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Aging Research in Yeast

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Aging Research in Yeast

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Chapter 1
Introduction

Michael Breitenbach, Peter Laun, and S. Michal Jazwinski

Abstract Aging in yeast is now a well researched area with hundreds of new research and review papers appearing every year. The chapters following in this book written by some of the leading experts in the field will give an overview of the most relevant areas of yeast aging. The purpose of this chapter is to give the newcomer an introduction to the field including some basic technical questions.

Keywords *Saccharomyces cerevisiae* · Replicative aging · Rejuvenation · Asymmetric segregation · Stem cells

General Introductory Remarks

Cells of the budding yeast, *S. cerevisiae*, have for several decades now been considered as the prototypic eukaryotic cells, ideally suited to study and uncover many of the basic phenomena of eukaryotic life. This is because of the unrivaled ease and speed of genetic and molecular genetic analysis in yeast, the small genome size (12 Mbp), the short doubling time (80 min on complex media), a fully developed system of sexual reproduction with stable haploid as well as diploid phases enabling complementation as well as recombination analysis (Dickinson and Schweizer 2004; Stansfield and Stark 2007).

Methods of "reverse genetics" are efficient and easy to handle making yeast one of only two model organisms of aging where exact gene replacement resulting in "knock in" strains can be routinely performed. The other cell type where this can routinely be achieved at present, although with a much higher investment of time and money, is ES cells of the mouse. In this way, any desired mutation can be introduced at will in haploid cells in the about 4800 non-essential yeast genes. In the remaining about 1200 "essential" yeast genes, the same is true, but a severe loss of function would lead to death, and these mutations have to be kept in a heterozygous state.

Knowing the yeast whole genome sequence and the functional annotation of yeast genes which has taken place over the last 15 years, and using high throughput

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methods and the many publicly available mutant and gene collections, including cDNA microarrays, whole genome screening procedures have become a powerful tool for yeast genetic research and have also been used for aging research.

However, of course, not every aspect of eukaryotic life can be modeled in yeast and an obvious example is development and cell differentiation, which exists in yeast, but is much more complex in higher multicellular organisms.

The questions which we are asking here are: are the cellular aging processes of yeast which are described in this book, relevant and similar in mechanism to the cellular aging processes observed in cultured higher cells and in higher organisms? What can we learn from yeast aging that is relevant to understand the aging processes of higher organisms? Can this lead to interventions in the aging process of humans that improve the lifespan and health span of humans? In order to answer these questions, we must understand the molecular genetic pathways relevant to aging both in yeast and in higher organisms and we have to compare the two systems with special emphasis on highly conserved genes playing a role in those pathways. Highly conserved genes, pathways, and external interventions would point to "public mechanisms of aging", while such genes and pathways that are found to influence aging only in a restricted number of organisms, are called "private mechanisms of aging" (Martin et al. 1996). One example for a public mechanism is caloric restriction (Jiang et al. 2000; Kaeberlein et al. 2005) while an example for a private mechanism of aging is provided by the extrachromosomal circles of ribosomal DNA (ERCs) (Sinclair and Guarente 1997) in yeast mother cell-specific aging. The model systems for organismic aging of higher organisms which are most highly developed are the mouse (important because it is so closely related to humans), *Drosophila melanogaster*, and *Caenorhabditis elegans*.

Yeast supplies us with two independent aging models which both have similarities to cellular aging processes in humans but have little to do with each other in terms of the genes which are involved (Laun et al. 2006). The main purpose of this Introduction is to present these two aging processes, to compare them with each other, and to evaluate them with regard to the aging processes in the human body for which they are claimed to be models.

Mother Cell-Specific (Replicative) Aging of Yeast Cells

Individual yeast cells of standard laboratory strains can produce only a limited number, typically 20–30, daughter cells during a lifetime (Mortimer and Johnston 1959). This process takes about 2–3 days on complex media at 28°C and is therefore one of the most rapid aging processes known. The lifespan of a cell is counted in generations (buds, daughter cells produced), but not in calendar time and is actually independent of calendar time (Müller et al. 1980). During the process, the mother cell becomes bigger with every generation and accumulates bud scars (Fig. 1.1). Mother cells change gradually in cycle duration (Egilmez and Jazwinski 1989) and many other biochemical parameters like ROS content (Laun et al. 2001) and protein carbonyl content (Aguilaniu et al. 2003), until they reach a final state of senescence

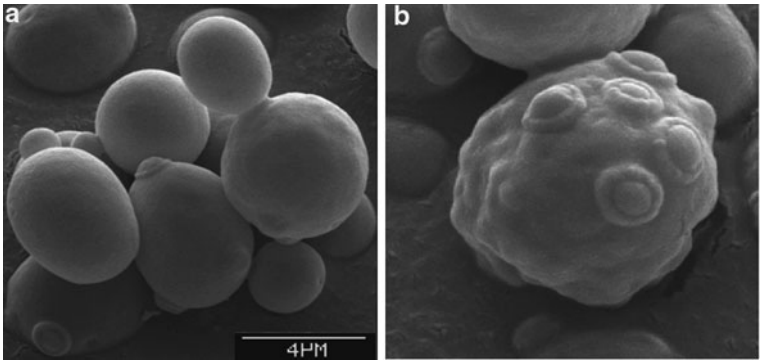

Fig. 1.1 Scanning electron microscopy pictures obtained by standard procedures after elutriation centrifugation (Laun et al. 2001) of a haploid strain (BY4741). **a** Fraction II young yeast cells. Note small size, the smooth surface and the infrequent bud scars. Virgin cells display no bud scars but only one birth scar. **b** Fraction V old mother cells of the same strain. Note the large size, the irregular surface and the multiple bud scars. Both pictures shown at the same magnification (unpublished data of the authors)

characterized by loss of cell cycle checkpoint mechanisms (Nestelbacher et al. 2000), loss of heterozygosity in diploid cells (McMurray and Gottschling 2004), and apoptosis (Laun et al. 2001). On the other hand, the daughter cells which are born to young and old mother cells, are rejuvenated: they differ in size only slightly (Klinger et al. 2010) and reset their clock to zero preventing clonal aging of the strain (Egilmez and Jazwinski 1989). A schematic representation of this process is shown in the now familiar "spiral" picture (Fig. 1.2). Only the daughters of very old mothers on glucose media inherit some of the "death factor" (damaged material) (Egilmez and Jazwinski 1989) and display a somewhat shortened lifespan (Kennedy et al. 1994). The mechanism by which rejuvenation is possible is of the highest interest and relevance but is not well understood yet today.

Mother Cell-Specific Aging Is a Stochastic Phenomenon

A cohort of about 60 cells, which is about the minimum for a statistically significant characterization of aging of a strain, displays a distribution of lifespans which follows the Gompertz law (Jazwinski et al. 1989) (Fig. 1.3). The median of this distribution function is the best single parameter to describe the lifespan of the strain in question. As shown in Fig. 1.3, single gene mutations are known which significantly increase the replicative lifespan of yeast.

In the case of replicative life span, the decrease in survival probability is exponential with increasing generations (cell divisions) completed (Gompertz 1825).

However, this relationship breaks down for the last survivors in an aging cohort due to the plateau in mortality rate at late ages (Jazwinski et al. 1998).

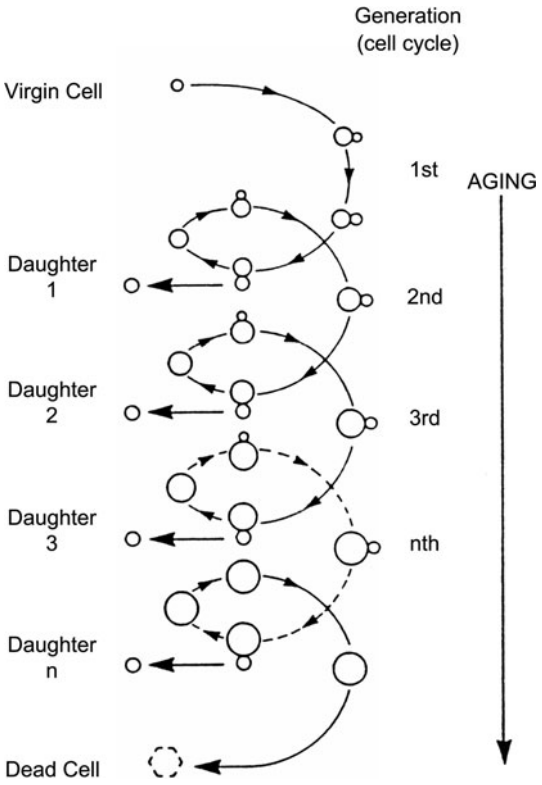

Fig. 1.2 Schematic of mother cell-specific aging (Jazwinski et al. 1989). Every cell division cycle is represented by one turn of the spiral. In every generation the mother cell grows and ages, while the daughter cell is rejuvenated and increases in size only slightly (Klinger et al. 2010). The terminally senescent mother cell can no longer produce a bud and eventually dies and lyses through apoptosis (picture taken from Jazwinski et al. 1989; with permission from Elsevier)

The morphological asymmetry of mothers and daughters of budding yeast is very obvious and it is now clear that many of the cellular components are asymmetrically segregated in this process, among them damaged proteins and organelles (Aguilaniu et al. 2003; Eldakak et al. 2010; Erjavec and Nystrom 2007; Klinger et al. 2010; Lai et al. 2002). The damaged material is retained in the mother while the fully functional cell components are transmitted to the daughter ensuring her rejuvenation. It is important to note that a similar process of asymmetric segregation take place even in cells where both progeny formed in a cell division cycle are morphologically equal (*E. coli*, *S. pombe*; Barker and Walmsley 1999; Nystrom 2007), and very probably in every living cell. Morphological and/or functional asymmetry is the basis of a theory of aging (Erjavec et al. 2008; but also Rashidi et al. (Chapter 14, this volume); Jazwinski 1993) that was explicitly tested in *S. pombe* (Barker and Walmsley 1999) before asymmetry had been recognized in this organism. Arguably, the asymmetric distribution and hence the differential transport of damaged cellular material ("waste") is necessary to prevent clonal aging which would eventually lead to death of all descendants of a cell, and therefore to the death of the species. Why is asymmetric segregation so important? Actually it is closely linked to the problem of selective degradation of damaged material in

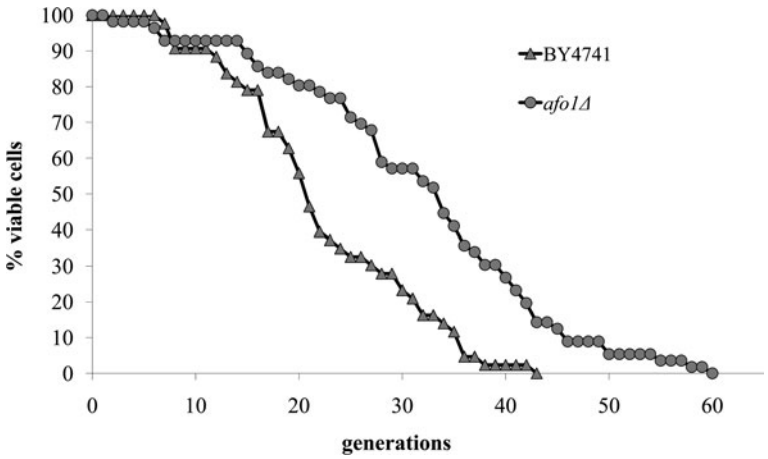

Fig. 1.3 Mother cell-specific lifespan distribution (replicative survival curve) of a wild type strain (BY4741, *triangles*) and an congenic mutant deleted for afo1 (*circles*), a gene coding for a mitochondrial ribosomal protein. The mutant is respiratory deficient and significantly long-lived (Heeren et al. 2009). About 40–50 individual cells each were micromanipulated to obtain the survival curves shown to obtain adequate statistics (picture modified after Heeren et al. 2009, with permission from Impact Journals LLC)

all cells. Numerous interlinked degradation pathways exist, but autophagy (only in eukaryotes) and the eukaryotic proteasome and its prokaryotic equivalent, the Lon protease, constitute the most important pathways. The overriding importance of the cellular degradation pathways to prevent cellular aging have been stressed by many authors (for example: Vellai 2009, and it is discussed at some length in a Chapter 4 in this book [Jazwinski]; Vernace et al. 2007). These processes are essential for life, but they do not work with 100% efficiency. We argue that deposition of the remaining damaged material in the mother cell is necessary in addition to functional autophagy and proteasomal degradation, to prevent clonal aging.

Comparison with "Hayflick Type Aging" of Human Cells

Leonard Hayflick discovered (Hayflick and Moorhead 1961) that human cells in culture (for instance, dermal fibroblasts or human umbilical vein endothelial cells, HUVEC) have a limited lifespan and undergo clonal aging resulting in death of all descendants of the primary cell. The aging HUVEC display remarkable similarity to aging yeast mother cells, in particular both cell types increase in size, produce ROS, collapse their actin cytoskeleton to large patches of F-actin, and undergo apoptosis (Breitenbach et al. 2003). However, other cell types do not apoptose after ceasing cell division but instead undergo a process called cellular senescence, remaining viable in a non-dividing state over a prolonged period of time. The discovery and

subsequent analysis of Hayflick aging was greeted with much enthusiasm, because the lifespan of a cell culture depends on the species and is proportional to the lifespan of the species. The in vitro lifespan correlates with the age of the donor individual, is characterized by telomere shortening, and can be elongated by several cell generations by ectopically expressing telomerase in the cultured cells (Blasco 2007; Bodnar et al. 1998). However, it is unclear to what degree the Hayflick phenomenon depends on the unphysiological oxygen partial pressure that was used in nearly all cell culture experiments and to what degree Hayflick aging occurs in the human body and actually limits lifespan. (There is, however, evidence that senescent cells accumulate during aging in human tissues. Work by Judy Campisi (Freund et al. 2010) suggests that these senescent cells have a pro-inflammatory and tissue proteolytic phenotype. This has an effect on neighboring cells, called the bystander effect). Measuring the Hayflick limit in low oxygen (3–5% similar to the oxygen partial pressure prevalent at peripheral tissues instead of the usual 21%) leads to a large increase in cell lifespan and perhaps immortality (Fehrer et al. 2007); a question most pertinent in the case of stem cells. These are open questions at present which are not the subject of our book.

Comparing Mother Cell-Specific Aging to the Aging of a Stem Cell Population

Presently, the role in aging and the changes in stem cell number and quality during the aging process of higher organisms are at the center of research interests. In this connection, it was noted early on that the process of mother cell-specific aging caused by asymmetric cell divisions is similar to the asymmetric divisions observed in stem cell populations of the human body (Lai et al. 2002). Cell divisions of stem cells are asymmetric and result in one rejuvenated new stem cell (akin to the yeast daughter cell) and one differentiated cell (progenitor cell) that has performed the first step towards a mature cell and eventually after many more cell divisions reaches a terminal state and no longer divides. The best studied among stem cell populations are the populations of hematopoietic stem cells of the red bone marrow and also muscle stem cells (satellite cells). There is at present much information available about changes in gene expression comparing stem cells and their differentiated derivatives, but nearly no information about asymmetric distribution of damaged material in the same process. However, studies of stem cell aging are now beginning and an increasing number of papers about the aging of stem cell populations appears, starting around 2005 (examples: Conboy and Rando 2005; Rando 2006). These papers also show the limits of the analogy between yeast mother cell-specific aging and stem cell aging: The yeast daughter cell rejuvenates completely; if it could not do so, the species would have died out long ago. The typical stem cell (muscle stem cell in the above example) needs a stem cell niche (which the yeast cell does not have) and ages, i.e. changes its quality during a lifetime with far-reaching consequences for the regeneration potential of the organ, stem cell therapy and related medical problems. There is only one cell type in the human body which

must by definition rejuvenate completely: this is the population of germ line stem cells which give rise to gametes. How they do it is a complete mystery at present.

