial dilutions of single yeast colonies bearing the indicated mutations were spotted onto the indicated media to monitor silencing, where COM is complete medium where all cells can grow, -trp is medium where cells that have not silenced the *TRP1* gene can grow, and FOA (5-fluoro-orotic acid) and CAN (canavanine) are media where cells that have silenced the *URA3* and *CAN1* genes, respectively, can grow. *Arrows to the right* indicate the relative level of silencing at each locus compared to wild type cells, with *up arrows* showing more silencing and *down arrows* showing less silencing

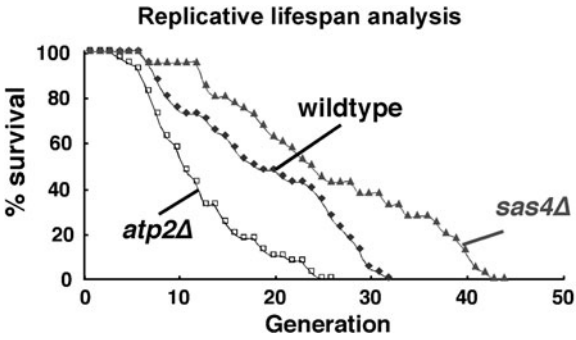

Fig. 13.3 The *atp2Δ* and *sas4Δ* mutants with the same silencing phenotypes (Fig. 13.2) have different replicative lifespan. Replicative lifespan assays (the number of times a cell can divide) were performed as described (Ray et al. 2003)

function. This mitochondria-to-nucleus signaling occurs in both yeast and mammals (Liu and Butow 2006). In yeast, the best understood retrograde signaling pathway involves 3 genes: *RTG2*, which encodes part of a chromatin remodeling complex, and *RTG1* and *RTG3*, which encode the subunits of the heterodimeric factor that activates transcription of many of the nuclear genes for mitochondrial proteins. However, transcriptional profiling experiments have shown that some genes whose transcription changes in response to loss of the mitochondrial genome do not require the *RTG2* gene for this change, dividing retrograde signaling into *RTG2*-independent and *RTG2*-dependent processes (Liu and Butow 2006). The

RTG2-dependent signaling pathway is also required for lifespan control in some, but not all, budding yeast laboratory strains (Kirchman et al. 1999). As mitochondria have an evolutionarily conserved role in aging (Kujoth et al. 2007; Sedensky and Morgan 2006; Wallace and Lott 2002), the requirement for the mitochondria-to-nucleus signaling pathway for lifespan control may impact human biology as well. The reason that *RTG2* controls lifespan in some strains is unclear, but may be related to the level of the *RTG2*-independent signaling pathway(s) (Liu and Butow 2006).

To determine if the *atp2Δ*-mediated changes in silencing occurred via *RTG2*-dependent or *RTG2*-independent retrograde signaling, we compared the silencing phenotypes of strains bearing single or double mutations in *ATP2* and *RTG2*. Briefly, if two mutations affect the same pathway, then the phenotype of the double mutant will be the same as one of the single mutants. The *RTG2* pathway is activated by loss of the mitochondrial genome (i.e. ρ^0 cells), and we found that while ρ^0 cells and cells bearing a deletion of *RTG2* (*rtg2Δ*) caused different silencing phenotypes, and cells with both defects had the *rtg2Δ* phenotype (Fig. 13.4a). These data demonstrate that our silencing assay can monitor the known *RTG2* mitochondrial-nuclear signaling pathway, and show that the *RTG2* gene is required for the silencing changes caused by loss of the mitochondrial genome. We then tested whether the *atp2Δ* mutation changed silencing via the *RTG2* pathway. We found that *atp2Δ* acts through a different pathway, because the *atp2Δ rtg2Δ* cells

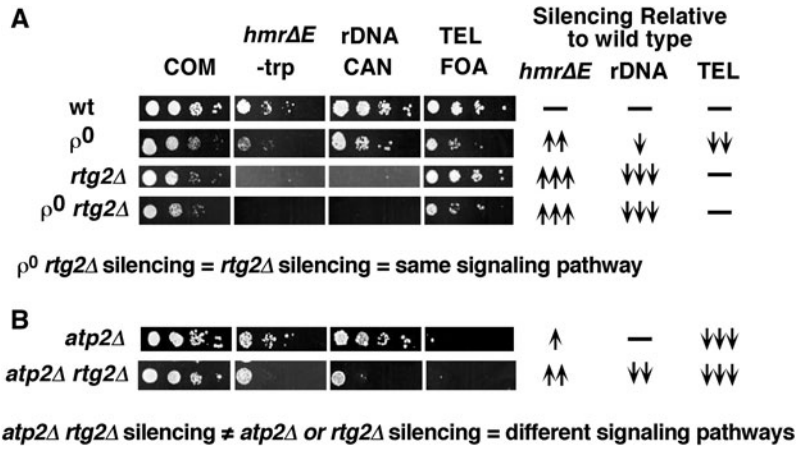

Fig. 13.4 The redistribution of silencing phenotype responds to mitochondrial state, and shows that the *atp2Δ* defect signals through a new pathway. **a** The silencing redistribution assay responds to the known *RTG2* control of cellular response to loss of the mitochondrial genome. Cells that have lost the mitochondrial genome are referred to as "ρ^0" cells. The silencing phenotypes of *rtg2Δ* and ρ^0 cells are different, but the *rtg2Δ* ρ^0 double mutant has the *rtg2Δ* phenotype. Thus, the silencing effect of ρ^0 cells is signaled through the *RTG2* pathway. **b** The silencing phenotype of the *atp2Δ rtg2Δ* double mutant is different from either single mutant, indicating that they affect silencing through distinct pathways

had a silencing phenotype that was different from either single mutant (Fig. 13.4b), indicating that two different effects were occurring in the double mutant. Thus, the *atp2Δ* mutation activates an *RTG2*-independent retrograde signaling pathway, and reveals a novel role for the F_1F_0-ATP synthase β-subunit in mitochondria-to-nucleus signaling.

Suppressor Mutants of the *atp2Δ* Silencing Phenotype Identify Chromatin Remodeling Genes

We hypothesized that the change in silencing caused by the *atp2Δ* mutation was due to the mitochondrial defect initiating a signal that results in changes in nuclear silencing. Based on the known examples of extracellular signals initiating multistep processes that end in changes in nuclear transcription (e.g. Slt2p phosphorylation of Sir3p), we hypothesized that mutants that eliminate components in this signaling pathway should re-establish wild type silencing in the *atp2Δ* mutant, although signaling mechanisms that do not follow this model were also possible. As a first step, we repeated the transposon mutagenesis in *atp2Δ* cells, this time screening for mutants that restored telomere silencing in the mutant background. As a similar approach had identified the signaling kinase that phosphorylates Sir3p (Ray et al. 2003), this approach had the potential to identify one or more proteins that signal between defective mitochondria and nuclear silencing.

The mutants we isolated affect histone modification (Sas2p, Sas3p, Rph1p) and chromatin remodeling (Isw1p), are yeast-specific silencing proteins (Sir1p, Sir3p) or are known to affect telomeric silencing (Rif1p, Yku80p). All of these genes encode proteins that change chromatin and would therefore directly affect silencing, and many altered silencing in the absence of the *atp2Δ* mutation (Lafon et al. 2007; Laroche 1998; Mishra and Shore 1999) (N. Roy, B.-R. Chen, J. Franco, X. Wang, A. Yakubenko, K.W. Runge, in preparation).

These suppressor mutants, while interesting from the perspective of the control of silencing, did not clearly identify the standard components of a signaling cascade such as transcription factors or kinases. Thus, several hypotheses are currently possible and under investigation. First, since many of these chromatin remodeling complexes are dependent upon ATP, cellular ATP levels could in effect be the signaling molecule that alters silencing. Second, one of these proteins may have an additional, and presently unknown, function that is responsive to mitochondrial function. For example, high throughput protein interaction screens have identified connections between mitochondrial proteins and telomeric proteins such as interactions between the telomeric protein Yku80p and the mitochondrial voltage dependent anion channel (called porin), a mitochondrial isocitrate dehydrogenase subunit (Idh1p) and a mitochondrial subunit of the pyruvate dehydrogenase complex (Ho et al. 2002), but whether these interactions have any functional significance is unknown. Finally, the simple

hypothesis that defective mitochondria in *atp2Δ* cells initiate a signal that changes in nuclear silencing may be more complex (e.g. a combination of different signals through multiple pathways). Experiments to distinguish these possibilities are underway.

Underexpression of *ATP2* Extends Lifespan, in Contrast to the Lifespan Shortening of the *atp2Δ* Mutation

The shortened lifespan of the *S. cerevisiae atp2Δ* strain (Fig. 13.3) seemed at odds with results from *Caenorhabditis elegans*, where RNAi knockdown of mitochondrial F_1F_0-ATPase subunits extends lifespan (Dillin et al. 2002; Lee et al. 2003). These contrasting results could be due to the difference between the complete lack of protein versus reduced levels of a protein. To test this possibility, we constructed a yeast strain that underexpresses the F_1F_0-ATPase β-subunit to determine if yeast that phenocopy the reduced expression in the RNAi-treated worms also phenocopy the lifespan extension.

The *ATP2* gene was placed under the control of a weak constitutive promoter to construct the allele *atp2**, and a single copy was integrated into the genome of a strain whose endogenous *ATP2* gene had been deleted. Western blot analysis confirmed that β-subunit (Atp2p) levels in mitochondria from the *atp2** strain were less than wild type, and that this strain grew more slowly on glycerol medium that requires full mitochondrial function for rapid growth compared to wild type cells (Runge, K.W., Yakubenko, A., Shtofman, R., submitted). Thus, the *atp2** strain was a yeast knockdown of F_1F_0-ATPase β-subunit expression.

The yeast knockdown of an F_1F_0-ATPase component phenocopied the lifespan extension of *C. elegans* RNAi knockdowns. The median lifespans, relative to wild type strain, were 47% for the *atp2Δ* mutant and 133% for the *atp2** knockdown mutant. Thus, both budding yeast and worms show lifespan extension when mitochondrial function is impaired by reducing expression. Given the large evolutionary distance between yeast and worms, this effect is likely to be broadly conserved among eukaryotes including humans. The significance of these results for humans derives from the data that the mitochondrial genomes of human skeletal muscle accumulate DNA damage over time, suggesting that mitochondrial function is increasingly impaired with increasing age (Wallace and Lott 2002). Thus, both the signaling pathway initiated by mitochondrial defects and the lifespan extension caused by reduced expression of mitochondrial components provide examples of what may occur in aging human cells with accumulating mitochondrial DNA defects, i.e. that cells from older humans may have activated a lifespan-extending signaling pathways in response to these functionally compromised mitochondria. Elucidation of these pathways in yeast and other model systems would provide another therapeutic target for the treatment of human aging and aging-associated diseases.