Chronological Aging of Stationary (Non-growing) Yeast Cells

This is the second aging model system which yeast offers us. It has been compared to the aging of postmitotic cells of the human body, most prominently to the aging of neurons in the central nervous system. Chronological aging, or the survival of stationary yeast cells is studied measuring clonogenicity of a stationary yeast culture, which is kept with shaking and aeration at 28°C in the spent medium for several weeks (see the chapters of Fabrizio and Longo (Chapter 4), Werner-Washburne et al. (Chapter 6) and of Piper (Chapter 7), this volume for a detailed description). Aliquots are plated out every day on complex media and the number of colonies is counted after 2 more days. Depending on the strain background and the media used, an exponential decay of clonogenicity is observed and typical half lives found in laboratory strains are between one and three weeks. Single gene mutations are known that lead to a large increase in chronological lifespan under otherwise identical conditions (Fig. 1.4). When the aging of postmitotic human cells and of stationary yeast cells are compared, some obvious similarities are observed, but also some obvious differences. In yeast chronological lifespan assays, the cells observed are starving and their survival depends to a large extent on the genetic response to starvation conditions resulting in a large number of morphological and physiological changes (see the Chapter 6 by Werner-Washburne et al. this volume). These changes were essential during evolution for survival of the species, because wild yeasts for the larger part of their life (for instance during winter in moderate climates!) are actually in non-growing (starving) conditions. It should be kept in mind, though, that yeast cells in the wild are largely diploid and undergo sporulation on starvation, yielding the long lived dispersal form called the spore. On the other hand, postmitotic human cells in grown-up individuals (neurons of the brain, myotubes of the muscle, and the

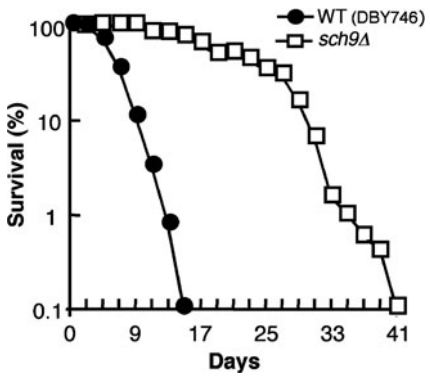

Fig. 1.4 Chronological survival of wild type DBY746 (*circles*) and congenic long-lived *sch9* deletion mutant (*squares*) (after Fabrizio and Longo 2003; with permission from John Wiley and Sons)

like) are not starved, but on the contrary at their peak metabolic activity; consider ATP production and respiration as an example. These cells therefore have to cope with different problems, like for instance damage removal by autophagy and other mechanisms. Dying stationary yeast cells undergo apoptosis (Herker et al. 2004). One of the most important findings in the field of chronological aging of yeast was the discovery of two quite different cell populations in stationary cultures (Fig. 1.5) (Allen et al. 2006), one of which, the replicatively young cells (daughters), are morphologically differentiated, display low metabolic activity and are very long-lived, while the other cells in stationary culture are prone to apoptosis, lyse, and can to some degree feed the other part and lead to adaptive re-growth (Fabrizio and Longo 2008).

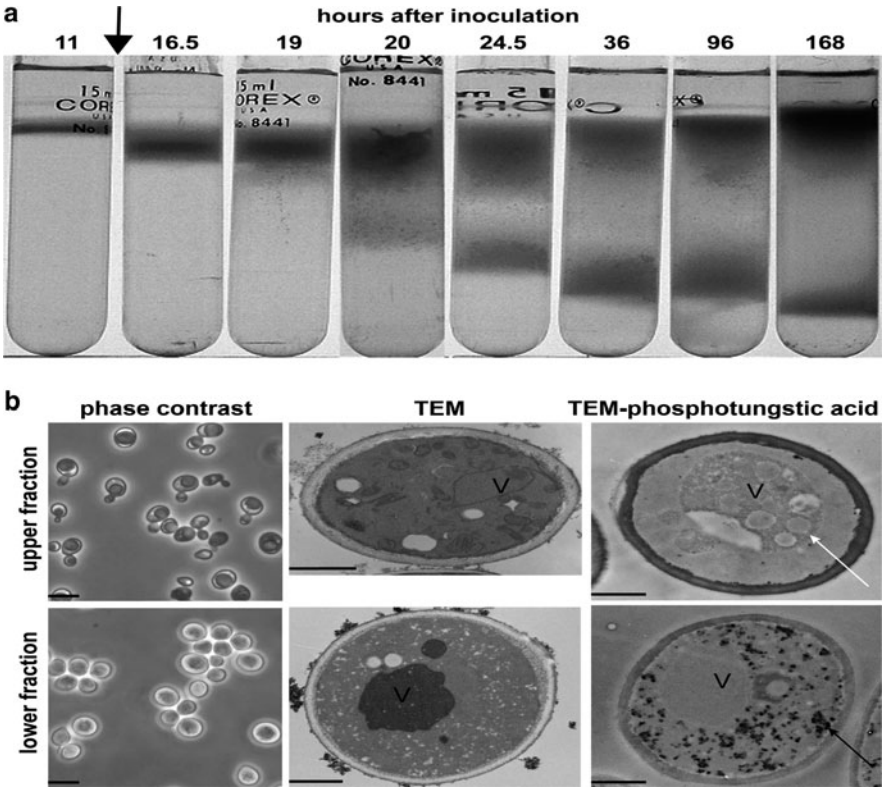

Fig. 1.5 Two distinct cell populations are formed in yeast cultures entering stationary phase. **a** Density-gradient separation of two distinct cell fractions in S288c cultures as a function of time after inoculation. Glucose exhaustion (*arrow*) occurred 12 h after inoculation. **b** Phase contrast, transmission EM (TEM), and phosphotungstic acid-stained transmission EM micrographs of upper and lower-fraction cells from stationary phase cultures (7 days after inoculation). The *white arrow* indicates vacuolar vesicles, and the *black arrow* indicates accumulated glycogen. V, vacuole. Bars: 10 μm (*left*); 1 μm (*middle* and *right*) (picture taken from Allen et al. 2006; Originally published in JCB. doi: 10.1083/jcb.200604072)

Caloric Restriction and the Role of Intermediary Metabolism

Over the last few years it became increasingly clear that basic carbon metabolism and intermediary metabolism plays a central role in the determination of lifespan. In rodents (mice, rats, and others) it was shown that allowing about 70% of the *ad libitum* food intake of a well-balanced diet containing all necessary components like essential amino acids, vitamins, and trace elements, led to an increase in lifespan and healthspan (as measured for instance by cardiovascular parameters) of about 50%. The incidence of cancer and cardio-vascular pathologies was much lower in the calorically restricted animals, comparing them with their age-matched *ad libitum* fed littermates. It is an open question, if such an intervention can slow down the aging process in primates (Anderson et al. 2009; Ingram et al. 2007) and humans, although promising reports on brain and heart aging in calorically restricted rhesus monkeys have been published (Kastman et al. 2010; McKiernan et al. 2011). Most recently, it has been reported that indeed CR may extend the lifespan of Rhesus monkeys (Colman et al. 2009). It is of course interesting, if the same intervention can positively influence the replicative as well as the chronological aging of yeast. In most papers, low glucose (0.5% instead of the usual 2%) was shown to enhance the RLS as well as the CLS of yeast. However, we would like to caution the reader that this intervention is not the same as reducing the total intake of an otherwise balanced diet. Lowering glucose is well known to relieve glucose repression in yeast, which for instance stimulates mitochondrial respiration substantially, while other essential components of the media remain the same. It is important to separate this increase in mitochondrial respiration from possible other consequences of low glucose by experiments with non-respiring strains, which has been done with controversial results (Kaeberlein et al. 2005; Lin and Guarente 2006; Lin et al. 2002). In one study, the influence of the whole range from 0.1 to 5% glucose on RLS was tested, and it was shown that low glucose as well as very high glucose leads to a substantial elongation of RLS (Kaeberlein et al. 2002). In another study, further decreases in glucose levels past the point at which the cells are released from glucose repression resulted in progressive increases in RLS (Jiang et al. 2000). Low glucose can also increase CLS (Chapter 5, this volume), but no systematic testing of the whole range of glucose concentrations is available, and most importantly, nobody has tested a balanced reduced diet for yeast. Reducing the availability of amino acids in the diet leads to an increase in lifespan (Houtkooper et al. 2010). This was shown for RLS in yeast as well (Jiang et al. 2000), and unpublished studies indicate that glutamate and aspartate play a central role (our own unpublished observations, SMJ). Recently this was shown for methionine in *Drosophila* (Grandison et al. 2009) and for tryptophan in yeast RLS (our own unpublished observations, MB). There are interesting gene-regulatory overlaps between caloric restriction and the retrograde response in yeast that may be related to the role of TOR (Wang et al. 2010 and they are discussed in a subsequent Chapter 4 by Jazwinski, this volume.).

After this introductory discussion of the two aging models of yeast and the influence of caloric restriction on yeast aging, we may again ask the question as to the

real driving force of organismic aging in higher organisms and what it possibly has to do with replicative and chronological aging of yeast.

The final word has not been said, but the aging processes seem to be multifactorial and multicausal with only some of the mechanisms known at this time. Survival of postmitotic cells on the one hand, and regenerative capacity provided by stem cell populations, together determine the lifespan of an organ.

The yeast RLS and CLS, we argue, may serve as models that stress the role that the handling of molecular damage by cells plays. Damage accumulation in cells produces a variety of stresses and the genetic program of stress response is what we observe as a "genetic program of aging". It can be expected that the processes of damage removal (autophagy and proteasomal degradation, and others), of apoptosis and of regeneration of tissues by activating stem cells, will be in the center of aging research in the future. The interaction of damage removal and asymmetric segregation of damage is a new and powerful paradigm in aging research. Yeast molecular biology and genetics will supply a substantial contribution to the open questions of future aging research.

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Chapter 2
Oxidative Stresses and Ageing

May T. Aung-Htut, Anita Ayer, Michael Breitenbach, and Ian W. Dawes

Abstract Oxidative damage to cellular constituents has frequently been associated with aging in a wide range of organisms. The power of yeast genetics and biochemistry has provided the opportunity to analyse in some detail how reactive oxygen and nitrogen species arise in cells, how cells respond to the damage that these reactive species cause, and to begin to dissect how these species may be involved in the ageing process. This chapter reviews the major sources of reactive oxygen species that occur in yeast cells, the damage they cause and how cells sense and respond to this damage.

Keywords Oxidative stress · Redox · ROS (reactive oxygen species) · Radical · Defence

Introduction

The concept of "oxidative stress" is commonly used in the current literature on aging of yeast cells and higher cells. It seems to be intuitively clear but is in reality hard to define and even harder to quantitate, because the redox-active metabolites of the cell are not in thermodynamic equilibrium, and it is often unclear how readily they exchange redox equivalents with each other and across the boundaries of subcellular compartments, which have different redox potentials. It is even harder to apply this concept to organismic aging of the most important currently used aging model animals, like *Drosophila*, *Caenorhabditis elegans*, and the mouse. This is because organs and tissues also differ in their redox potentials and in the management of oxidative stress. In the words of many authors in the aging research field, oxidative stress is loosely defined as "the accumulation of reactive oxygen species (ROS)". A more logical definition of oxidative stress posits that oxidative stress is a period of deviation from the normal value of the intracellular redox environment. In the cytoplasm of a non-stressed living yeast cell, this the redox potential is very reducing, recent data indicate in yeast cells this is about –310 mV

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(Ayer et al. 2010), and is maintained by elaborate homeostatic mechanisms. Any deviation that cannot be immediately repaired is detrimental to the cell, leading either to reductive or oxidative stress (Lipinski 2002; Trotter and Grant 2002). The problem with this concept is the difficulty in determining the intracellular (or intra-organellar) redox potential. There are now several possibilities through the development of green fluorescent protein derivatives such as ro-GFP and through metabolomics, measuring by HPLC/MS methods the concentration of all relevant metabolites (some of which are discussed later) and using the Nernst equation. Such methods have become available only recently through the current increased interest in metabolomics. In subsequent chapters this potential involvement of reactive oxygen species (ROS) and oxidative stress in aging is discussed in more detail. This chapter discusses some of the major ROS present in cells, and how cells respond to them.

An overview of the most important ROS is given in Fig. 2.1a. However, most ROS are short lived and do not accumulate. For instance, the half-life of hydroxyl radicals, the most reactive ROS produced in the cell, is in the range of ns (reactions of the hydroxyl radical are diffusion-controlled). Superoxide under physiological conditions found in living cells (presence of SOD) has a half-life in the range of ns also, although it is longer-lived in the absence of degrading systems (Di Mascio 2007; Sies 1993). Hydrogen peroxide (H_2O_2), peroxynitrite (HOONO) and other non-radical ROS also arise from the primary ROS, by various chemical and metabolic pathways (Fig. 2.1b, c). What do accumulate are the downstream reaction products of the ROS. These include organic peroxides (such as fatty acid peroxides), protein carbonyls, oxidatively inactivated proteins; and reactive aldehydes

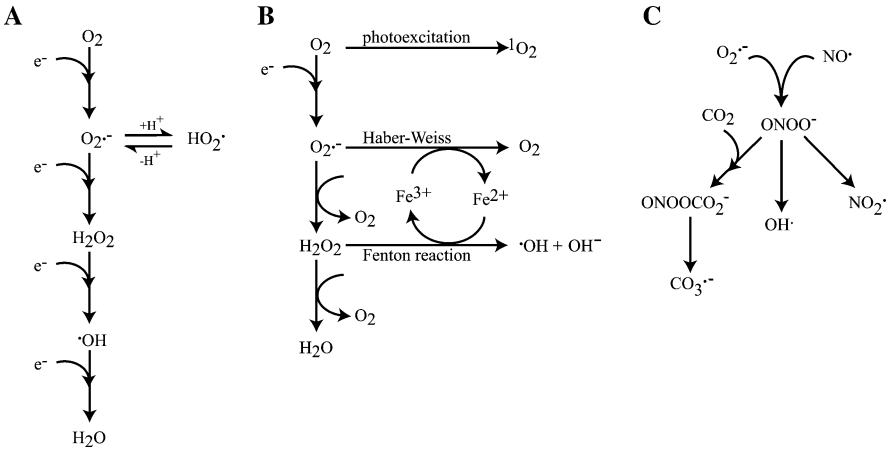

Fig. 2.1 Reactive species generated in biological systems. **a** The range of various reduced states from O_2 to H_2O_2. Note that the *arrows* do not denote reactions, but merely the number of electrons needed to go from oxygen to the reduction state. **b** Reactive oxygen species generated in biological systems from the one-electron reduction of O_2. **c** Reactive nitrogen species (RNS) and resulting radical formation

such as malondialdehyde (Turton et al. 1997) and 4-hydroxy-nonenal (HNE) (Cipak et al. 2006), which are toxic and mutagenic. HNE formation depends on the presence of poly-unsaturated fatty acids, which wild type yeast does not produce, but expressing just one additional plant fatty acid desaturase in yeast leads to HNE formation, with the expected consequences of formation of the toxic HNE (Cipak et al. 2006). A large number of such reaction products are known, but only a few of them have been studied in detail with respect to their metabolism, degradation, and their relationship with the aging process. Some of the (over 100) oxidative post-translational modifications of proteins, lipids and nucleic acids are physiological and reversible, but some are apparently pathologic and irreversible and can be removed only by degradation of the modified molecule. They can, indirectly, lead to cell death. For very few of these modifications the adequate analytical methods for quantification are available, although there has been a detailed analysis of protein carbonyls (Aguilaniu et al. 2003; Erjavec et al. 2008; Reverter-Branchat et al. 2004), which are conveniently recognizable at the cellular level. At the molecular level, protein carbonyls are, however, heterogeneous (Briggs et al. 2002; Levine and Stadtman 2001).

Reactive Oxygen Species

Through evolution, aerobic organisms have developed an efficient way to harvest energy from organic compounds using oxygen via respiration in the mitochondrion. However, some electrons escape from the respiratory chain during the reduction of oxygen to water and this leads to generation of reactive oxygen species (ROS), mainly the superoxide anion, $O_2^{\bullet -}$ (Boveris and Cadenas 1982; Boveris 1984) (see the chapter on mitochondria and aging, this volume). $O_2^{\bullet -}$ is also produced from microsomal metabolism (Reinke et al. 1994) and the respiratory burst produced by phagocytes as a defence mechanism against bacteria (Babior 1984). Although the normal operation of the mitochondrial respiratory chain is the main source of ROS production under physiological conditions, changes in mitochondrial morphology (Scheckhuber et al. 2007) and disruption of actin dynamics (Gourlay and Ayscough 2005, 2006) can exacerbate ROS production. There are two main methods to measure $O_2^{\bullet -}$, electron spin resonance (ESR) and fluorescence measurements using molecules, which after oxidation through superoxide emit a defined fluorescence. Electron spin resonance can be performed in vivo, using spin traps like DEPMPO and still is the gold standard for measuring superoxide (Heeren et al. 2004; Nohl et al. 2005) because it produces specific resonances that depend on the presence of the superoxide itself in the adduct. Among the fluorescence-based methods for measuring superoxide, dihydroxyethidium (DHE) oxidation stands out as the most reliable (Benov et al. 1998). The oxidation of DHE yields two fluorescent products: 2 hydroxyethidium (EOH), which is more specific for superoxide; and, the unspecific ethidium. Therefore only HPLC in combination with fluorescence detection of EOH can approximate superoxide release. The method is complicated by the fact

that DHE is also oxidised to EOH by other species such as peroxinitrite and xanthine oxidase, therefore, a combination of different methods: DEPMPO/ESR (highly specific, less sensitive), CPH/ESR (highly sensitive, less specific) and DHE/HPLC (average sensitivity/less specific) is recommended (Gille and Staniek, personal communication).

The endoplasmic reticulum, which provides the environment for protein folding is also another main source of ROS production. The ROS generated from the ER mainly come from oxidative protein folding especially during disulphide bond formation (Tu and Weissman 2004). Two electrons are transferred to the protein disulphide isomerase Pdilp, then to the flavoprotein-containing Ero1p during this process and under aerobic conditions, oxygen acts as the terminal electron acceptor with the probable generation of hydrogen peroxide (Tu et al. 2000; Tu and Weissman 2002). In addition, ER stress caused by conditions such as hypoxia and viral infection, which disrupts ER homeostasis, also produce ROS including superoxide (Tan et al. 2009).