Development of a Chronological Lifespan Assay for *S. pombe* that Allows the Direct Selection of Long-Lived Mutants

In addition to the effect of the F_1F_0-ATPase β-subunit knockdown described above, altering a subset of processes in distantly related species such as budding yeast, *C. elegans* and *Drosophila melanogaster,* has revealed multiple evolutionarily conserved pathways in lifespan control (Hamilton et al. 2005; Kaeberlein et al. 2005; Lee et al. 2003; Lin et al. 1998; Powers 3rd et al. 2006), such as reducing the amount of calories in the environment (caloric restriction) and mutating the Akt kinase (Bishop and Guarente 2007; Dilova et al. 2007; Paradis and Ruvkun 1998; Sinclair 2005). While these systems have been extremely powerful and the approaches to high-throughput screening for long-lived mutants have been thoughtful and elegant, we believed that the aging field would benefit from a system in which one could directly select for long-lived random mutants as opposed to individually screening large banks of defined mutants. We have therefore developed a chronological aging assay for the fission yeast *Schizosaccharomyces pombe* that is amenable to the direct selection of long-lived mutants (Chen and Runge 2009).

The advantages of *S. pombe* are that this species is as distant from *S. cerevisiae* as humans are from *C. elegans* (Hedges 2002), so one can test for evolutionarily conserved lifespan extending pathways by comparing these two very different yeasts. *S. pombe* also has a sequenced genome, powerful molecular genetics, a strong international research community and a number of processes, e.g. RNA splicing, requirement of a mitochondrial genome for survival, DNA repair, telomere function and RNAi, that are distinct from *S. cerevisiae* and more similar to those in humans (Moreno et al. 1991; Wood et al. 2002). While these differences elucidate the value of *S. pombe* as a model system, they also indicate that it would be inappropriate to simply transfer the existing *S. cerevisiae* assays to *S. pombe* without some form of independent validation. We therefore established a new aging assay in *S. pombe* that recapitulates the evolutionarily conserved properties of lifespan extension by caloric restriction and lifespan shortening by overnutrition.

Establishment of a Chronological Aging Assay in *S. pombe*

Two types of lifespans are routinely measured in the budding yeast *S. cerevisiae*: Replicative lifespan (RLS), which is the number of times an individual cell can divide prior to senescence, and chronological lifespan (CLS), which is the length of time cells can survive in stationary phase when most nutrients in the medium are exhausted and growth is minimized. RLS is considered to be a model for human stem cells that divide throughout life, while CLS is a model for terminally differentiated, post-mitotic cells such as neurons and muscle. RLS can be measured with relative ease in *S. cerevisiae* because cell division is asymmetric and the small, newly formed daughter cell can be distinguished from the larger, older mother cell (Guarente and Kenyon 2000; Sinclair et al. 1998). *S. pombe* is a fission yeast whose early divisions are symmetric, so the two daughters are morphologically identical

and not easy to distinguish. It has been reported that some asymmetry in *S. pombe* cell division does occur and is easily observable in later divisions (Barker and Walmsley 1999; Erjavec et al. 2008; Minois et al. 2006), but the difficulty in observing these differences in early divisions makes establishing an *S. pombe* replicative lifespan assay in a rigorous way difficult. Consequently, we and others have sought to establish a CLS assay in *S. pombe*.

It should be noted that extensive work on the cell cycle in *S. pombe* has established that placing cells into medium that lacks a nitrogen or carbon source causes cell cycle exit and can allow cells to survive for many months (Shimanuki et al. 2007; Su et al. 1996). While these experiments are revealing with respect to the differences between rapidly growing and stationary phase G0 cells, this transition is quite different from cells that grow in medium and gradually exhaust a subset of the nutrients. In the wild, rapidly growing cells would most likely exhaust the most limiting nutrient first, and cells would need to stop growing and survive in the presence of a complex but nutritionally incomplete medium. Consequently, the approach to yeast CLS assays has been to grow cells to stationary phase in liquid medium and observe how long cells in this medium can survive and grow when returned to fresh medium.

The central question in developing a CLS assay for *S. pombe* is what criteria should be used to determine if a CLS protocol is a valid assay? In keeping with the goal of a model system to reveal evolutionarily conserved processes, one essential point is that the approach must recapitulate the well-established responses to conditions known to control lifespan. We therefore tested some of the known media used in the *S. pombe* field for one of the most well-conserved properties of lifespan control: lifespan extension by under nutrition and lifespan shortening by overnutrition.

Caloric restriction, or under nutrition, is an intervention known to cause a delay in the onset of aging phenotypes and a significant increase in lifespan in rodents, while higher levels of calories are associated with shorter lifespan (McCay et al. 1935). These observations have been recapitulated in *S. cerevisiae*, *C. elegans* and *Drosophila* (Bishop and Guarente 2007; Dilova et al. 2007; Guarente 2008). An additional property of these long-lived organisms is resistance to stress: a higher proportion of a population of long-lived yeast, nematodes and flies can survive lethal stresses compared to their normal counterparts (Gems and Partridge 2008; Kennedy et al. 1995; Masoro 2005, 2007; Sinclair 2005). Thus, lifespan extension by caloric restriction and the increased stress resistance of long-lived cells are evolutionarily conserved properties of aging in both unicellular and multicellular eukaryotes. One would therefore expect that a valid CLS assay for *S. pombe* should show these evolutionarily conserved responses to changes in nutrient levels.

The methodology for the CLS assay that fulfilled these criteria is shown in Fig. 13.5. Cells were tested in several media, including the commonly used Edinburgh Minimal Medium (EMM) and Synthetic Dextrose (SD) medium. It was surprising to find that the commonly used EMM medium did not respond in an evolutionarily conserved manner, as cells grown in 5% glucose (i.e. with higher levels of calories) lived longer than cells grown in EMM medium with the standard 2%

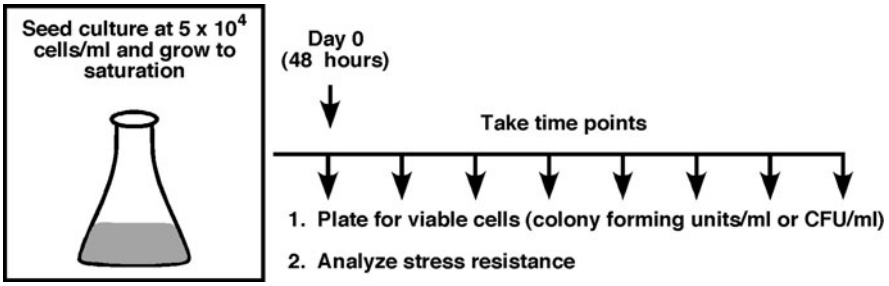

Fig. 13.5 Basics of the *S. pombe* CLS assay. Recently grown *S. pombe* cells are resuspended in 30 ml of medium at a low density (5×10^4 cells/ml) in a 125 ml flask and grown at 30°C under rotation for the course of the experiment. After 2 days of growth, cells reach their maximum density, which is marked as day 0 in the experiment. The density of viable cells per ml is determined by plating dilutions of cells on medium where all cells can grow, and counting the number of colonies after 4 days (this number does not change if cells are grown for longer periods of time). Synthetic dextrose medium provided a CLS assay that recapitulated all of the evolutionarily conserved features of aging that we tested

glucose (Chen and Runge 2009). Thus, increased calories prolonged lifespan, in contrast to what is observed in other species (Piper and Partridge 2007). Therefore, this medium was inappropriate for elucidating evolutionarily conserved mechanisms of lifespan control.

It is important to note that while increased glucose concentration has been reported to increase RLS in the budding yeast, this high-glucose-induced lifespan extension appears to be due to increased osmotic stress (Kaeberlein et al. 2002). In contrast to RLS, high osmotic stress shortened CLS in *S. cerevisiae* (Murakami et al. 2008). Thus, the extension of *S. pombe* CLS by high glucose in EMM appears to be an unusual phenomenon that does not reflect conserved mechanisms in the biology of aging, arguing against the use of EMM medium in *S. pombe* aging assays.

Synthetic Dextrose or SD medium proved to recapitulate the evolutionarily conserved features of lifespan shortening by overnutrition (high glucose levels) and lifespan extension by caloric restriction (low glucose levels). The standard 3% glucose medium did not artificially extend or shorten CLS by caloric restriction or overnutrition. The overnutrition condition (5% glucose) had a median lifespan only 40% of the 3% normal condition and a lower glucose concentrations of 0.3 and 0.1% showed the same long lifespans that were more than twice as long as the standard condition (Fig. 13.6a) (Chen and Runge 2009). Remarkably, the phenomenon of reaching a maximum lifespan as calories are further decreased is the same in rodents (Weindruch et al. 1986), so *S. pombe* grown in different SD media recapitulate the lifespan control seen in mammals. The longest-lived *S. pombe* from calorically restricted medium also showed increased resistance to heat and oxidative stress (Chen and Runge 2009), showing that the long-lived yeast showed the increased stress resistance seen in other long-lived organisms. Thus, the CLS in SD media was validated by evolutionary conservation of the response to overnutrition, caloric restriction and stress resistance of long-lived cells.