H_2O_2, another ROS, is generated from the breakdown of $O_2^{\bullet -}$ by superoxide dismutases (SODs) and from oxidases and β-oxidation of fatty acids in peroxisomes. The hypochlorite produced from H_2O_2 by the action of myeloperoxidase in neutrophils during phagocytosis can act on free amines to form chloramines, which are also toxic to cells. The most dangerous ROS is the highly reactive hydroxyl radical, $^{\bullet}OH$, which reacts indiscriminately with most cellular constituents (Beckman et al. 1994; Halliwell 1995; Scandalios 1987) and can lead to formation of a wide range of carbon-centred free radicals (Fig. 2.2a). This radical is generated from the Fenton reaction catalyzed by reduced transition metal ions such as Fe^{2+}, which are oxidised in the process (Fig. 2.1b). The reaction is exacerbated by the simultaneous presence of $O_2^{\bullet -}$ or other reductants including L-ascorbate, which can reduce the Fe^{3+} to Fe^{2+}. Therefore the mechanisms involved in metal ion homeostasis (for Cu and Fe ions) play important role in cellular defences by minimizing formation of ROS.

In plants, both mitochondrion and chloroplast can be the source of ROS production and of these the chloroplast may be more active. Fungal metabolites and air pollutants can generate singlet oxygen $^1O_2$ in the presence of light (Scandalios 1987). In plants, singlet oxygen is produced by photo-excited chlorophyll and can cause membrane lipid peroxidation, photo-oxidation of amino acids and DNA damage. In additions to ROS, cells can also generate reactive nitrogen species (RNS) from reaction of the nitric oxide radical $NO^{\bullet}$ with the superoxide anion to produce peroxynitrite ($ONOO^-$). Peroxynitrite is very reactive and its reactions eventually enhance formation of radicals such as nitrogen dioxide ($NO_2^{\bullet}$) and the carbonate radical ($CO_3^{\bullet -}$) (Fig. 2.1c). These reactive species can nitrate aromatic amino acid residues (Beckman et al. 1994), produce DNA lesions (Wiseman and Halliwell 1996) and oxidise thiols (Buchczyk et al. 2000). At the outset it should be understood that there is no such thing as a single oxidative stress. Rather there are different forms of oxidative stress that arise depending on the ROS that is being generated in the cell. This became clear from the results of screening of the effects

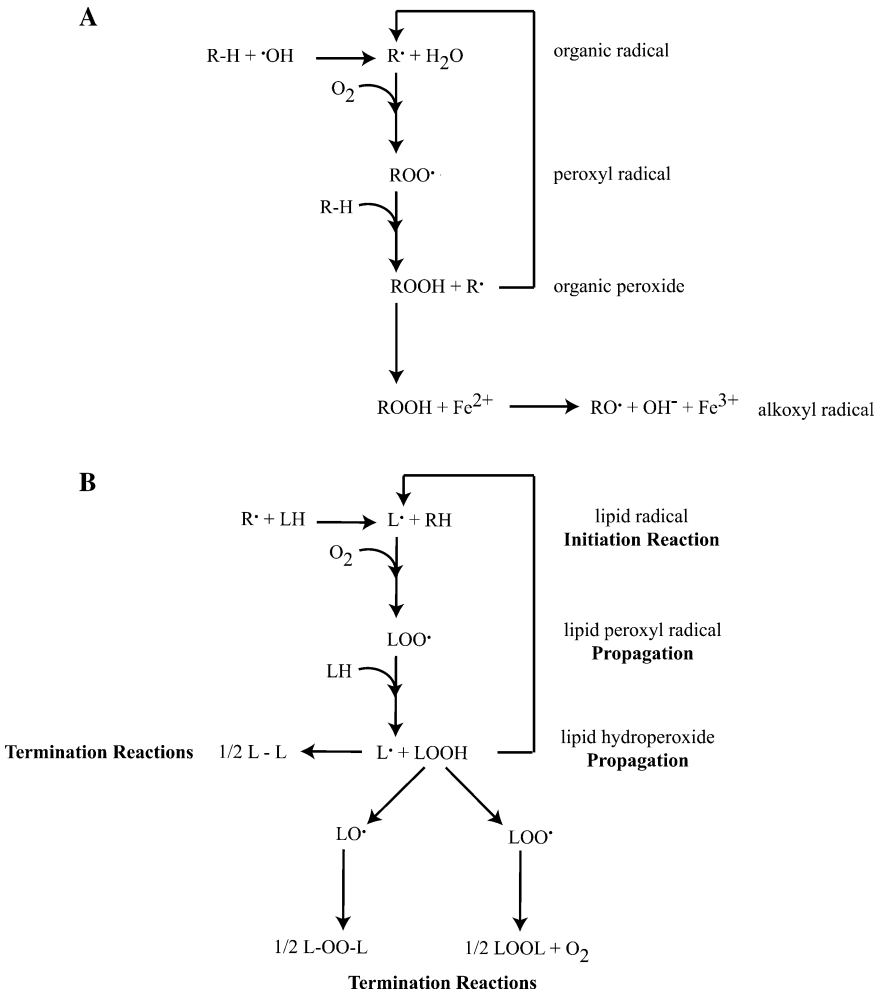

Fig. 2.2 Lipid autoxidation and free radical generation. **a** Carbon-centered free radicals generated by interactions with reactive oxygen species. RH indicates a compound that can accept an electron to become a radical. **b** Lipid peroxidation chain reactions; LH indicates a polyunsaturated fatty acyl residue

of a range of different ROS, or ROS-generating agents on the deletion mutants in the genome-wide deletion collection – for each ROS there was a different and distinctive spectrum of mutants that were sensitive to that compound (Thorpe et al. 2004). In analysis of the roles of ROS and RNS in processes such as ageing it is therefore important to identify which species is involved, and not rely on the use of a single oxidant such as hydrogen peroxide as a general oxidant.

Reactions between these reactive species and cellular components produce many secondary ROS and other radicals. Their reactivity varies significantly. Damage to

DNA caused by treatment with ROS has been implicated in mutagenesis and carcinogenesis (Ames and Gold 1991; Joenje et al. 1991). Treatment with paraquat (which generates $O_2^{\bullet -}$) and H_2O_2 can also lead to intrachromosomal recombination and significant levels of interchromosomal recombination at high doses (Brennan et al. 1994). While DNA damage may be a contributor to cell death, it might not be a main one, since mutants that are affected in DNA repair do not feature strongly in the set of mutants that are sensitive to a range of different ROS-generating reagents (Thorpe et al. 2004), although this outcome may be a reflection of the redundancy that exists in the DNA repair pathways. Moreover there is considerable overlap or redundancy in cellular antioxidant functions, and it requires deletion of all five peroxiredoxin genes (involved in detoxification of hydroperoxides) to generate strains with greatly increased mutation rates (Wong et al. 2004).

Protein damage caused by $^{\bullet}OH$ leads to cross-linking, fragmentation and oxidation of amino acyl residues, particularly aromatic side chains and cysteine (Stadtman 1992). The protein hydroperoxides formed are reactive and upon decomposition release free radicals leading to further protein modification and unfolding (Gebicki et al. 2002). Damage to amino acids leads to formation of hydroxylated derivatives and oxidation of aromatic amino acid residues can produce reactive phenoxy radicals (Aeschbach et al. 1976; Fu et al. 1995). FeS-proteins are very susceptible to $O_2^{\bullet -}$, as evidenced by the methionine and lysine auxotrophy of the double *sod1 sod2* mutant lacking superoxide dismutase (SOD) activity (Liu et al. 1992).

Hydrogen peroxide also leads to reversible oxidation of reactive cysteine residues in some proteins to form disulphides or sulfenic acid residues, or irreversible oxidation to sulphinic or sulphonic acids. In the presence of reactive nitrogen species there can be *S*-nitrosylation as well. Some 200 proteins that have oxidised cysteine residues have been identified in cells exposed to H_2O_2. These include many of the antioxidant enzymes that act as scavengers of ROS, including the Cu/Zn SOD Sod1p, the peroxiredoxins Tsalp, Tsa2p, Ahplp and Prxlp, a glutathione peroxidase Gpx2p, thioredoxin reductase Trrlp, protein disulphide isomerase Pdilp and the methionine sulfoxide reductase Mxrlp (Le Moan et al. 2006). In addition, a number of stress chaperones, enzymes involved in carbohydrate, energy and amino acid metabolism, proteins involved in translation and proteolytic degradation were susceptible to cysteine oxidation. Many of the same proteins can undergo reversible disulphide formation with glutathione (glutathionylation) as a protective measure (Grant et al. 1999).

Unsaturated fatty acyl groups are the most susceptible to $^{\bullet}OH$ and the protonated form of $O_2^{\bullet -}$, can initiate autocatalytic lipid peroxidation to form reactive lipid radicals and lipid hydroperoxides (Gunstone 1996) (Fig. 2.2b). Lipid hydroperoxides are among the most toxic hydroperoxides to yeast cells (Alic et al. 2004; Evans et al. 1998). These toxic molecules can cause significant damage to cell membranes (Evans et al. 1998). When lipid peroxides are broken down, they can produce reactive aldehydes such as malondialdehyde and (in organisms with multiply unsaturated fatty acids – not *Saccharomyces cerevisiae*) 4-hydroxynonenal, which can contribute to the carbonylation of proteins (Esterbauer et al. 1991). As discussed in subsequent chapters, carbonylation has also been implicated in protein

degradation and ageing (Nystrom 2005). Proteins that are vulnerable to carbonylation following exposure of cells to H_2O_2 include glyceraldehyde-3-phosphate dehydrogenase isozymes, aconitase, pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase, Hsp60p, fatty acid synthase and Cu-Zn SOD (Costa et al. 2002).

Cellular Defences

The recent rapid development in genomic technologies has provided advanced tools including deletion mutants for every non-essential gene (Winzeler et al. 1999) and data for protein-protein interactions (Lehne and Schlitt 2009; Salwinski et al. 2004; Schwikowski et al. 2000), synthetic lethality of mutations (Tong et al. 2004), and transcription factor binding (Ren et al. 2000; Zhu et al. 2009) that can be used to identify the genes and functions that are important for responses and resistance to stress. The use of these approaches has led to a much more detailed insight into how cells respond to ROS and other stresses.

Aerobic organisms are constantly exposed to many different ROS and their toxic products generated from both endogenous and exogenous sources. For some ROS such as $O_2^{\bullet -}$ and H_2O_2 there is some understanding that they may act as signaling molecules at low concentration. At higher concentrations these are generally very detrimental to cells. Therefore organisms have evolved a very wide range of both enzymatic and non-enzymatic cellular defence mechanisms against the deleterious effects of ROS. These include constitutive redox protection systems buffering the cell against sudden exposure to oxidants as well as inducible systems that include modulation of gene expression and metabolism to up-regulate antioxidant and repair systems and down-regulate growth functions to allow the cells time to repair damage (Dawes 2004; Gasch et al. 2000).

Non-enzymatic Defence Systems

The non-enzymatic antioxidant functions include low molecular mass redox-active molecules such as glutathione, D-erythroascorbate (the 5-carbon analogue of ascorbate) and ubiquinol. The water-soluble tripeptide glutathione (GSH; γ-glutamyl-cysteinyl-glycine) is the most abundant low molecular mass thiol in yeast cells. It can be oxidised to form the disulphide GSSG by a range of oxidants, including H_2O_2 and disulphides, and hence GSH makes up a substantial proportion of the cellular redox buffering capacity (protein thiols also constitute a fairly high proportion of the redox buffering capacity). GSH has a range of functions in addition to its many roles in protecting against oxidative stress; these include protein folding, amino acid transport and metabolism and secretion of various xenobiotic compounds (Dawes 2004). The rate of reaction of H_2O_2 with GSH is slow relative to that with some of the enzymatic defence systems, especially the peroxiredoxins (if the yeast enzymes have similar activity to their mammalian homologues) (Peskin et al. 2007). The main antioxidant activity of GSH probably arises from its role in maintenance of cellular

reducing potential at a very low value, its role as a substrate in a number of enzymic detoxification and repair enzymes, or its action as an antioxidant at high doses of H_2O_2 when other antioxidant functions are swamped. The *gsh1* mutant (lacking the first enzyme in the committed pathway to GSH) is viable, but is sensitive to H_2O_2 and a range of other ROS (Lee et al. 2001).

Saccharomyces cerevisiae does not synthesise significant amounts of L-ascorbate, which in other organisms has a strong antioxidant activity as a scavenger of free radical species including superoxide anion, lipid peroxy radicals and the hydroxyl radical. On the other hand *S. cerevisiae* and a number of other fungi synthesise the five-carbon analogue D-erythroascorbic acid. This molecule has some role in defence against H_2O_2 since mutants lacking the last gene (*ALO1*) in the biosynthetic pathway are sensitive to the ROS and over-expression of *ALO1* increases resistance to H_2O_2 (Huh et al. 1998).

Yeast also lacks tocopherols found in higher eukaryotes, and a likely contender for the main lipid-soluble antioxidant is ubiquinol (coenzyme Q) – the yeast version has a side chain with six isoprenoid residues rather than the ten found in humans. Mutation of any of the genes in the coenzyme Q biosynthetic pathway leads to the respiratory petite phenotype as expected for a disruption of respiration. The *coq3* mutant is very sensitive to polyunsaturated fatty acids compared to the wild type, and since the sensitivity is rescued by the addition of antioxidants reacting with free radicals it has been suggested that ubiquinol does play a role in protection against the products of lipid autoxidation (Bossie and Martin 1989).

Enzymatic Defences

The wide range of ROS generated in cells has led to evolution of a large number of enzymes to detoxify the ROS or repair the damage caused by them, and the role of these enzymes and their regulation have previously been reviewed extensively (Dawes 2004). Many of those that have been identified to date are listed in Table 2.1. These enzymes are localised to various cellular compartments and hence the cells have different strategies for removal of ROS or repair that are specific to different compartments. Some of the different mechanisms for dealing with the main ROS species and their damage are summarised for the cytoplasm (Fig. 2.3), the mitochondrion (Fig. 2.4) and the peroxisome (Fig. 2.5).

There is no enzyme known that can detoxify the hydroxyl radical, which reacts very rapidly with the nearest molecule and is therefore unlikely to accumulate in cells. The superoxide radical anion is removed by dismutation to hydrogen peroxide and oxygen catalysed by superoxide dismutases (SODs). *Saccharomyces cerevisiae* encodes two SOD enzymes, the more abundant Cu/Zn-containing Sod1p is located mainly in the cytoplasm, but a small proportion is also transported to the inter-membrane space of the mitochondrion. The less abundant Mn-containing Sod2p is found in the mitochondrial matrix (Gralla and Kosman 1992). Expression of both *SOD1* and *SOD2* is induced by growth on non-fermentable substrates,

Table 2.1 Primary genes involved in redox homeostasis and antioxidant defence in *Saccharomyces cerevisiae*

Systematic name	Common name	Function
Peroxiredoxins		
YIL010W	*DOT5*	Nuclear thiol peroxidase
YBL064C	*PRX1*	Mitochondrial peroxiredoxin with thioredoxin peroxidase activity
YLR109W	*AHP1*	Cytoplasmic thioredoxin peroxiredoxin
YDR453C	*TSA2*	Cytoplasmic thioredoxin peroxiredoxin
YML028W	*TSA1*	Cytoplasmic thioredoxin peroxiredoxin
Transcription factors		
YML007W	*YAP1*	Basic leucine zipper (bZIP) transcription factor
YHR206W	*SKN7*	Nuclear response regulator and transcription factor
YMR037C	*MSN2*	Transcriptional activator related to Msn4p
YKL062W	*MSN4*	Transcriptional activator related to Msn2p
Glutathione system		
YPL091W	*GLR1*	Cytosolic and mitochondrial glutathione oxidoreductase
YJL101C	*GSH1*	Gamma glutamylcysteine synthetase catalyzes the first step in glutathione biosynthesis
YOL049W	*GSH2*	Glutathione synthetase; catalyzes the second step in glutathione biosynthesis
YKL026C	*GPX1*	Phospholipid hydroperoxide glutathione peroxidase
YBR244W	*GPX2*	Phospholipid hydroperoxide glutathione peroxidase
YIR037W	*GPX3*	Phospholipid hydroperoxide glutathione peroxidase
YCL035C	*GRX1*	Cytoplasmic di-thiol glutaredoxin
YDR513W	*GRX2*	Cytoplasmic di-thiol glutaredoxin
YDR098C	*GRX3*	Nuclear shuttling monothiol glutaredoxin
YER174C	*GRX4*	Nuclear shuttling monothiol glutaredoxin
YPL059W	*GRX5*	Mitochondrial monothiol glutaredoxin; required for iron-sulfur cluster biogenesis
YDL010W	*GRX6*	Cis-golgi localized monothiol glutaredoxin
YBR014C	*GRX7*	Cis-golgi localized monothiol glutaredoxin
YLR364W	*GRX8*	Glutaredoxin; localizes to the cytoplasm
YJL212C	*OPT1*	Proton-coupled oligopeptide transporter of the plasma membrane; transports glutathione
YIR038C	*GTT1*	Endoplasmic reticulum-associated glutathione-*S*-transferase
YLL060C	*GTT2*	Glutathione-*S*-transferase possibly mitochondrial