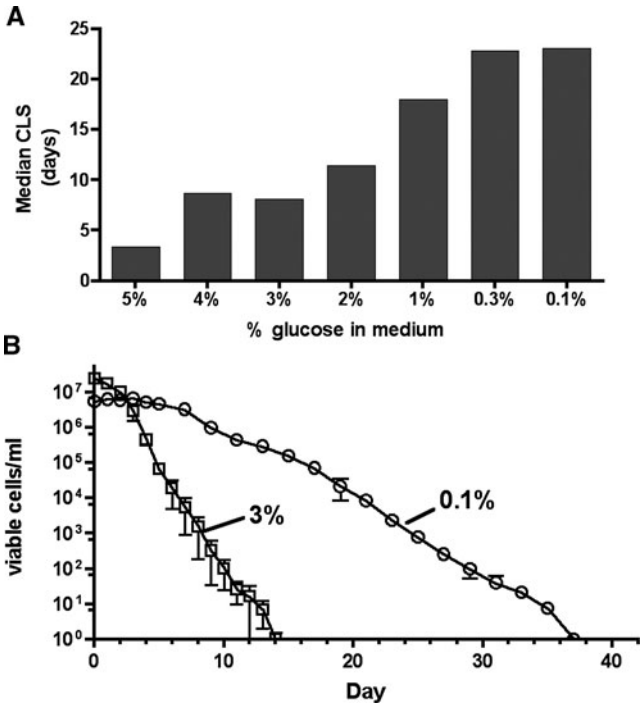

Fig. 13.6 The *S. pombe* CLS assay shows lifespan shortening under overnutrition and lifespan extension under caloric restriction. **a** The median CLS of wild type *S. pombe* in SD media with different glucose concentrations as described in Chen and Runge (2009). The standard 3% glucose condition does not artificially extend nor shorten lifespan, while overnutrition (5% glucose) shortens lifespan and caloric restriction (≥2% glucose) extends lifespan as seen in other species. Median lifespans for these assays are the point when $\log_{10}$ of the viable cells/ml is reduced to 50% of its original value (Chen and Runge 2009). **b** Representative CLS curves of two conditions showing that the number of viable cells/ml decreases uniformly without significant regrowth until all cells are dead (Modified from Chen and Runge 2009)

One unique feature of our CLS is that cells show a uniform decline in viability until all cells in the culture are dead (Fig. 13.6b). This assay represents a major departure from most previously published CLS assays in yeast and all other CLS assays performed in *S. pombe*, where viability is monitored over a decrease from 100% alive to 1 or 0.1% alive (e.g. Fabrizio and Longo 2003; Fabrizio et al. 2001; MacLean et al. 2001; Mutoh and Kitajima 2007; Ohtsuka et al. 2008; Roux et al. 2006, 2009; Wei et al. 2008; Weinberger et al. 2007; Zuin et al. 2008)). One advantage of following this smaller range of viability is that it allows a simple comparison of yeast CLS assay results with other lifespan assays of other organisms, which usually monitor scores to thousands of individuals, instead of the hundreds of millions of individuals in a yeast culture. In addition, analysis beyond 3 logs can be a problem because large numbers of individuals remain alive when only 0.1% of the culture is viable ($\sim 10^6$ cells), and it has been shown in budding yeast that some cells can

regrow as other cells die (Fabrizio et al. 2004) (Franco, J. and Runge, K.W., unpublished observations). These results complicate the selection in that the cells that survive the longest may not be those with the longest lifespan but those best at scavenging nutrients from dead cells, or those bearing secondary mutations that allow survival. While a number of important discoveries have been made with the budding yeast system in the face of this limitation (e.g. Fabrizio et al. 2001; Powers 3rd et al. 2006), the ability to age a culture with a uniform decline in viability until almost all of the cells are dead means that the *S. pombe* system affords a new opportunity to directly select for long-lived cells from a large pool of random mutants.

While our work was in progress, several other labs reported chronological lifespan assays for *S. pombe* that followed the *S. cerevisiae* protocol using EMM, SD or the complex rich medium YE (Mutoh and Kitajima 2007; Ohtsuka et al. 2008; Roux et al. 2006, 2009; Zuin et al. 2008, 2010). Using both SD and EMM media, Ohtsuka et al. were successful in isolating a multicopy plasmid that could extend *S. pombe* lifespan in their assay (Ohtsuka et al. 2008), which is quite remarkable since such plasmids vary in copy number (Brun et al. 1995), and thus the expressed protein levels vary between different cells in the population. While the function of the 80 aa ORF that Ohtsuka et al. cloned is currently unknown, their data suggest that lifespan extending genes whose effects operate above a certain threshold of overexpression can be identified from plasmid libraries. Consistent with this supposition, this plasmid-based assay has been used to clone several genes that extend CLS when present in multiple copies in both *S. pombe* and *S. cerevisiae* (Azuma et al. 2009; Ohtsuka et al. 2009; Roux et al. 2010). Two other labs have also reported lifespan extension by caloric restriction in SD or YE media (Roux et al. 2009; Zuin et al. 2010). The ultimate test for the utility of our assay and these others will be their ability to elucidate new lifespan controlling pathways that are also conserved in humans.

Requirements for the Direct Selection of *S. pombe* Mutants with Long CLS

In order to identify new longevity controlling pathways in *S. pombe*, one requires a method that applies the power of yeast genetics to directly select long-lived mutants. One important consideration in the identification of long-lived mutants is that lifespan is assayed in populations, not individuals, and some genetically identical members of the population die early while others die later. Consequently, in a mixed culture of mutants that have wildtype or extended lifespans, some cells with wildtype lifespan will most likely survive along with the long-lived mutants. While some mutations may increase lifespan so much that a culture can be aged until all of the cells with normal lifespan are dead and only the long-lived mutants survive, mutations that increase median lifespan by 10 to 15% are also very interesting (e.g. *clk-1* in *C. elegans* or *Drosophila* treated with resveratrol (Lakowski and Hekimi 1996; Wood et al. 2004)). Consequently, a method that can identify

mutations that increase lifespan by relatively small amounts as well as by large amounts is necessary for a thorough analysis.

A second consideration is that when trying to identify those mutants with a lifespan that is 15% or greater than wild type in a large population of random mutants, the culture would need to be sampled before all individuals are dead to isolate the full range of mutants. For example, some mutants may "rectangularize" the lifespan curve such that the cells survive for a long period of time (and thus have a longer median lifespan) but all of these cells are dead at the same point as wild type cells (Fig. 13.7a). This type of lifespan is akin to a human living a very active and healthy life up to death, making identification of such mutants both interesting and significant. However, a culture bearing these mutants will also have surviving wild type cells that have not yet died. If the long-lived mutant strain is one of thousands of mutants in a culture, then the thousands of surviving mutants with normal lifespan will still out-number the surviving long-lived mutant when the culture is sampled (Fig. 13.7b). Thus, a rapid method is needed to identify the long-lived mutant in this pool of cells with normal lifespan.

A third consideration is the identification of the mutation in the long-lived mutant. The typical yeast method of cloning the wild type gene by complementation with a library of plasmids is not practical. One would have to identify the few transformants in a population that have the shorter, wild type lifespan compared to the thousands other transformants that have the long lifespan of the mutant. Consequently, the lifespan-extending mutations must be readily identifiable.

The solution our lab has chosen is to create an *S. pombe* insertion mutant library where each mutant bears a "barcoded" DNA insertion vector randomly integrated

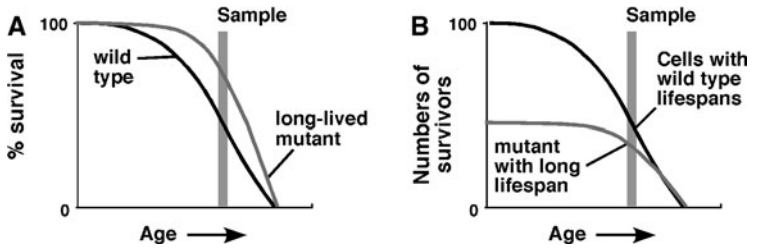

Fig. 13.7 Hypothetical survival curves of a long-lived mutant whose median lifespan is extended and whose maximum lifespan is equal to wild type. The *gray bar* labeled "Sample" indicates a point in the CLS when viability is monitored and colonies of surviving cells are isolated. **a** Examples of wild type cells and a mutant that "rectangularizes" the lifespan curve to provide a longer median lifespan (described in the text). If survival is on a log scale (e.g. Fig. 13.6b), then the number of viable mutant cells in the indicated Sample could be 100-fold higher than wildtype. **b** Relative amounts of surviving cells with 1-long lived mutant in a pool of 1000 mutants with normal lifespan. Even if the proportion of the long-lived mutant is increased by 100-fold in the total culture, this long-lived mutant will still only make up 10% of the surviving cells. Thus, aging the culture to near the end of the lifespan can amplify the fraction of long-lived mutants, but an additional mechanism to identify the long-lived mutants from the high background of cells with normal lifespan is required

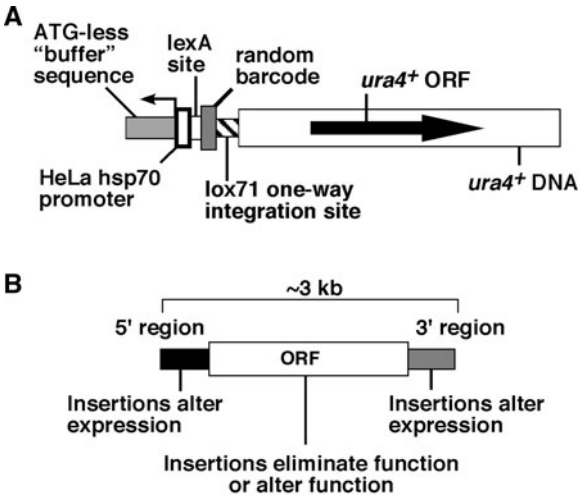

Fig. 13.8 Construction and rationale for the *S. pombe* DNA insertion library. **a** A map of the DNA insertion vector. The insertion vector contains the *ura4*$^+$ gene for selection in *S. pombe*, a random barcode containing 27 random nucleotides, a *lexA* binding site for tethering transcriptional enhancers upstream of a HeLa cell hsp70 promoter that functions in *S. pombe* (Prentice and Kingston 1992) and a buffer sequence with no ATG codons. DNA transformed into *S. pombe* can be degraded prior to integration, and the ATG-less buffer sequence and *ura4*$^+$ gene serve to preserve the barcode sequence in *ura4*$^+$ transformants. The hsp70 promoter can drive transcription of a nearby gene or gene fragment to alter its regulation. The vector also contains a mutant lox71 site of the cre-lox recombination system, which strongly favors site-specific integration with plasmids bearing the mutant lox66 site (described in detail in: Albert et al. 1995; Araki et al. 1997). These features allow the DNA insertion site to be identified by TAIL-PCR using the vector sequences (Liu et al. 1995), by Inverse or splinker PCR (Devon et al. 1995; Innis et al. 1990) using the unique barcode sequence as primer or by integrating a plasmid with a lox66 site and bacterial origin of replication into the lox71 site and cloning the flanking DNA in *E. coli*. **b** Insertions into different regions of an *S. pombe* gene can generate different phenotypes. Insertions into the exons and introns that give rise to the final mRNA ORF may eliminate the function of the final protein or generate a truncated product with an altered function (e.g. removal of a regulatory region). Insertion into the 5' or 3' sequence may alter promoter activity or mRNA stability, respectively, altering the final levels of the protein

into the genome (Fig. 13.8). Because the DNA insertion vector marks the mutation, one can readily identify the mutated gene and test if this mutation caused the longer lifespan. Each insertion contains a unique, random sequence that serves as a molecular barcode, so the relative proportion of each mutant in the culture is reflected by the relative proportion of each barcode. A long-lived mutant whose relative proportion increases late in the CLS (Fig. 13.7b) will be reflected by a similar increase in the proportion of its barcode. Thus, analysis of the barcodes can identify the long-lived mutant, and the unique barcode provides a PCR primer for identifying the cells that carry this insertion.