Table 2.1 (continued)

Systematic name	Common name	Function
Thioredoxin system		
YDR353W	*TRR1*	Cytoplasmic thioredoxin reductase
YHR106W	*TRR2*	Mitochondrial thioredoxin reductase
YLR043C	*TRX1*	Cytoplasmic thioredoxin
YGR209C	*TRX2*	Cytoplasmic thioredoxin
YCR083W	*TRX3*	Mitochondrial thioredoxin
NADPH regeneration		
YOR374W	*ALD4*	Mitochondrial aldehyde dehydrogenase
YPL188W	*POS5*	Mitochondrial NADH kinase
YNL009W	*IDP3*	Peroxisomal $NADP^+$-dependent isocitrate dehydrogenase
YPR074C	*TKL1*	Transketolase; similar to *TKL2*
YER073W	*ALD5*	Mitochondrial aldehyde dehydrogenase
YDL066W	*IDP1*	Mitochondrial $NADP^+$-specific isocitrate dehydrogenase
YJL121C	*RPE1*	D-ribulose-5-phosphate 3-epimerase
YKL029C	*MAE1*	Mitochondrial malic enzyme
YNL241C	*ZWF1*	Glucose-6-phosphate dehydrogenase
YPL061W	*ALD6*	Cytosolic aldehyde dehydrogenase
YLR174W	*IDP2*	Cytosolic $NADP^+$-specific isocitrate dehydrogenase
YHR183W	*GND1*	6-phosphogluconate dehydrogenase
YLR354C	*TAL1*	Transaldolase
YBR117C	*TKL2*	Transketolase; similar to *TKL1*
Antioxidant		
YGR088W	*CTT1*	Cytosolic catalase T
YDR256C	*CTA1*	Peroxisomal catalase A
YJR104C	*SOD1*	Cytosolic copper-zinc superoxide dismutase
YHR008C	*SOD2*	Mitochondrial manganese superoxide dismutase
YKR066C	*CCP1*	Mitochondrial intermembrane space localised Cytochrome C peroxidase

oxygen or superoxide-generating agents such as paraquat, while *SOD2* is also strongly up-regulated as cells enter diauxic growth or become starved (Flattery-O'Brien et al. 1997; Gralla and Kosman 1992). Mutants lacking Sod1p are viable, but have reduced growth rates under aerobic conditions. The double *sod1 sod2* mutant grows slowly in air, requires methionine and lysine for growth and has an increased mutation rate (Liu et al. 1992).

There is a wide range of enzymes capable of detoxifying hydroperoxides. Catalases are reportedly specific to H_2O_2 and unable to accommodate larger hydroperoxides in their catalytic sites (Dawes 2004). *Saccharomyces cerevisiae* has

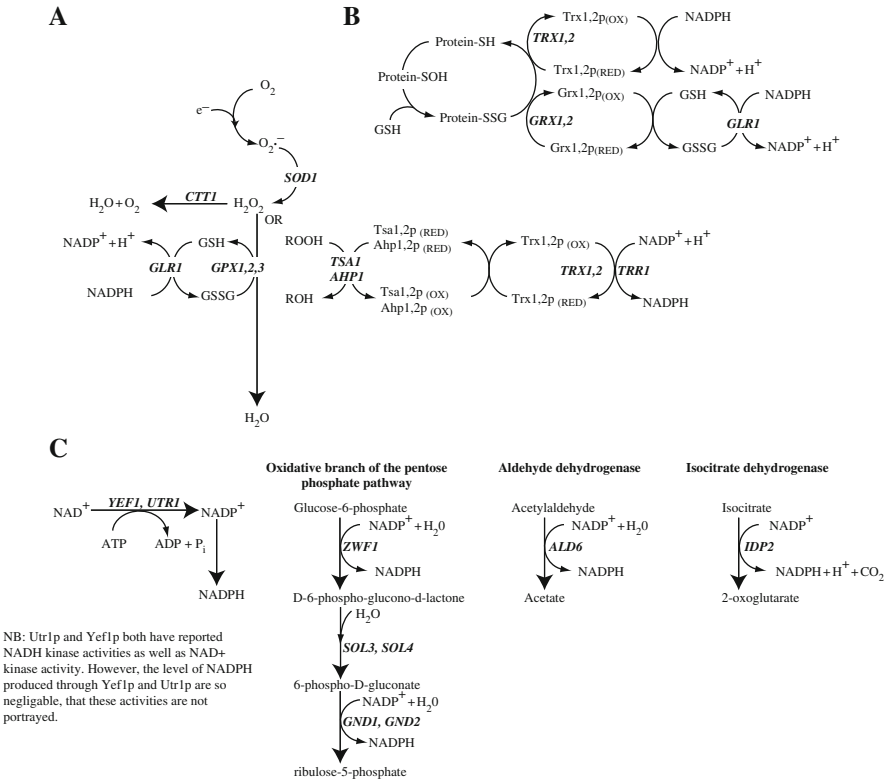

Fig. 2.3 Reactive oxygen species and antioxidant defence systems in the cytosol. **a** ROS and defence. The main reactive oxygen species include the superoxide anion radical and hydrogen peroxide and organic peroxides (ROOH) that are detoxified to water via the Cu,Zn-superoxide dismutase, catalase or glutathione systems. Hydrogen peroxide and organic peroxides (ROOH) can also be detoxified to an alcohol (ROH) by the thioredoxin system. **b** Maintenance of reduced protein-thiol groups in the cytosol. Oxidised protein-SH groups can be reduced either by the glutathione-based system (glutaredoxin1,2, glutathione reductase, glutathione and NADPH) or the thioredoxin system (thioredoxin1,2, thioredoxin reductases and NADPH). Thioredoxins/glutaredoxins catalyse the reduction of other proteins and are oxidised. Oxidised thioredoxins are reduced directly by thioredoxin reductases using electrons supplied by NADPH. Oxidized glutaredoxins are reduced by glutathione using electrons supplied by NADPH. **c** Pathways involved in NADPH regeneration in the cytosol. NADPH is primarily produced as a product of $NADP^+$-dependent reactions. Yeast genes are denoted in *bold* and *italic uppercase* and the protein product of the gene is designated by *Roman type*, with the first letter capitalized and suffix "p"

two catalases. The cytosolic catalase T (encoded by *CTT1*) is inducible by oxygen, heat, osmotic and oxidative stress, copper ions and availability of several nutrients (Bissinger et al. 1989). The peroxisomal catalase (encoded by *CTA1*) is induced by oxygen, growth on respiratory substrates and fatty acids, and is repressed by glucose (Ruis and Hamilton 1992). While disruption of *CTT1* has been reported to lead to sensitivity to H_2O_2, disruption of either or both catalase genes did not

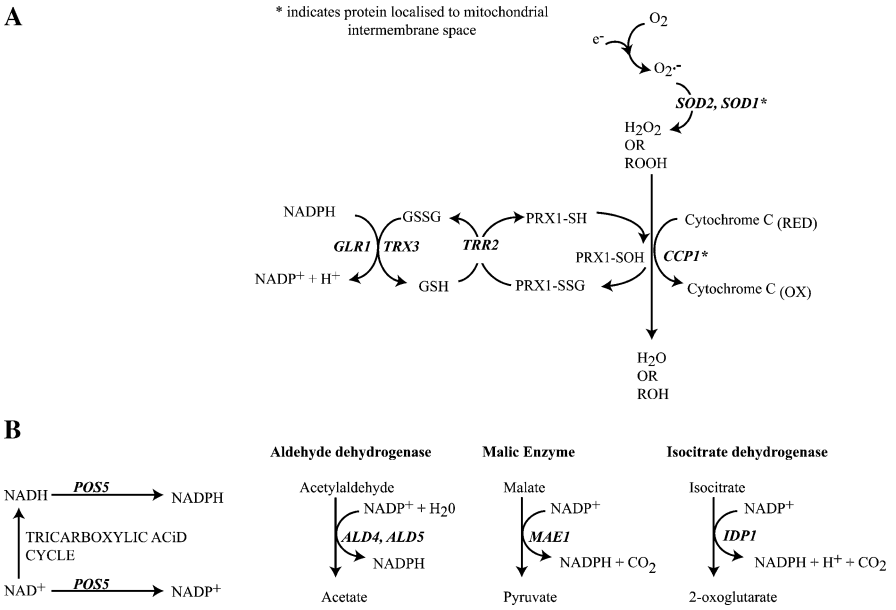

Fig. 2.4 Reactive oxygen species and defence systems in the mitochondria. **a** The main reactive oxygen species include the superoxide anion radical and hydrogen peroxide and organic peroxides (ROOH) that are detoxified to water or alcohols (ROH) via Mn-superoxide dismutase or the peroxiredoxin-thioredoxin-glutathione system coupled to cytochrome-c peroxidase. **b** Pathways involved in NADPH regeneration in the mitochondria. NADPH is produced either by phosphorylation of NADH or as a product of $NADP^+$-dependent reactions catalysed by aldehyde dehydrogenase, the malic enzyme or isocitrate dehydrogenase. Yeast genes that encode the enzymes are in *bold italics*

affect the growth rate of the strain during exponential growth under non-stressed conditions nor seriously affect the sensitivity to H_2O_2. During stationary phase the double mutant is more sensitive to H_2O_2 than the wild-type (Izawa et al. 1996). Comparison of the sensitivity of mutants affected in the catalases and in glutathione metabolism has shown that in exponentially growing cells glutathione has a more important role than the catalases in responding to H_2O_2 (Grant et al. 1998). This is consistent with the long-held view that in mammalian cells the glutathione peroxidases have a greater role in detoxification of H_2O_2. In fact, kinetic data for purified enzymes would indicate that where the peroxiredoxins (discussed below) are present in a cellular compartment, they would have a more important role than either the glutathione peroxidases or catalases in breaking down H_2O_2. The peroxiredoxins use thioredoxin as the reduced substrate rather than glutathione (Peskin et al. 2007).

S. cerevisiae cells contain several classes of peroxidases, depending on their specific reducing substrates which include glutathione, thioredoxin or cytochrome c. These enzymes have a role in repair as well as detoxification since many have the ability to repair damage to proteins that have oxidised thiols as indicated in Fig. 2.3b. Yeast cells have three glutathione peroxidases (Gpx1-3) encoded by *GPX1-3*. These

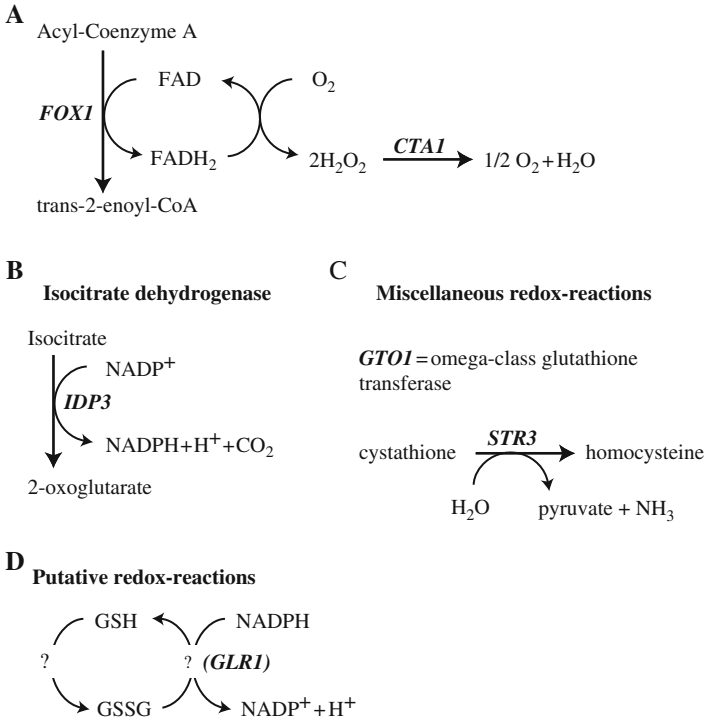

Fig. 2.5 Reactive oxygen species and defence systems in the peroxisome. **a** The only known reactive oxygen species produced in the peroxisome is hydrogen peroxide, as a by-product of beta-oxidation that is subsequently detoxified by the peroxisomal catalase. **b** The isocitrate dehydrogenase reaction is the only known pathway of NADPH regeneration in the peroxisome. **c** Miscellaneous enzymes that may be involved in oxidative stress defence in the peroxisome. **d** Schematic of the glutathione system in the peroxisome. *Question marks* denote as yet unidentified enzymes. Yeast genes encoding the enzymes are in *bold italics*

lack the selenocysteine group found at the catalytic site in other Gpxs, but all have peroxidase activity (Inoue et al. 1999), in fact they are unusual in that they are probably lipid hydroperoxide peroxidases, they are monomeric, can associate with membranes and are capable of reducing lipid hydroperoxides in membranes (Avery and Avery 2001). Of the deletants, only the *gpx3* mutant is sensitive to peroxides, which is probably due to the fact that the enzyme is also the sensor of H_2O_2 damage in cells (Delaunay et al. 2002). The *GPX* genes are differentially regulated, *GPX1* is induced on glucose starvation, *GPX2* by oxidative stress and *GPX3* is reported to be constitutive (Inoue et al. 1999).

The thioredoxin peroxidases (peroxiredoxins) are a family of cysteine-dependent peroxidases that react rapidly with H_2O_2 and other alkyl hydroperoxides, including amino acid hydroperoxides and peroxy residues in oxidised proteins, and in mammalian systems the peroxiredoxins may be the most relevant anti-oxidant systems for removing hydrogen peroxide under normal conditions (Peskin et al. 2010).

Saccharomyces cerevisiae has at least five peroxiredoxins encoded by *TSA1*, *TSA2, PRX1*, *AHP1* and *DOT5* (Dawes 2004). All peroxiredoxins contain a conserved peroxidatic cysteine residue in the active site, which is oxidised to a sulfenic acid residue by the hydroperoxide. In 2-Cys enzymes this sulfenic acid residue initially reacts with another cysteine residue to form an intra-molecular disulfide bridge, which forms a substrate for subsequent reduction by the thioredoxin system (Fig. 2.3b). In 1-Cys peroxiredoxins lacking the second conserved cysteine there is an alternative reduction system – for the yeast mitochondrial Prx1p this reduction is mediated via glutathionylation of the catalytic cysteine residue and subsequent reduction by glutathione catalysed surprisingly by the mitochondrial thioredoxin reductase, Trr2p (Greetham and Grant 2009). Tsa1p is a cytoplasmic and ribosome-associated 2-Cys enzyme, and in addition to its peroxidase activity, under oxidative stress it can self-associate to form a high molecular mass complex with chaperone activity, which can also contribute to repair of protein damage (Trotter et al. 2008). Tsa2p and Ahp1p are also located in the cytoplasm; in its active form Ahp1p is covalently attached to the ubiquitin-related protein Urm1p (Goehring et al. 2003). Deletion of either *TSA1* or *TSA2* leads to some hypersensitivity to hydrogen peroxide and nitrosative stress and the *tsa1 tsa2* double mutant is even more sensitive (Wong et al. 2002), while deletion of *AHP1* leads to sensitivity to t-butyl hydroperoxide (Goehring et al. 2003). *DOT5* encodes a 2-Cys peroxiredoxin that is located in the nucleus. The enzyme is more active against alkyl hydroperoxides, is induced on respiratory substrates and is required for starvation survival (Cha et al. 2003). Interestingly, deletion of all five peroxiredoxins does not lead to loss of viability. The multiple deletant grows slowly, has induced levels of other antioxidant enzymes and has a significantly increased rate of mutation (Wong et al. 2004).

The mitochondrion lacks catalase, and in addition to be the source of a relatively large proportion of the $O_2^{\bullet -}$ generated in cells, it is also the site of assembly of the very oxidant sensitive FeS complexes. The antioxidant functions in the mitochondrion are augmented by the cytochrome c peroxidase, which is located in the mitochondrial inter-membrane space and encoded in the nucleus by the *CCP1* gene. Deletion of this gene does not affect viability of cells under aerobic conditions, even on respiratory substrates, but leads to increased sensitivity to H_2O_2 (but not to paraquat) and formation of petites on respiratory substrates (Jiang and English 2006). It has been suggested that Ccp1p has a role in signaling oxidative stress via the Skn7p transcription factor (Charizanis et al. 1999).

A recent report has shown that the glutathione transferases (Gtt1p and Gtt2p) are also important for protecting the cells against H_2O_2 stress by reducing formation of lipid peroxides as well as products of protein carbonylation (Mariani et al. 2008).

The above discussion is mainly concerned with removal of ROS or their toxic products. There are, however, repair functions, which can remove the damage from molecules. Alkyl hydroperoxides (formed from lipids or proteins) are reduced by the peroxiredoxins, and the peroxidases forming the corresponding alcohol, which is usually less toxic. One class of damage that is important is that caused to reactive protein thiol groups, which are among the most readily oxidised residues in proteins. Reactive cysteinyl residues can be oxidised to disulphides (via two protein

cysteines), or to form mixed disulphides between protein thiols and a number of low molecular-mass thiols such as glutathione (*S*-thiolation). This does not require enzymatic action, and can serve a protective function by preventing further irreversible oxidation of the thiol group. Thiols can also be oxidised successively to the sulfenic (SOH), sulfinic (SO_2H) or sulfonic (SO_3H) acid derivatives (Grant et al. 1999). In most cases cysteine oxidation to the sulfenic acid can be reversible through the action of a number of enzymes (especially the thioredoxins and glutaredoxins), while the subsequent oxidation to sulfinic or sulfonic acids is not. One exception is the sulphiredoxin enzyme (encoded in *S. cerevisiae* by *SRX1*) that can reduce the cysteine sulfinic acid residue formed at the active site of the peroxiredoxin Tsa1p (Biteau et al. 2003).

The cell has two classes of low molecular mass proteins with thiols at the reactive site that play many roles in the cell, not least the repair of oxidatively damaged thiols in proteins, as well as in maintenance of cellular reducing potential. These are the thioredoxins and glutaredoxins, which show structural similarity and which share a number of functions. Both proteins can exist in the reduced or oxidised forms. For glutaredoxin, the oxidised form is reduced by reaction with glutathione to generate GSSG, which is subsequently reduced by NADPH catalysed by glutathione reductase (encoded by *GLR1* in yeast). Oxidised thioredoxin is reduced directly by NADPH in a reaction catalysed by thioredoxin reductase (the cytoplasmic form is encoded by *TRR1* and the mitochondrial by *TRR2*). Both thioredoxin and glutaredoxin are also directly involved in nucleic acid biosynthesis as the hydrogen donors to ribonucleotide reductase, and in sulphur metabolism (Trotter and Grant 2003). In yeast there are three thioredoxins – two (Trx1p and Trx2p) are located in the cytoplasm (Gan 1991) and Trx3p in the mitochondrial matrix (Pedrajas et al. 1999). The cytoplasmic redoxin system is not essential for growth, since the triple mutant *trx1 trx2 trr1* can grow, although the double *trx1 trx2* mutant is affected in cell cycle progression and requires cysteine and methionine due to loss of the reducing power for sulphate assimilation (Muller 1991). The *TRX2* gene is regulated by the Yap1p transcription factor and its activity is important for the inactivation of Yap1p as the cell recovers from H_2O_2 stress (see later discussion of Yap1p this chapter). The *trx2* deletion mutant has increased sensitivity to hydroperoxides during stationary phase (Garrido and Grant 2003). One of the important roles of the cytoplasmic thioredoxin system is setting the cytoplasmic reducing environment of the cell as determined by the GSH/GSSG couple. Deletion of both thioredoxins 1 and 2 led to the greatest shift in cellular reducing potential of any of the antioxidant mutants tested in exponential phase, and deletion of thioredoxin reductase 1 had the same effect in stationary phase (Drakulic et al. 2005).

Glutaredoxins are heat-stable glutathione-dependent disulphide oxidoreductases (Holmgren and Aslunf 1995), which have some overlap in their function with the thioredoxins, including the ability to donate hydrogen to ribonucleotide reductase. The importance of the glutaredoxins in repair of ROS damage is due to their ability to catalyse the cleavage of mixed disulphides between GSH and proteins (Chrestensen et al. 1995). In yeast there are eight glutaredoxins, Grx1p-8p, encoded by *GRX1-8*, with *GRX1-5* being the most well studied in yeast. Grx1p and Grx2p

show strong homology to each other and have a pair of cysteines at the active site. The three monothiol glutaredoxins, Grx3-5p, have only one cysteine at the catalytic site. Grx1p and Grx2p have glutathione peroxidase activity in addition to thiol transferase activity (Collinson et al. 2002). Grx1p is located in the cytoplasm, Grx2p has two isoforms, one cytoplasmic and the other mitochondrial (Pedrajas et al. 2002). All combinations of the *trx1, trx2, grx1* and *grx2* mutations are viable except the quadruple mutant lacking any of the cytoplasmic thioredoxins and glutaredoxins (Trotter and Grant 2003) indicating the strong overlap in the essential functions of glutaredoxins and thioredoxins. Of the monothiol glutaredoxins the best characterised is Grx5p, which is located in the mitochondrion and which has an important role in synthesis or assembly of iron/sulphur centres (Rodriguez-Manzaneque et al. 2002). Deletion of *GRX5* leads to sensitivity to oxidative stress. Grx3p and Grx4p are located in the nucleus and have been shown to be important for the iron inhibition of the transcription of the Aft1p transcription factor regulating iron homeostasis in *S. cerevisiae*, deletion of both proteins leads to constitutive expression of the iron regulon (Ojeda et al. 2006).

A critical component of many oxidative damage repair and detoxification systems is the generation of NADPH to provide reducing equivalents. There are a number of important systems for NADPH synthesis in cells, these are illustrated in Figs. 2.3, 2.4, and 2.5. In the cytosol the major source of NADPH is the pentose phosphate pathway, and mutations that affect this pathway lead to sensitivity to many ROS as well as inability to adapt to ROS treatment (Ng et al. 2008). Other enzymes that may contribute to NADPH synthesis in the cytosol include the acetaldehyde dehydrogenase encoded by *ALD6* and the isocitrate dehydrogenase encoded by *IDP2*. In the mitochondrion the NADH kinase encoded by *POS5*, isocitrate dehydrogenase (*IDP1*), the malic enzyme (*MAE1*) and the aldehyde dehydrogenases (*ALD4, ALD5*) may also be involved.

Additional proteins such as heat shock proteins (Kalmar and Greensmith 2009) and DNA damage repair enzymes (Salmon et al. 2004) are also important for repairing the damage caused by oxidative stress.

Cellular Responses to ROS

Physiological management of oxidative stress situations includes metabolic readjustments, which occur within a very short time (seconds to minutes) and represent non-genomic consequences of the oxidative stress (Gruning et al. 2010; Ralser et al. 2009). On a somewhat longer time scale, the transcriptome of the stressed cells changes and responds by activating the antioxidant defence pathways and, at higher doses, the cell death pathway of yeast (Gasch et al. 2000; Laun et al. 2005). One surprising aspect of the stress response is that on demand the cells can cope with excessive oxidative or reductive stress by getting rid of a surplus of oxidation/reduction equivalents by non-classically secreting oxidised, or in case of reductive stress, reduced glutathione (Heeren et al. 2004; Perrone et al. 2005; Suzuki and Ohsumi 2007).

Yeast cells respond to oxidative stress in dose-dependent manner. At low doses below the level at which cell death occurs, the cells adapt to become more resistant to a subsequent dose that would otherwise be lethal and also to some other oxidants (Collinson and Dawes 1992; Evans et al. 1998; Flattery-O'Brien et al. 1993; Jamieson 1992; Turton et al. 1997). At higher doses, the cells delay cell division (Alic et al. 2001; Flattery-O'Brien and Dawes 1998; Nunes and Siede 1996; Lee et al. 1996) and induce antioxidant defence and repair mechanisms (Dawes 2004; Gasch et al. 2000). In the presence of very high doses, the cells initiate apoptosis (Madeo et al. 1997, 2002).

Adaptation

Cells are capable of adapting to treatment with low doses of a range of ROS including H_2O_2 (Collinson and Dawes 1992) and menadione (which generates $O_2^{\bullet -}$) (Flattery-O'Brien et al. 1993; Jamieson 1992) and the products of lipid peroxidation such as linoleic acid hydroperoxide (LoaOOH) (Evans et al. 1998) and malondialdehyde (Turton et al. 1997). This adaptation is observed in both human cells (Kim et al. 2001) and yeast (including petite strains which lack a mitochondrial genome) (Collinson and Dawes 1992; Evans et al. 1998; Flattery-O'Brien et al. 1993; Jamieson 1992; Turton et al. 1997) and increases cell survival in a subsequent challenge. The adaptive response elicited by a particular ROS can in some cases confer increased resistance to another form of ROS. For example, adaptation to H_2O_2 protects against most other ROS (Asad et al. 1997; Christman et al. 1985; Dukan and Touati 1996; Nunoshiba et al. 1991). Heat stress causes oxidative stress (Davidson et al. 1996) and stimulates antioxidant defences (Kim et al. 2006) which may be why heat shocked cells are resistant to most ROS or compounds producing ROS, including H_2O_2 (Spitz et al. 1987), menadione (Flattery-O'Brien et al. 1993) and LoaOOH (Evans et al. 1998). By contrast, pre-treatment with low doses of H_2O_2 does not confer thermotolerance (Collinson and Dawes 1992). Previously it was reported that pretreatment with H_2O_2 leads to superoxide tolerance but not vice versa (Flattery-O'Brien et al. 1993), while others reported that treatment with sublethal dose of menadione leads to H_2O_2 tolerance (Jamieson 1992; Lee et al. 1995) in a GSH-dependent manner but not vice versa (Fernandes et al. 2007). Although the latter are conflicting results, it is clear that cross-adaptation can occur and there is hierarchy with regards to this process. The cross-adaptation could be a result of the generation of secondary ROS during pre-treatment with some oxidants to the concentration that can elicit an adaptive or a damage response.

Although physiological adaptation to oxidative stress was discovered relatively early, the mechanisms involved in adaptation to specific ROS are not fully understood. Different ROS produce distinct adaptive responses, which require de novo gene expression and protein synthesis, and the responses are transient lasting for about 1.5 h (Collinson and Dawes 1992; Davies et al. 1995; Flattery-O'Brien et al. 1993). Genes involved in the adaptive response were predicted to be a subset of those that are induced in the acute response (Costa and Moradas-Ferreira 2001). However,

this is not the case for the adaptation to linoleic acid hydroperoxide (LoaOOH). At low (adaptive) doses there is an up-regulation of metabolic systems for synthesis of NADPH and export of LoaOOH from the cell and a mild down-regulation of protein synthesis gene expression, but somewhat surprisingly there is a down-regulation of the genes encoding more general oxidant defence enzymes including those for thioredoxin 2 and glutaredoxin 1. Induction of these more general oxidative and general stress response genes did not occur until the maximal adaptive dose was reached (Alic et al. 2004). This led to the speculation that the major antioxidant enzymes are not induced until there is a threshold dose, which may be that which overcomes the redox buffering capacity of the cell. Adaptation to LoaOOH is observed only in the presence of the glutathione peroxidases encoded by *GPX1* and *GPX2*, and *GPX1* is induced by LoaOOH (Dawes 2004; Gasch et al. 2000). During LoaOOH adaptation, transcription is regulated in part by Pdr1p or Pdr3p, which are two homologues transcription factors that recognize the pleiotropic drug-resistance elements controlling the synthesis of multidrug resistance transporters.

Genome wide analysis of the set of deletion mutants identified 286 mutants that are sensitive to H_2O_2 (Thorpe et al. 2004). Further sub-screening of those mutants sensitive to H_2O_2 identified seven genes that when deleted led to a marked reduction in the adaptive response to H_2O_2 (Ng et al. 2008). These genes can be divided into two categories: (i) genes encoding transcription factors such as Yap1p (one of eight yeast homologues of the human AP-1 family of proteins), which is the major oxidative stress transcription factor in yeast, Skn7p which partners Yap1p to regulate genes encoding antioxidant enzymes and the more general transcription co-activator Gal11p; and, (ii) genes that are involved in the generation of NADPH via either the pentose phosphate pathway or in the mitochondrion. Similar adaptation to H_2O_2 also occurs when anaerobically grown cells are exposed to oxygen for a short time (Beckhouse et al. 2008). Although inhibition of glutathione metabolism has been reported to reduce adaptation, we have not found loss of adaptation in the mutants that are unable to synthesise glutathione. Yap1p and Yap2p were shown to play a role in adaptation to H_2O_2 but not to $O_2^{\bullet -}$ in a previous study (Stephen et al. 1995). The critical role of Yap1p and of NADPH generation systems could indicate that adaptation is due to mainly to activation of Yap1p, and that it is maintained in the cell as long as Yap1p remains activated after an initial oxidative insult. This period can last up to 1.5 h – and from an in vitro analysis of the activation of Yap1p by H_2O_2, in the presence of Gpx3p (to transmit the oxidation signal to Yap1p), thioredoxin 2, thioredoxin reductase and NADPH it appears that Yap1p can be oxidised to form multiple intramolecular disulphide bonds, some of which are relatively refractory to reduction by the thioredoxin system, leading to an extended activation of Yap1p (Okazaki et al. 2007).

Most recently, a combination of genetic screening of the *S. cerevisiae* gene deletion collection and analysis of mRNA expression profiles has identified the involvement during adaptation to H_2O_2 of two additional transcription factors, Mga2p and Rox1p (Kelley and Ideker 2009). These two transcription factors were found to respond earlier than the known oxidative response transcription factors

Yap1p and Skn7p and regulate targets involved in ergosterol metabolism, zinc homeostasis and fatty acid metabolism. The discovery of the role of these additional transcription factors supports the suggestion that membrane integrity is increased as part of the adaptation to H_2O_2 (Branco et al. 2004).

Cell Cycle Arrest

Cell cycle delay or arrest upon exposure to ROS occurs, possibly to allow the cells to repair damage that would otherwise be deleterious to cell survival. Progression through the cell cycle is regulated by coordinated gene expression under the control of a small number of transcription regulators which are sequentially activated (Burhans and Heintz 2009). Different ROS lead to cell cycle arrest at different checkpoints, mostly at G1 or late S/G2. The response is through stress signalling and inhibition of gene expression that is required for cell cycle progression (Burhans and Heintz 2009).

Treatment with H_2O_2 leads to cell cycle arrest in G_2 and this is dependent on the Rad9p-dependent DNA damage checkpoint pathway (Flattery-O'Brien and Dawes 1998). However, the superoxide generators, paraquat and menadione, as well as exposure to hyperbaric oxygen, cause a pronounced G1 arrest independent of Rad9p (Flattery-O'Brien and Dawes 1998). The differential effect of H_2O_2 and menadione on cell cycle delay results from expression of two small co-expressed groups of genes that are under the control of the transcription complexes Mcm1-Fkh2-Ndd1 (Shapira et al. 2004). This shows similarity to the oxidative stress response in mammalian cells (Kops et al. 2002), in which the highly conserved cell cycle regulator fork-head proteins are also involved in H_2O_2-induced cell-cycle arrest.

The expression of cyclins and activation of cyclin-dependent kinases (CDK) are important for the transitions from one phase to another during cell division (Burhans and Heintz 2009). The *sod1* mutant grows slowly due to an increased time spent in the G1 phase of the cell cycle and in the presence of excess oxygen they arrest in G1 phase resulting from inhibition of transcription of *CLN1* and *CLN2* genes encoding the auto-regulated cyclins involved in progression to S phase (Lee et al. 1996).

The products of lipid peroxidation including 4-hydroxynonenal (Wonisch et al. 1998) and LoaOOH also cause G1 arrest (Alic et al. 2001). Screening of LoaOOH-sensitive strains from the genome-wide deletion collection identified 47 deletants that were unable to cause cell cycle delay upon treatment with LoaOOH (Alic et al. 2001; Fong et al. 2008). One of the interesting deletants that did not undergo cell cycle delay in response to LoaOOH lacked Swi6p. Swi6p is the G1/S-phase specific transcription factor that (in combination with either Swi4p or Mbp1p transcription factors) activates the periodic expression of G1 cyclins required for the transition from G1 into S phase of the cell cycle. While Swi6p regulates cell-cycle arrest following DNA-damage through phosphorylation by Rad53p (Sidorova and Breeden 1997), its involvement in regulating cell-cycle arrest in response to oxidative stress is not dependent on the DNA-repair pathway (Flattery-O'Brien and Dawes 1998). Instead Swi6p regulates cell-cycle delay by functioning as a sensor and transducer

for oxidative stress since it has an intrinsically reactive cysteine-404 residue that is oxidised to a sulfenic acid by LoaOOH. Mutation of the reactive cysteine-404 residue to an alanine abolishes the cell cycle delay caused by the oxidant, but not cell cycle progression. This leads to altered transcription of the cyclin genes that are required for triggering of S phase (Chiu et al. 2011). Based on microarray data, the heat shock response and glucose transport are also involved in Swi6p-dependent cell cycle delay (Fong et al. 2008). Additionally, three homologous genes, two of which (*OCA1* and *SWI4*) encode putative protein phosphatases, may also be involved in stress signaling and cell cycle progression upon treatment with LoaOOH (Alic et al. 2001).