The barcodes were designed so that they could be amplified by PCR and easily oligomerized. Thus, a single DNA sequencing reaction could provide data on

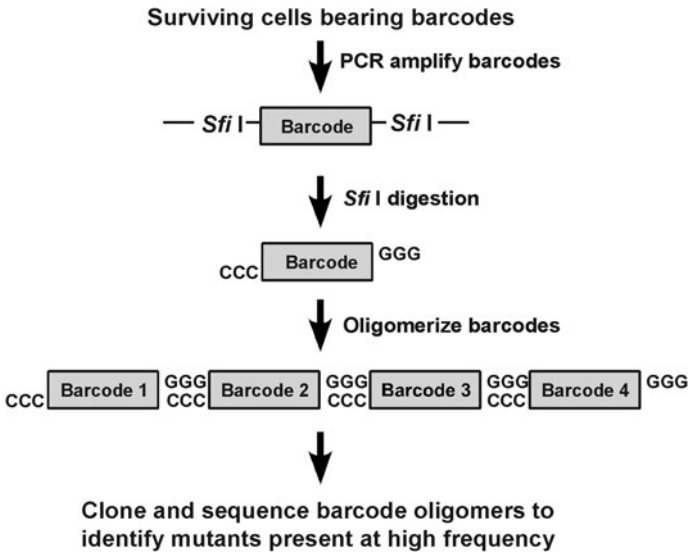

Fig. 13.9 Using barcodes to identify long-lived mutants. Each barcode is bordered by two *Sfi* I sites. *Sfi* I recognizes GGCCAGGGxAGGCC, where "x" shows the cleavage site that leaves a 3 nt overhang. The *Sfi* I sites in the insertion vector yield fragments that can be ligated into head-to-tail oligomers, and oligomers of 6–10 barcodes can be easily isolated and cloned for sequencing. One can therefore sample a large number of barcodes from the population of surviving cells and identify those that are present at high frequency. The barcodes sequences were designed as 27 bases of random sequence interspersed with Adenine residues at defined positions such that the barcodes contain no *Sfi* I, *BamH* I or *Sma* I sites, so digestion of the PCR fragments with *Sfi* I can recover all of the barcodes in the sample

multiple barcodes (Fig. 13.9). By starting with cultures where all mutants are present in approximately equal amounts, those barcodes that are present in a higher proportion (e.g. 10% of the total sequenced) are easily identified in an aged culture. A major advantage of this approach is that the barcode sequences do not need to be known prior to performing the experiment. By using sufficiently long, random barcodes to construct the insertion mutants, each of the barcodes will be unique as the chance of isolating two mutants with the same barcode is extremely small (e.g. the chance of two out of 1000 mutants having the same barcode from a library of $\sim 10^5$ barcodes is about $1/10^3$).

One notable advantage of this approach is that one can isolate several types of mutations in both genes that are dispensable for growth and in genes that are required for growth. Each *S. pombe* gene can be considered to have 3 regions where insertion can give rise to a phenotype (Fig. 13.8b). Insertions within the exons and introns that give rise to the final ORF could eliminate gene function and yield a null phenotype. However, some of these insertions, as well as insertions into the 5' and 3' regulatory regions, could alter protein function or final protein levels. As described above for *ATP2* in *S. cerevisiae* and a large number of *C. elegans* RNAi

mutations, reducing the level of protein without eliminating it can extend lifespan. Consequently, the insertion mutagenesis approach has the potential to identify a wide variety of lifespan extending mutations.

We have generated 10,000 such insertion mutants to date, with the individual mutants arrayed in 384-well plates (Chen, B.-R., Hale, D., Ciolek, P. and Runge, K.W., in preparation). To determine if the DNA insertion mutants were random, we assayed ~3600 of them for easily scorable phenotypes. We found 89 mutants: 30 were unable to grow on minimal medium (consistent with a mutation in a biosynthetic enzyme), 19 had changes in a colony color assay (reflecting mutations in mitochondrial function and/or adenine biosynthesis), 13 could grow on medium that causes loss of the mitochondrial genome (suggesting a mutation that allows *S. pombe* to lose mitochondrial genome and live) and 8 were unable to grow at 36°C (consistent with altered expression of a gene required for growth at higher temperature). No mutant had more than one phenotype, and a least one mutant was found in every test we tried. Thus, the variety of mutants are consistent with random integration of the DNA insertion vector throughout the genome.

We should note that a collection of deletion mutants is currently available from the private South Korean company Bioneer (Kim et al. 2010). Unlike the haploid ORF deletion mutant collection that was constructed in *S. cerevisiae* with public funds and sold at a reduced cost (~$3500), the *S. pombe* collection is more expensive (~$13,000), only 60% of the genes are deleted in the haploid strains, the deletions do not remove the entire ORF in some cases (i.e. over 600 mutants retain 20% of their coding sequences and over 200 mutants retain more than 40% (Kim et al. 2010)) and the barcode sequences for each deletion were not provided for several years after the library was first sold. Since the identity of the barcodes was published, we have found a number of discrepancies in the documentation of the mutant strains and their barcodes versus what is present in the collection. In spite of these shortcomings, the release of the data for most of the mutants in the collection does provide an opportunity for adapting this collection for the isolation of long-lived mutants from the pool of total mutants. These considerations also highlight the fact that additional genome-wide resources that combine the variety of mutations in our mutant library with the defined location of each mutation in the Bioneer library would be quite useful for large-scale mutant screening.