Transcriptional Regulation

Adaptation and the subsequent repair mechanisms require regulation of gene expression. Many repair and antioxidant defence systems including catalases, SODs and enzymes involved in glutathione metabolism, thioredoxins and glutaredoxins are found to be up-regulated following exposure to ROS. Genome-wide transcriptional analysis has shown that several hundred genes are either induced or repressed on exposure of cells to moderate to high environmental stresses including heat, several ROS, starvation, osmotic and salt stress (Causton et al. 2001; Gasch et al. 2000). Although no two stress conditions lead to an identical pattern of gene expression, a very large group of genes were found to respond similarly and transiently across most of the stresses. This common response has been known as either an environmental stress response (ESR) or a common environmental response. The common response genes include those involved in carbohydrate metabolism, breakdown of ROS, cellular redox control, heat-shock proteins, protein degradation, lipid metabolism, cell wall modeling, vacuolar functions, autophagy and signaling pathways. Many of the ESR genes are regulated by the transcription factors Msn2p/Msn4p, which are controlled by the protein kinase A (PKA) signaling pathway that responds to nutritional status. Many are also regulated by the protein kinase C (PKC) pathway, which is involved in signalling cell integrity (Gasch et al. 2000). Therefore it is suggested that ESR regulation may play a role in integration of the PKA response to nutritional signals and the PKC response to impaired secretion. In addition it was proposed that the response to stresses including ROS may involve the induction of genes that are associated with changes in either cell wall or membrane permeability or loss of protein integrity, which might require up-regulation of pathways that supply energy for ATP-dependent processes such as the activity of molecular chaperones (heat shock proteins) in assisting protein folding (Causton et al. 2001).

In addition to the genes of the ESR, there are others that are either induced or repressed by oxidative stress and specifically by a particular ROS (Alic et al. 2004). Based on screening of the genome-wide collection of deletion mutants in non-essential genes, more than 600 genes were found to be required for full resistance to a range of oxidants/compounds producing ROS (Thorpe et al. 2004;

Tucker and Fields 2004). Only 12 mutants were sensitive to all the compounds tested whereas many of the rest of the mutants were sensitive to a single oxidant. This shows that the responses are very dependent on the nature of the oxidant. Deletion of the genes involved in mitochondrial functions as affecting the synthesis or assembly of components of the respiratory chain (in particular complexes III and IV) and protein synthesis (mitochondrial ribosomal subunits) leads to sensitivity in mainly to H_2O_2. The mutants that are sensitive to menadione include those affected in the pentose phosphate pathway indicating the importance of NADPH (or of antioxidant functions that require NADPH) in the detoxification of $O_2^{\bullet -}$. Therefore no single oxidant represents general oxidative stress. The genes identified from deletant studies on the sensitivity to a particular ROS shows little correlation with those whose transcripts are altered following treatment with the same ROS (Alic et al. 2004; Gasch et al. 2000). This shows that deletant studies identify constitutive functions that are required before exposure to ROS to increase survival while transcription studies shows those that are likely to be concerned with either repair or removal of damage.

One important defence mechanism against oxidative stress is maintenance of the cellular redox environment. In higher eukaryotic cells, there are numerous redox couples, which include reduced glutathione/oxidised glutathione (2GSH/GSSG), reduced thioredoxin/oxidised thioredoxin, NADPH/$NADP^+$, protein-SH/protein-SS-R and ascorbate/dehydroascorbate (Schafer and Buettner 2001; Wheeler and Grant 2004). These molecules play different roles in buffering cellular redox potential. Of them, the thiol-containing tripeptide glutathione is the most important. The redox potential (E_h) of 2GSH/GSSG follows the trend proliferation < differentiation < apoptosis < necrosis (Filomeni et al. 2002). Disruption of glutathione homeostasis, particularly depletion of glutathione leads to serious consequences for ROS production, cell degeneration, ageing and apoptosis (Fernandes and Holmgren 2004; Holmgren 2000). Genes, cellular processes and environmental factors important for intra/extracellular glutathione homeostasis were identified in a genome wide-study, and the cellular processes included late endosome/vacuolar functions, nitrogen/carbon source signaling, mitochondrial electron transport, ubiquitin/proteosomal process, transcriptional regulation, ion transport and cellular integrity pathways (Perrone et al. 2005).

Early work from a number of groups analysing the promoters of many of the genes encoding anti-oxidant enzymes indicated that the response to ROS was mediated by a set of transcription factors, including: the relatively specific oxidative stress-response factor, Yap1p (Moye-Rowley et al. 1989); Skn7p (which plays an auxiliary role with Yap1); the more general stress response factors, Msn2p and Msn4p (Msn2/4p); and, the Hap1p and the multimeric Hap2,3,4,5p factors which activate genes in response to the switch to respiratory metabolism. As discussed earlier, in addition to the antioxidant defence systems, the roles of copper ion homeostasis and ion uptake regulated by Ace1p (Carri et al. 1991; Gralla et al. 1991) and Aft1p (Pujol-Carrion et al. 2006) respectively are also important for oxidative stress responses. The responses of individual antioxidant genes are rarely under the control of a single transcription factor. Each gene has its own mixture of promoter

motifs for binding a combination of different transcription factors tailoring regulation to the physiological conditions. For example, expression of the *TRX2* gene encoding thioredoxin 2 is regulated by Msn2/4p after heat shock, but by Yap1p after treatment with H_2O_2 (Gasch et al. 2000). Two sets of genes were identified in the H_2O_2 stimulon by proteomic analysis (Godon et al. 1998; Lee et al. 1999). One set of genes, including those involved in many of the cellular antioxidant processes, requires Yap1p in conjunction with the auxiliary transcription factor Skn7p while the others, such as those needed for NADPH synthesis, only depend on Yap1p.

As indicated above, comparison between the transcriptional responses to high and low doses of LoaOOH highlighted that induction and/or repression of genes during oxidative stress are dose dependent. At low doses of LoaOOH, the cells elicit a more subtle response that affects metabolic functions, which include increasing the export of LoaOOH from the cells and generation of NADPH (Alic et al. 2004). Interestingly the major oxidant defence mechanisms such as *TRX2* and *GRX1* are down-regulated under this condition. Cell cycle delay and induction of antioxidant genes are observed only at high doses (Alic et al. 2001). Since aerobic cells are constantly exposed to low concentrations of lipid peroxides, it is may be advantageous to repair the damage caused by low doses without impairing the capacity of the cell to replicate.

The response of gene expression to ROS is controlled not only at the level of transcription but also by the dynamics of mRNA decay. Systematic analysis on a genomic scale of the changes in rate of transcription and mRNA concentration for individual genes using genomic run-on methodology has indicated that changes in mRNA decay rates are as important as those in transcription rate during adaptation to oxidative stress (Molina-Navarro et al. 2008).

Translational Control

Inhibition of protein synthesis occurs following most stresses, and this is true after most forms of ROS stress with a decrease in transcription of many genes involved in the protein synthesis machinery (Gasch et al. 2000). However, recent elegant work from the Grant laboratory has shown that in *Saccharomyces cerevisiae* the rate of protein synthesis decreases rapidly after treatment of cells with H_2O_2 (Shenton et al. 2006). Some of this inhibition occurs at the level of translation initiation, and is dependent on the Gcn2p protein kinase, which phosphorylates the α subunit of the eukaryotic initiation factor eIF2. There appears to be another component of the decreased rate of protein synthesis that is Gcn2p-independent since there was still some inhibition in a *gcn2* mutant. The data from polysome analysis were consistent with this inhibitory effect occurring at either the elongation or termination step of translation. Microarray analysis of monosome- and polysome-bound mRNA showed that a particular set of mRNAs were preferentially bound to ribosomes following stress, and these included mRNAs for stress protective molecules such as thioredoxin reductase 1 (Trr1p) and superoxide dismutase

2 (Sod2p). These experiments were extended to show that the Gcn4p transcription factor mediating the general control of amino acid biosynthesis response is translationally up-regulated by H_2O_2 and that Gcn4p is required for hydroperoxide resistance, indicating that there is an overlap of this regulatory system into the domain of transcription. Similar responses have also been observed after treatment of cells with other oxidants including cumene hydroperoxide, diamide and cadmium (Mascarenhas et al. 2008). In addition to the Gcn2p-dependent response, the eIF4E-binding protein Eap1p is also required for regulation of translation initiation during cadmium and diamide stress. Based on the analysis on the genes whose expression is altered by H_2O_2, it is clear that extensive metabolic reconfiguration is needed for optimal survival of oxidative stress.

Apoptosis

Here we provide a short overview of the role of ROS in apoptosis, for a more extensive treatment of the relation of apoptosis with yeast aging the reader is referred to Laun et al. (Chapter 10, this volume). At high doses of oxidants, cells initiate cell death by a form of apoptosis and at extreme doses by necrosis (Teramoto et al. 1999). Apoptosis was first observed in a *cdc48* mutant that lacks an essential gene involved in the translocation of ubiquitinylated proteins from the ER to the proteasome for degradation (Madeo et al. 1997). The process is characterised by the flipping of phosphatidylserine from the inner to outer layer of the cell membrane, chromatin condensation, accumulation of DNA strand breaks, nuclear fragmentation and formation of apoptotic bodies. Subsequently, apoptosis was also found in the *gsh1* mutant lacking the ability to synthesise glutathione and in cells exposed to other ROS including H_2O_2 (Madeo et al. 1999) and superoxide (Fabrizio et al. 2004) indicating that ROS can trigger apoptosis. Moreover, elevated levels of ROS are found in the cells undergoing apoptosis triggered by other conditions such as NaCl stress (Wadskog et al. 2004), acetic acid (Ludovico et al. 2002), high levels of mating pheromone, certain drugs such as amiodarone (Pozniakovsky et al. 2005) and chronological ageing (Herker et al. 2004).

Yeast has a caspase-like protein (although not one with an aspartyl residue at its active site like the mammalian caspases) encoded by the *YCA1* gene, a caspase regulating serine protease (Madeo et al. 2002) and apoptosis inducing factor Aif1p (Wissing et al. 2004). The involvement of Yca1p in apoptosis is not well understood. Over-expression of Aif1p in yeast promotes apoptosis in the presence of an apoptotic level of H_2O_2 (Wissing et al. 2004) while the mammalian counterpart shows both pro- and anti-apoptotic potential (Lipton and Bossy-Wetzel 2002; Vahsen et al. 2004). The exact mechanism of how apoptosis is initiated by ROS still needs to be carefully investigated. Most of the studies on ROS related apoptosis used H_2O_2. As mentioned earlier, no one oxidant is representative of a general oxidant and therefore further careful analysis is needed on what damage is caused by each oxidant and how the cells respond to it.

Other Cellular Responses

In addition to the responses described above, recent studies have identified other unique cellular mechanisms that form part of the cellular responses to oxidative stresses. One such mechanism involves post-translational changes rerouting flux through metabolic pathways to promote survival (Ralser et al. 2007). During this process a number of enzymes are modified causing rapid and reversible changes in their enzymatic activity (Biswas et al. 2006). Krobitsch and colleagues showed that during oxidative stress, the activities of several glycolytic enzymes, including glyceraldehyde 3-phosphate dehydrogenase (GAPDH), are inhibited in order to channel the carbohydrate flux from glycolysis to the pentose phosphate pathway to generate NADPH which provides the reducing power for most antioxidants and redox regulatory enzymes, including the glutathione/glutaredoxin and thioredoxin systems (Ralser et al. 2007). Earlier work has shown that GAPDH is a major target of *S*-thiolation by glutathione. There are three GAPDH isozymes in yeast encoded by *TDH1-3*, and deletion of either *TDH1* or *TDH3* leads to loss of viability. Tdh3p, but not Tdh2p is a major target of glutathionylation following treatment with H_2O_2, which reduces the activity of both enzymes. However, only Tdh3p activity is restored after a recovery period indicating that the glutathionylation is reversible and protects the enzyme. The *tdh3* deletion mutant is very sensitive to a lethal dose of H_2O_2, indicating that glutathionylation of Tdh3p is required for survival during conditions of oxidative stress. In contrast, the non-thiolated Tdh2p is required during chronic exposure to a low level of oxidants under conditions in which the Tdh3p would be *S*-thiolated and inactive (Grant et al. 1999).

tRNA cleavage, which is conserved in eukaryotic cells, is also found to increase during oxidative stress and in yeast this cleavage is catalysed by release of the endonuclease RNase (Rny1p) from the vacuole into the cytosol (Thompson and Parker 2009). Overexpression of Rny1p also promotes cell death even in unstressed cells. The other interesting observation is the induction of prion formation at higher frequency under stressful conditions including treatment with H_2O_2 (Tyedmers et al. 2008). This phenotype is heritable if it provides advantage to cell survival.

Sensing Stress

Although many transcription factors involved in stress responses are identified, how oxidants are sensed and the signals are transmitted are not yet fully understood. The most well studied mechanism is the role of cysteine residues and disulphide bonds in redox sensing seen with the mechanism whereby the Yap1p transcription factor is activated.

Yap1p

In yeast, Yap1p regulates many key antioxidant genes (Gasch et al. 2000; Godon et al. 1998; Grant et al. 1996; Lee et al. 1999) and its subcellular location

is important for regulation of Yap1p-targeted genes (Kuge et al. 1997). Under unstressed conditions, Yap1p interacts with the nuclear exportin Crm1p through the nuclear export signal (NES) in the C-terminal domain of Yap1p and is exported out of the nucleus (Kuge et al. 1998; Yan et al. 1998). Exposure to H_2O_2 induces changes in the redox state of two cysteine residues in Yap1p, C598 (in the NES) and C303 forming an intramolecular disulphide bond, which masks the NES (Delaunay et al. 2000; Wood et al. 2004). As a result, the Yap1p NES is dissociated from Crm1p and Yap1p remains exclusively in the nucleus. The modification of the C-terminal cysteine residues C598, C620, and C629 are required for ROS-independent activation of Yap1p (Azevedo et al. 2003; Kuge et al. 2001). The accumulation of Yap1p in the nucleus causes alteration of transcription of approximately 70 genes including *GRX2*, *CTT1* and *SOD1* (Gasch et al. 2000; Godon et al. 1998; Grant et al. 1996; Lee et al. 1999). A mutation that affects the C-terminal region of Yap1p and causes constitutive nuclear localisation of Yap1p does not lead to increased tolerance to H_2O_2 (Coleman et al. 1999). This indicated that in addition to sub-cellular localisation, the nature of disulphide bonds formed in the cysteine-rich regions of Yap1p also affects the expression of Yap1-targeted genes possibly through the changes in the binding affinity for the promoters of the genes that are involved in tolerance to a particular stress. In vivo analyses have indicated that inactivation of Yap1p to its reduced state is most likely effected by thioredoxin (Carmel-Harel et al. 2001; Izawa et al. 1999). Since Yap1p controls the synthesis of thioredoxin 2 (Kuge and Jones 1994) and thioredoxin reductase (Lee et al. 1999), which promote the formation of reduced thioredoxin, there is an autoregulatory loop restoring normal conditions on recovery from the oxidative damage.

Although the presence of H_2O_2 leads to changes in Yap1p, H_2O_2 is directly sensed by the peroxiredoxin Gpx3p through a cysteine residue (C36) (Delaunay et al. 2002; Toledano et al. 2004). H_2O_2 converts this residue to a sulfenic acid, which then reacts with Yap1p to form an intermolecular disulphide bond between C36 of Gpx3p and C598 of Yap1p. The disulphide exchange reaction that follows leads to the formation of a C303-C598 disulphide bond in Yap1p and regenerates reduced Gpx3p. Gpx3p-dependent responses to oxidative stress involve induction not only of Yap1p-dependent antioxidant defence genes but also other proteins participate in various cellular mechanisms such as biogenesis of cellular components, cell cycle and energy metabolism which are independent of Yap1p. The sensing via Yap1p of thiol-reactive electrophiles including *N*-ethyl-maleimide, 4-hydroxynonenal, Cd^{2+} and diamide is independent of Gpx3p (Azevedo et al. 2003; Kuge et al. 2001). These compounds activate Yap1p through modification of the C-terminal residues that are different to those affected by H_2O_2.

Swi6p

As described under the above section on cell cycle delay, the transcription factor Swi6p has recently been shown to be involved not only as a transducer, but also as a sensor, in an oxidative stress response that coordinates oxidative stress sensing with

cell cycle delay (Chiu et al. 2011; Fong et al. 2008). Deletion of this gene abolishes cell cycle delay induced by LoaOOH, and as in the above system the initial event is oxidation of a reactive cysteine (C404) residue to a sulfenic acid.

Skn7p

About one half of the genes, including those encoding major antioxidant functions such a *TRX2*, *TRR1*, *GPX2* and *CCP1,* that are activated by oxidative stresses under the control of Yap1p also require the cooperation of the second transcription factor Skn7p, which plays a similar cooperative role in some other stress responses (He and Fassler 2005; Lee et al. 1999). Unlike the cysteine-based redox sensor Yap1p, none of the cysteines in the receiver domain of Skn7 are required for the oxidative stress response; instead Skn7 is phosphorylated following exposure to oxidation (He et al. 2009). This oxidant-dependent phosphorylation is abolished in the absence of Yap1p. Skn7 is constitutively nuclear, and its association with Yap1p to form a complex is also important for the Skn7p response to oxidants. Therefore the authors proposed that the association of Yap1p with Skn7p in the nucleus is a prerequisite for Skn7p phosphorylation and activation of oxidative stress response genes.

Msn2/Msn4p

The common set of genes involved in the general stress response is mainly regulated by the transcription factors Msn2p and Msn4p (Msn2/4p) (Causton et al. 2001; Gasch et al. 2000). The phosphorylated Msn2/4p transcription factors are localized in the cytoplasm during optimal growth conditions (Beck and Hall 1999; Gorner et al. 1998). Under stress conditions, they are imported into the nucleus and the nuclear export is dependent on the Msn5p nuclear export factor (Durchschlag et al. 2004). Although it is still a puzzle how the oxidative stress signal is received and triggers the nucelo-cytoplasmic trafficking of Msn2/4, it has been shown that both cytoplasmic thioredoxins, Trx1p and Trx2p, are essential for nuclear accumulation of Msn2/4 under H_2O_2 treatment and the oxidised thioredoxins are essential for signaling the presence of H_2O_2 (Boisnard et al. 2009). On the other hand, Yak1p kinase activates Msn2/4 by phosphorylation in a PKA-dependent regulation (Lee et al. 2008).

Heterogeneity

In addition to the genotype of the cells, the phenotypic heterogeneity that is evident among individual cells within isogenic cultures is also important for cellular responses to oxidative stress (Sumner and Avery 2002). For examples, treatment with a high dose of oxidant does not lead to complete inviability, but instead a

population of cells remains competent for growth. This differential stress sensitivity is driven by non-genetic heterogeneity, which could result from differences in cell cycle progression, cell age, mitochondrial activity, ultradian rhythms (metabolic oscillations), epigenetic regulation and stochastic variation. These aspects have been reviewed in detail (Sumner and Avery 2002). The variability in copper resistance has been observed in individual cells and has been related to the cell cycle and age-dependent regulation of Cu, Zn-superoxide dismutase (Sod1p) (Sumner et al. 2003). Similarly, the heterogeneity in GSH content within the cell population leads to differing stress resistance to cadmium and H_2O_2 (Smith et al. 2007). Metabolic oscillations and heterogeneity in GSH content can be suppressed by over-expression of Gts1p, and this decreases variation in stress resistance.

Oxidative Effects and Ageing

Owing to the long lifespan, ageing studies in mammals are mostly performed in human or mouse fibroblasts culture. But the relevance of the findings in the cell culture to the organismal ageing process is still debatable (Campisi and d'Adda di Fagagna 2007). As a second approach, organisms which age rapidly and can be manipulated easily both genetically and environmentally are used for ageing research. These include fruit flies (Helfand and Rogina 2003) and worms (Houthoofd and Vanfleteren 2007; Olsen et al. 2006). The single celled eukaryotic *S. cerevisiae* (Kaeberlein et al. 2007; Piper 2006) also serves such a purpose and studies performed in yeast have provided enormous data on molecular mechanisms that accompany ageing. In *S. cerevisiae*, two types of ageing are studied: replicative and chronological. The former also known as mother cell-specific ageing is defined as the number of times each cell can divide before senescence and the latter studies how long the yeast cell can remain viable in the non-dividing (post-mitotic) state under nutrient-depleted conditions.

Since Harman proposed the free-radical theory of ageing, which states that ageing is caused by accumulation of macromolecular damage caused by free radicals (Harman 1956), researchers have been trying to find the link between the build up of ROS and the ageing process. In agreement with Harman's theory, ROS production from mitochondria, as well as biochemical markers of oxidative damage, are found to be elevated with age (Aguilaniu et al. 2003; Laun et al. 2001; Reverter-Branchat et al. 2004; Sitte et al. 2000). These observations are true from yeast, to mice and human cells. Some conditions that increase intracellular ROS accumulation shorten the lifespan and those that reduce intracellular ROS extend lifespan. Deletion of SODs (Barker et al. 1999; Guarente 2001; Kaeberlein et al. 2005a; Unlu and Koc 2007; Wawryn et al. 1999) and increased exposure to oxygen lead to shortening of lifespan (Nestelbacher et al. 2000). On the other hand, overexpression of catalases (Dai et al. 2009; Schriner et al. 2005) and SODs (Harris et al. 2003) increases lifespan. Some mutants with extended lifespan are also found to be resistant to oxidative stress (Lin et al. 1998). However, examples to the contrary also

exist, especially for replicative lifespan (Breitenbach et al. Chapter 3, this volume; Mesquita et al. 2010).

An increase in intracellular oxidative stress and in ROS is detected in both chronologically and replicatively aged cells in the absence of any external stressors (Fabrizio et al. 2004; Herker et al. 2004; Laun et al. 2001). The limitation of using yeast cells to study replicative ageing is the difficulty in obtaining truly old cells. One half of the cells in a rapidly growing culture are virgins, 1/4 one-division-old mothers, 1/8 two-division-old mothers, and so on. However, several approaches have been employed to enrich for old cells. The most accurate method is micromanipulation of daughter cells away from mother cells and counting the number of generations produced by individual mother cells (Mortimer and Johnston 1959). However, this method is not suitable for high throughput screening or most analytical techniques. Sucrose density gradient can also be applied to separate young and old yeast cells (Egilmez et al. 1990). Some use magnetic bead technology to immobilize yeast cells, wash away the daughter cells and re-culture the immobilized cells in fresh media and this process is repeated a few times (Smeal et al. 1996). Although this approach give cells with similar age, it is not possible to isolate significant numbers of senescent cells. The more recent elutriation centrifugation technique fractionates the cells based on size and is described in detail by Laun et al. (2001). For high throughput screening, an engineered strain, which only allows mother cells to divide when cells are grown on glucose can be used (Jarolim et al. 2004). In this strain, the essential gene *CDC6* is under the control of mother cell specific *HO*-promoter and the final optical density of the culture is directly proportional to the number of the cell divisions the strain has undergone. The daughter cells arrest growth at the G1 phase of the cell cycle.

The intriguing process that occurs during yeast budding is that the mother cells retain damaged proteins caused by oxidative stress (asymmetric segregation) and the biological clock of the daughter cell is set to zero (Aguilaniu et al. 2003). The levels of carbonylated proteins and other forms of oxidatively damaged proteins are higher in mother cells than in the daughter cells (Aguilaniu et al. 2003; Klinger et al. 2010). The proteins targeted for oxidation during aging were found to be similar in both chronological and replicative ageing (Reverter-Branchat et al. 2004). Both stress-resistance proteins (Hsp60p and Hsp70p) and the enzymes involved in glucose metabolism appeared to be carbonylated in aged cells and those grown in high glucose concentration. This supports the view that damage caused by oxidative stress could be one cause of ageing. A functional actin cytoskeleton is important for the unequal distribution of the oxidized proteins and aggregates (Aguilaniu et al. 2003; Erjavec et al. 2007; Liu et al. 2010) and increase in actin dynamics reduces ROS and extends lifespan (Gourlay et al. 2004). In addition to the restricted distribution of the damaged proteins, the daughter cells are also equipped with enhanced catalase activity to combat damage (Erjavec and Nystrom 2007). Transcriptional analysis of the old cells versus young cells also shows that metabolism shifts from glycolysis to gluconeogenesis in aged cells (Lesur and Campbell 2004; Lin et al. 2001) and expression of certain stress- and damage-responsive genes is elevated (Lesur and Campbell 2004). Despite several observations that protein carbonyls and

other biochemical markers of damage are accumulated in older cells, there is still no clear proof that the damaged proteins are the cause of ageing (Muller et al. 2007). It is interesting to note that chronologically old cells have accumulated death factors which can limit the replicative potential of those cells (Ashrafi et al. 1999; Piper et al. 2006). This is one indication that replicative and chronological aging of yeast cells share some similarity despite the fact that the genome-wide analysis of both processes using the yeast deletion collection has revealed little overlap (Laun et al. 2006). However, this interesting observation depends on the carbon source and is not seen when yeast are grown on glycerol or ethanol (Piper et al. 2006).

As mentioned earlier, mitochondria are the major source of ROS. It was also suggested that inefficient respiration or defective mitochondrial activity found in aged (Ermini 1976; Terman et al. 2003) cells may have caused increased generation of ROS in old cells. However, high levels of ROS and carbonylated proteins are detected in yeast cells that are less than 10 generations old (Aguilaniu et al. 2003; Lam et al. 2011). Treatments with different uncouplers of oxidative phosphorylation produce contradictory results: CCCP (carbonyl cyanide 3-chlorophanylhydrazone) causes an increase in ROS and shortens the replicative lifespan (Stockl et al. 2007) while dinitrophenol reduces ROS and increases lifespan (Barros et al. 2004). We have also found that ROS appeared after mitochondrial damage, which is probably triggered by a high level of glucose metabolites during chronological ageing. Therefore the role of mitochondria in aging is still not completely clear (Breitenbach et al. Chapter 3, this volume).

The assumption that eliminating the respiratory chain would also prevent oxygen toxicity also turns out to be wrong. A study of a respiratory-deficient ρ° mutant showed that there are cytoplasmic oxygen-dependent reactions that could give rise to oxygen toxicity (Rosenfeld and Beauvoit 2003; Rosenfeld et al. 2002). The chronological lifespan of the ρ° mutant is shorter than that of the wild-type cells (Fabrizio et al. 2010). However, the replicative lifespan of ρ° mutants may be shorter (Berger and Yaffe 1998; Powell et al. 2000), equal (Heeren et al. 2009) or longer than that of rho-plus wild type cells, depending on the retrograde response (Jazwinski, Chapter 4, this volume) or back-signaling (Heeren et al. 2009). Analysis of the deletion mutants that affect both replicative and chronological ageing has highlighted the importance of respiratory functions for both forms of aging (Laun et al. 2006). It was also shown that stationary phase cells pre-adapted to respiratory carbon source maintain their replicative capacity on glucose media compared to non-adapted cells (Piper et al. 2006).

One of the first genes identified by screening stress-resistant mutants for enhanced longevity was *SIR4*, which encodes a member of the Sir complex that mediates transcriptional repression at telomeres and the silent mating-type loci *HML* and *HMR* (Kennedy et al. 1995). Later another member of the Sir complex (*SIR2*) was found to play a prominent role in ageing (Guarente 2001). The second gene linked to yeast ageing in the initial screen was *UTH1* (Kennedy et al. 1995) which is required for induction of apoptosis in yeast by expression of mammalian Bax protein and for mitophagy (Camougrand et al. 2003; Kissova et al. 2004). Deletion of *UTH1* increases replicative lifespan (Kennedy et al. 1995) and the gene has also

been shown to be involved in the oxidative stress response (Bandara et al. 1998; Camougrand et al. 2004). The link between longevity and the multifunctional Uth1p was intriguing at that time. Its importance for longevity may be based mechanistically on its role in apoptosis, autophagy, mitochondrial morphology, oxidative stress resistance or a combination of these processes. The importance of autophagy and maintenance of mitochondrial morphology in ageing processes are increasingly evident (Bergamini et al. 2007; Droge 2004; Vellai 2009).

Currently both caloric restriction (CR) (Masoro 2005, 2009; Mehta and Roth 2009; Osborne et al. 1917) and inhibition of growth signaling (TOR pathways) (Kaeberlein et al. 2005b; Powers et al. 2006; Vellai et al. 2003) are very well accepted as the conditions that increase lifespan in both aging processes of yeast. Exactly how these conditions lengthen either lifespan is still not clear. The common responses that are up-regulated in both conditions are starvation response and autophagy. Autophagy recycles damaged organelles and provides nutrients for the cells when they are in need (Cuervo 2004; Klionsky 2005; Klionsky and Emr 2000; Suzuki and Ohsumi 2007). It seems that cells grown under the conditions that favor low metabolic rates survive longer than those with high metabolic rates. Functional stress resistance genes are still important for lifespan extension under these conditions. For example, the lifespan extension of *ras2, cyr1* and *sch9* mutants requires the *SOD2* gene (Fabrizio et al. 2001, 2003).

There are some controversial results that lead to conclusion that lifespan can be extended without reduction of ROS. Certain yeast mutants live longer although they have increased intracellular ROS (Kharade et al. 2005) and inhibition of ROS production did not extend lifespan in *Drosophila* (Miwa et al. 2004). Previously it was shown that providing antioxidants in the diet is beneficial for cell survival and cognitive performance in rodents (Floyd 1991). In addition, the biological antioxidant glutathione is also found to be necessary to ensure the benefits of caloric restriction during ageing in yeast (Mannarino et al. 2008). However, exposure to the antioxidants α-tocopherol and coenzyme Q_{10} shortens the replicative lifespan of *S. cerevisiae*, possibly because α-tocopherol treatment leads to an increase in lipid peroxidation (Lam et al. 2010).

It is still inconclusive if ROS are the primary determinant factor in ageing. Whether the short-lived cells died early due to accelerated ageing or due to defects unrelated to ageing is still not known. For our point of view, ageing is well programmed. However this does not mean that the "program of ageing" has been positively selected for in evolution and that the aging processes constitute an adaptive trait that enabled species to survive during evolution. This hypothesis is still highly controversial in the current discussion. We hold the view that, based on existing genetic data, what we perceive as a "genetic program of ageing" through numerous genetic studies of aging is in reality the genetic program, or rather multiple genetic programs of stress response. Age-related diseases such as Parkinson's and Alzheimer's may be a result of early damage caused by the build up of ROS during the ageing process or via the diet or environment. Cells possess mechanisms to remove and replace oxidised lipids, proteins and nucleotides and repair damage however if the damage exceeds the cell's capacity, early cell death is bound

to happen. Nevertheless, studies on ageing including accumulation of oxidatively damaged molecules in aging cells, and the cells' responses to this during ageing can be beneficial for the understanding of age-related diseases.

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Chapter 3
The Role of Mitochondria in the Aging Processes of Yeast

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Abstract This chapter reviews the role of mitochondria and of mitochondrial metabolism in the aging processes of yeast and the existing evidence for the "mitochondrial theory of aging". Mitochondria are the major source of ATP in the eukaryotic cell but are also a major source of reactive oxygen species (ROS) and play an important role in the process of apoptosis and aging. We are discussing the mitochondrial theory of aging (TOA), its origin, similarity with other TOAs, and its ramifications which developed in recent decades. The emphasis is on mother cell-specific aging and the RLS (replicative lifespan) with only a short treatment of CLS (chronological lifespan). Both of these aging processes may be relevant to understand also the aging of higher organisms, but they are biochemically very different, as shown by the fact the replicative aging occurs on rich media and is a defect in the replicative capacity of mother cells, while chronological aging occurs in postmitotic cells that are under starvation conditions in stationary phase leading to loss of viability, as discussed elsewhere in this book. In so doing we also give an overview of the similarities and dissimilarities of the various aging processes of the most often used model organisms for aging research with respect to the mitochondrial theory of aging.

Keywords Mitochondria · Mutation · DNA repair · Somatic mutation theory · Hypoxia

Introduction

Mitochondria came into existence at the base of eukaryotic evolution and, arguably, are a prerequisite for multicellular development due to the improved energy metabolism of cells that contain mitochondria (Lane and Martin 2010). Mother Nature has "played" with mitochondria like with any other invention she has made

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in the biosphere. This has led to additional functions of mitochondria besides ATP production, like the function in programmed cell death with its close relationship with aging (see below) and the development of the capacity to exchange signals with the nucleo-cytoplasmic system of protein synthesis and the capacity of the cell to degrade mitochondria in case of severe loss of function. Fortunately, nearly all of these mitochondrial functions have been conserved during eukaryotic evolution. Yeast is well suited to study the functions of mitochondria in aging due to its rapid growth and cell division cycle, its highly developed genetics, the fact that respiration-deficient mutants are viable and can be easily obtained, and, in particular, the availability of methods for introducing nearly every genetic change at will in the chromosome as well as in the mitochondrial genome.

But what can yeast cells really tell us? As has been argued above, because of the conservation of mitochondrial functions in evolution, there is reason to believe that the results obtained with yeast genetics can be relevant for answering the one question that is behind all other questions: Why do we humans age? Can we manipulate, slow down or perhaps stop the intrinsic aging process that appears to be operative in nearly all living things and, of course also in humans?

To get started, we will review here the mitochondrial theory of aging (TOA) in its historical context. But also this theory does not supply us with a single or single most important cause of aging. Mitochondrial defects that occur naturally during aging lead to an increase in internal oxidative stress or an imbalance of redox homeostasis. But the genetic and pharmacological interventions that were applied to cells and organisms to influence their redox environment, in most cases led to only moderate changes in the lifespan.

A Short History of the Mitochondrial Theory of Aging

High energy ionizing radiation (X-rays, gamma rays) produces radicals in aqueous solutions and in living cells. The combination of ionizing radiation and oxygen is much more efficient in radical production, cell killing, and mutagenesis than either treatment alone, leading to the concept of oxygen toxicity in biology. These are typical discoveries of the decade from 1950 to 1960, when "radiation biology" was in its heyday and nuclear reactors were built. Irradiation of living cells, particularly in the presence oxygen, was shown to lead to defects phenotypically similar to those of aging cells. It is important to note that, contrary to the early beliefs, this does not mean that the very low level of natural high energy background radiation is the cause of natural aging, but it was still an important stimulus for the development of aging theories and for directing the research of subsequent decades.

Gerschman (1954) showed that oxygen radicals actually can be formed in living cells. A list of the most important reactive oxygen species (ROS) formed in living cells following the primary production of superoxide radical anion and of the enzymes converting and metabolizing them, as well as the pathophysiology caused by these ROS is given in another chapter (Aung-Htut et al. Chapter 2, this volume). It is now appropriate to broaden the concept of ROS and to speak of "RONS" (reactive

oxygen and nitrogen species) because of the importance of nitric oxide (NO, which is a radical) and peroxynitrite, a highly reactive non-radical species (for review: Halliwell et al. 1987; Halliwell and Gutteridge 1984, 1988).