Proof-of-Principle Experiment to Isolate Long-Lived *S. pombe* Mutants from the Insertion Mutant Collection

To determine the feasibility of using our insertion mutant collection to identify mutations that extend lifespan, we have performed a pilot experiment in which 3400 insertion mutants were pooled into a single culture and aged in our standard assay. When the density of viable cells had dropped from ~10^7 cells/ml to ~10^3 cells/ml, 400 surviving colonies were picked to stock plates, the barcodes from the surviving cells were obtained and their frequencies were determined as described in

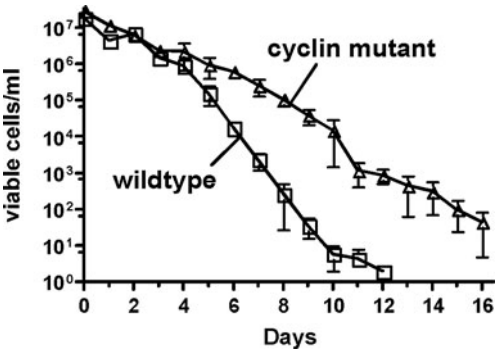

Fig. 13.10 The CLS of a long-lived *S. pombe* mutant that was isolated by direct selection using the insertion mutant library and barcode screening strategy. The mutation was later verified by recreating it in a wild type strain and showing that two independently recreated mutants also had longer lifespan. Thus, the barcoded insertion mutant strategy shows great promise for the identification of evolutionarily conserved lifespan-extending mutations using the *S. pombe* system

Fig. 13.9. One barcode, and thus its associated mutant cell, constituted ~10% of the all barcodes sequenced in the experiment. An oligonucleotide corresponding to the barcode was synthesized and used to identify the colonies containing the barcode by PCR, and then to subsequently determine the insertion site and mutated gene. The insertion was in the middle of the ORF of the gene with a high sequence similarity to a class of *S. cerevisiae* cyclins for the cyclin-dependent kinase Pho85p (Carroll and O'Shea 2002; Matsumoto and Wickner 1993; Measday et al. 1997).

This mutant was shown to have a longer lifespan when tested in a culture containing only this mutant in the standard assay, and creating this mutation in a wild type strain produced the long-lived phenotype (Fig. 13.10). Thus, the selection strategy was able to isolate a mutant with a longer lifespan. These data raise the interesting possibility that the mutated cyclin gene allows *S. pombe* to enter stationary phase more easily and thus survive longer after nutrients are exhausted, and tests of this idea are underway.

Promises and Problems of Unbiased Genetic Approaches to Identify Lifespan Extending Pathways

The molecular genetics of the budding and fission yeast systems have provided important tools for directly testing existing models of aging. The ability to easily alter the genome, coupled with a large background of information on the physiology of each system, provides a great platform for testing hypotheses. The budding yeast system has the added benefit of an extensive array of publicly available, affordable resources and tools that have been successfully used to identify new genetic pathways whose alteration can extend lifespan. Screens for mutants with increased stress resistance (Fabrizio et al. 2001) or ability to regrow after extended periods of storage

(Kennedy et al. 1995; Powers 3rd et al. 2006) have made important contributions to the yeast aging field in both the identification of new pathways (e.g. sirtuins) and additional insight into evolutionarily conserved ones (e.g. Akt and Tor). Our own screen for budding yeast mutants that alter gene silencing has led us to investigate a new signaling pathway from mitochondria to the nucleus and the consequences of varying the expression of components of the mitochondrial F_1F_0-ATPase.

While these screening approaches have generated interesting lines of research that can occupy many labs for years, all of the screens are strongly biased. The screens for stress resistance or changes in silencing require that the lifespan controlling mutations share these phenotypes. The screens for regrowth using the yeast ORF deletion strain set only examine complete loss of gene function, and so miss those lifespan extending phenotypes caused by underexpression or point mutations (e.g. the lifespan extending *sir4-42* mutation (Kennedy et al. 1995)). While our newly developed insertional mutant library in *S. pombe* addresses some of these issues, all potential lifespan extending mutations will not be represented (e.g. point mutations that alter a regulatory phosphorylation site). These considerations illustrate that while these broad genetic approaches can identify a diverse array of pathways, some lifespan controlling pathways will be missed. Thus, the list of pathways identified by these approaches will be limited.

Currently, this limitation is not a problem because the number of genes to investigate is quite large. However, this situation may change as the fields of bioinformatics and genomics continue to advance. As more computational tools are developed to compare the interactions of proteins, transcription networks and the growth of double mutants (Charbonnier et al. 2008; Ge et al. 2001; Li et al. 2004; Rual et al. 2004; Walhout et al. 2002; Yu et al. 2004), it seems likely that similar tools can be adapted to cross-reference phenotypes and classes of mutations that correlate with changes in lifespan. If such tools can be developed, then comprehensive mutant screens that test as many types of mutations as possible should help reveal how different lifespan controlling pathways connect to one another to create a normal lifespan, and how to perturb them to cause the largest lifespan extension with the least alteration of other phenotypes.

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