The "oxygen radical" theory of aging (TOA) (Harman 1956) and the "somatic mutation" TOA (Szilard 1959; Orgel 1963) are closely related because oxygen radicals are mutagenic and the two theories were created at about the same time based on the experimental findings of the 1950s. In 1956, Denham Harman proposed that aging, which is a gradual loss of function in all constituents of living cells, might be caused by the chemical damage due to oxygen radicals, and that the molecular damage which is formed downstream of the short-lived primary radical species might accumulate during the lifetime of a cell and of an organism. All of these theories are interconnected and all of them had to wait for decades before the predictions contained in them could be tested by precisely aimed experiments.

Around 1965–1970, the location, metabolic importance and molecular details of the respiratory chain of the inner mitochondrial membrane gradually were elucidated (Racker 1977; Mitchell 1961). Later, mainly through the work of Britton Chance (for review see: Chance et al. 1979) it was shown that mitochondria not only transform oxygen directly to water (in complex IV of the respiratory chain) but also transform oxygen by an "unwanted" one electron process into the superoxide anion radical. Superoxide is a comparatively long-lived and slow-reacting radical species (but still more reactive and short-lived than most non-radical molecules). An important step in understanding oxygen radical biology was the discovery of superoxide dismutase (SOD) by Fridovich and his students (McCord and Fridovich 1969) at about the same time. Later it was shown that eukaryotic cells produce relatively large amounts of two different superoxide dismutases underscoring the physiological importance of this part of oxygen radical metabolism. The manganese SOD (Sod2) is present in the mitochondrial matrix while the copper/zinc SOD (Sod1) is located mainly in the cytoplasm but some is also found in the mitochondrial intermembrane space.

Based on the finding that most oxygen radicals derive from mitochondria, Harman in 1972 developed a new TOA, now called the "mitochondrial theory of aging" (Harman 1972). Subsequently, the major points of "leakage" of electrons from the respiratory chain were found to be complex III, and complex I. Therefore in yeast, which lacks complex I, complex III is the only physiological point of superoxide formation in the respiratory chain. However, in yeast, in place of complex I, NADH reducing equivalents can be fed into the respiratory chain by Nde1 or Nde2 (external NADH) or Ndi1 (internal NADH) without proton pumping, and these proteins were also found to be capable of producing superoxide (Luttik et al. 1998; Marres et al. 1991; Li et al. 2006). It is controversial which percentage of the electron flow ends up in superoxide, but a rough estimate is 1% under in vivo conditions (Cadenas and Davies 2000).

The present view of the role of ROS in aging includes a signalling function of the ROS and the importance of non-mitochondrial sources of superoxide besides the well-known mitochondrial source. There is now overwhelming evidence for a

physiological role of ROS (oxygen radicals and the molecules derived from them) in cellular signalling. This adds a number of new aspects to the oxygen TOA. The state of current knowledge is slightly different for the fungal model systems of aging and for the model systems based on mammalian cells and organisms. In mammals, the physiological function of ROS produced by mitochondria and NADPH oxidases (NOXs) has been well researched (Buetler et al. 2004) and these ROS (most probably hydrogen peroxide) control growth and proliferation, and also in some specialized cells, cell differentiation and the production of some specialized molecules, for instance the hormone, thyroxine. In this scenario, overexpression or activation of NOX enzymes can lead to hypermitogenic arrest and to apoptosis, pointing to a direct involvement of ROS generated by NADPH oxidases in the aging process, although many questions remain. In fungi, the signalling function of ROS is less well researched (Takemoto et al. 2007) and in the unicellular hemiascomycetous yeasts (*S. cerevisiae* and *S. pombe*), the current literature view is that an NADPH oxidase does not exist.

Some Experiments Designed to Test the Mitochondrial TOA

Simple theory would predict that supplying the cells with biological antioxidants would increase the lifespan. However, adding biological antioxidants (for instance reduced glutathione) to wild type yeast cells does not increase the replicative lifespan. Addition of the lipophilic antioxidants alpha-tocopherol or ubiquinone reduces the lifespan of *Saccharomyces cerevisiae* (Lam et al. 2010). However, adding glutathione in 55% oxygen does increase the lifespan of a yeast strain to that observed in the wild type in ambient air. One additional step is to use a yeast strain deleted for both catalases, and now the lifespan elongation by glutathione is again observed, even in ambient air (Nestelbacher et al. 2000) (see Fig. 3.1).

The mitochondrial TOA is an offshoot of the oxygen radical TOA. While there is some truth in the oxygen radical or mitochondrial TOA, the above experiments indicate that it is not easy to improve the lifespan of the wild type in ambient air, presumably because the dynamic redox equilibrium of the cell has been set at an optimal value during evolution and deviations that lead to an excess of either oxidants or reductants are detrimental to the cell. The redox equilibrium is determined largely by the most important redox buffer of the cell, which is the GSH/GSSG couple. Other important redox couples of living cells are NADPH/$NADP^+$ and NADH/NAD^+, or the relevant SH groups on redox-active proteins. These are, however, far from equilibrium and do not easily exchange with each other (Schafer and Buettner 2000).

If a number of long-lived mutant strains are tested for resistance to oxidants, most of them are resistant to at least one oxidant, but many exceptions exist, as is obvious from a comparison of whole genome deletion mutant data aimed at oxidative stress resistance (Thorpe et al. 2004), a partial data set for RLS (Kaeberlein et al. 2005; Steinkraus et al. 2008; see also Kaeberlein, Chapter 12, this volume) and a complete data set for CLS (Powers et al. 2006; Fabrizio et al. 2010). However, the correlation

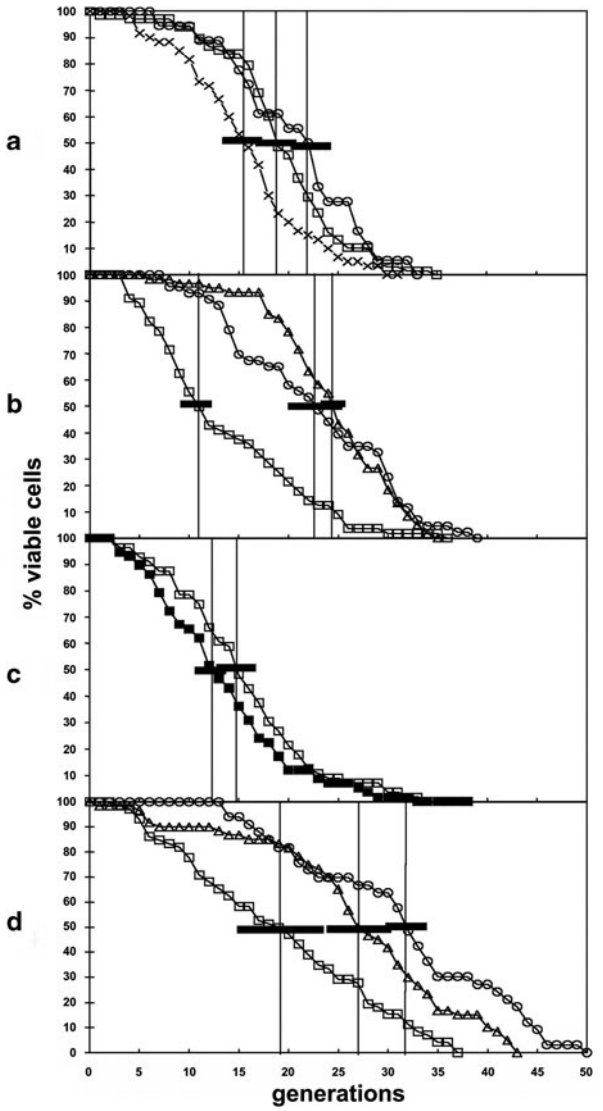

Fig. 3.1 Replicative life-span determination. Percent viable cells are plotted against cell division number (generations). Sample size in all cases was 50–60 virgin cells. *Perpendicular dotted lines* indicate median life spans. SD of the median life spans at a confidence level of 95% which were calculated by applying Kaplan–Meier statistics are indicated by *error bars*. To decide whether two given survival distributions are significantly different at a 95% confidence level, Breslow, Tarone-Ware, and log-rank statistics were used. All experiments were done on SC plates. Additions were as indicated below. **a** Strain W303eA: *circles*, ambient air; *squares*, 55% oxygen; *crosses*, 20 μM of paraquat. **b** Strain W303eA *ctt1:: URA3*, *cta1:: URA3*: *circles*, ambient air; *squares*, 55% oxygen; *triangles*, 55% oxygen, 1 mM GSH. **c** *open squares*, strain W303eA, *ctt1::URA3*; *closed squares*, strain W303eA, *cta1:: URA3*. 55% oxygen. Note that the control experiments for (**c**) are included in (**a**) (isogenic WT, 55% oxygen) and (**b**) (isogenic double mutant, 55% oxygen). **d** Strain JC482: *Circles*, ambient air; *squares*, 55% oxygen; *triangles*, 55% oxygen, 1 mM GSH (from Nestelbacher et al. 2000; with permission from Elsevier)

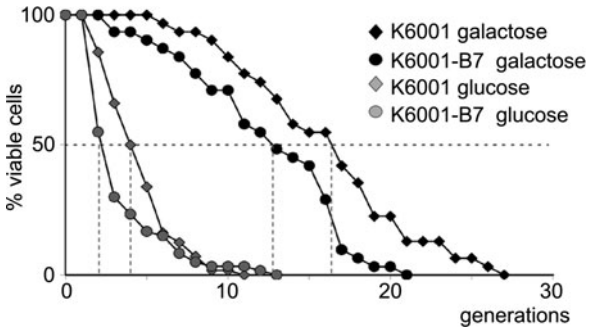

Fig. 3.2 K6001-B7 has a shortened replicative lifespan. Daughter cells from K6001 and K6001-B7 were continuously removed by micromanipulation and counted and analyzed statistically. The shortened lifespan of K6001-B7 in both glucose and galactose media was tested statistically significant using Mantel-Cox, Breslow as well as Tarone/Ware statistics (from Timmermann et al. 2010, with permission from Impact Journals LLC)

between oxidative stress resistance and longevity is better for CLS than for RLS. Comparable correlative evidence exists for long-lived mutants of *C. elegans* (Park et al. 2009).

If, on the other hand, the mutants obtained from an unbiased whole genome screen for resistance to oxidants are tested for their replicative lifespan, many of them are normal or even short-lived. One example analysed in detail was the phenotype of the dominant point mutation B7 in the yeast peroxyredoxin Tsa1, which was found to be short-lived (Timmermann et al. 2010) (see Fig. 3.2). Mutants in triose phosphate isomerase would be another example. They have an increased NADPH/$NADP^+$ ratio, are resistant to oxidants, but are short-lived in both CLS and RLS (Ralser et al. 2006).

Genetics and Biochemistry of Human and Yeast Mitochondria

Mitochondria are the main producers of ATP in most eukaryotic cells, but also house a large number of essential metabolic pathways or, in some cases, parts or steps of pathways. It is unknown why during the evolutionary history of these endosymbiontic organelles, specific parts of metabolic pathways of the endosymbiont had to be retained in these versatile organelles while others were lost to the nucleocytoplasmic system. There is nowadays no serious doubt in the once controversial endosymbiont hypothesis stating that mitochondria evolved by reduction and gene loss from an original hypothetical prokaryotic symbiont in a monophyletic way. Only a small part of the original gene set of the symbiont was retained in the mitochondria while the largest part moved to the nuclear chromosomes. Among the “retained” genes (13 protein coding genes in human cells, 8 in yeast cells)

are genes coding for the largest and most hydrophobic protein components of the respiratory and ATP synthase complexes of the inner mitochondrial membrane. In addition, the ribosomal RNAs and the 22 tRNAs needed for mitochondrial translation had to be retained. The rest of the about 700 (yeast) and about 1500 (mammals) mitochondrial proteins are encoded in the nuclear genome, synthesized on cytoplasmic ribosomes and imported into mitochondria in rather complicated but well-known ways (Schmidt et al. 2010; Baker et al. 2007). Only about 300 of the 4800 viable yeast gene deletions lead to "*petite*" (non-respiring) mutants that are unable to grow on non-fermentable carbon sources and only about half of them are directly involved in respiration (Merz and Westermann 2009). What is puzzling from an evolutionary viewpoint is that some (for instance mammalian) mitochondrial genomes are extremely small and streamlined, while others (yeast, plants) are bigger, very variable in size, and encode slightly different sets of proteins of the respiratory complexes. A microorganism with a much larger number of mitochondrially encoded proteins was discovered (Lang et al. 1997), which is therefore perhaps closer to the original endosymbiont than most modern mitochondria. The genetic code used in mitochondria differs from the universal code, and the mitochondrial code used by humans is not the same as that used by yeast.

Yeast and human cells are inviable without mitochondria, however they are viable without mitochondrial respiration, as exemplified by the so-called rho-zero mutants which by definition are devoid of mitochondrial DNA, but still contain mitochondria. Rho-zero mutant cells of yeast, which can easily be isolated either after spontaneous loss of the mitochondrial genome or after treatment with ethidium bromide, grow slowly on glucose and other fermentative substrates like raffinose, but not at all on "respiratory substrates" like glycerol, ethanol, or lactate. These mutants must import ATP (produced from fermentation) into their mitochondria, which is possible by means of a functional adenine nucleotide transporter. All respiratory deficient mutants of yeast as well as rho-zero cells are chronologically short lived (Fabrizio et al. 2010), but the RLS of these non-respiring cells can be substantially longer than that of the corresponding wild type, depending on the retrograde response in those cells (Kirchman et al. 1999, see the Chapter 4 by Jazwinski, this volume). Certain mutations in mitochondrial ribosomal proteins and in the apparatus regulating mitochondrial translation are also replicatively long-lived (Heeren et al. 2009; see below).

Human rho-zero cells can be isolated by ethidium bromide treatment and can be maintained in cell culture easily, but rho-zero cells or any cells completely devoid of mitochondrial respiration cannot differentiate in the context of a chimeric embryo and cannot form an embryo. We mention these cells because loss or large deletions of the mitochondrial genome as well as mitochondrial point mutations clearly occur during aging in the mouse and in humans. There is no detailed published evidence for the frequency of mitochondrial mutations during replicative or chronological aging of yeast cells (compare, however, Muller 1971), but relatively recently the group of Gottschling showed that the daughters of old mothers indeed tend

to become petite (Veatch et al. 2009). Long-lived respiratory deficient mutations were among the last survivors in replicative aging in certain strains (Kirchman et al. 1999). However, re-examination of this experimental result showed that the respiratory-deficient mutant cells pre-existed in the sample, they were not generated during aging (S.M. Jazwinski, personal communication).

The frequency of chromosomal mutations in replicatively old cells was studied in more detail. The group of Gottschling showed an increase of gene conversions (loss of heterozygosity) at the *MET15* locus in diploid cells during replicative aging (McMurray and Gottschling 2003; Veatch et al. 2009), and the group of Longo showed an increase in canavanine-resistant clones during chronological aging (Longo 1999) and an increase in chromosomal rearrangements caused by mitochondrial oxidative stress leading to DNA lesions and the activation of translesion synthesis repair pathways (Madia et al. 2009; Parrella and Longo 2008) indicating an increase in chromosomal mutations during chronological aging.

Further Evidence for the Role of Mitochondria in the Aging Processes: Mitochondrial Mutations (Point Mutations vs. Large Deletions)

The mitochondrial TOA (see above) was historically conceived because mitochondria were recognized as a source of ROS leading to the idea of a vicious cycle in mitochondrial ROS metabolism. Mitochondrial ROS according to this idea would produce point mutations, which would produce defective respiratory complexes, which would again produce more ROS and so on. This was plausible, although not really proven (Muller et al. 2007). A general decline of mitochondrial function in aging was shown in higher organisms (for instance Boveris and Navarro 2008). In yeast, accumulation of ROS-positive mitochondria (Laun et al. 2001) and a strong decline in aconitase activity in old wild type mother cells (Klinger et al. 2010) was shown in mother cell-specific aging. Similarly, aconitase is deactivated during chronological aging in wild-type and *sod2*-deleted yeast (Fabrizio et al. 2001; Parrella and Longo 2008) and mitochondria showing increased ROS accumulate during chronological aging (Herker et al. 2004; Buttner et al. 2006; Mesquita et al. 2010; Buttner [unpublished data]) (see Fig. 3.3).

It seems clear that a decline of mitochondrial activity takes place during both aging processes observed in yeast, but so far there is only evidence for the accumulation of mitochondrial point mutations or large deletions in replicative aging (Veatch et al. 2009). Mitochondrial mutation would also be worth investigating in chronological aging. There is presently intensive research going on pertaining to the role of mitochondrial mutations in mammalian aging (Khrapko et al. 2006; Trifunovic et al. 2004; Vermulst et al. 2007, 2008; Williams et al. 2010).

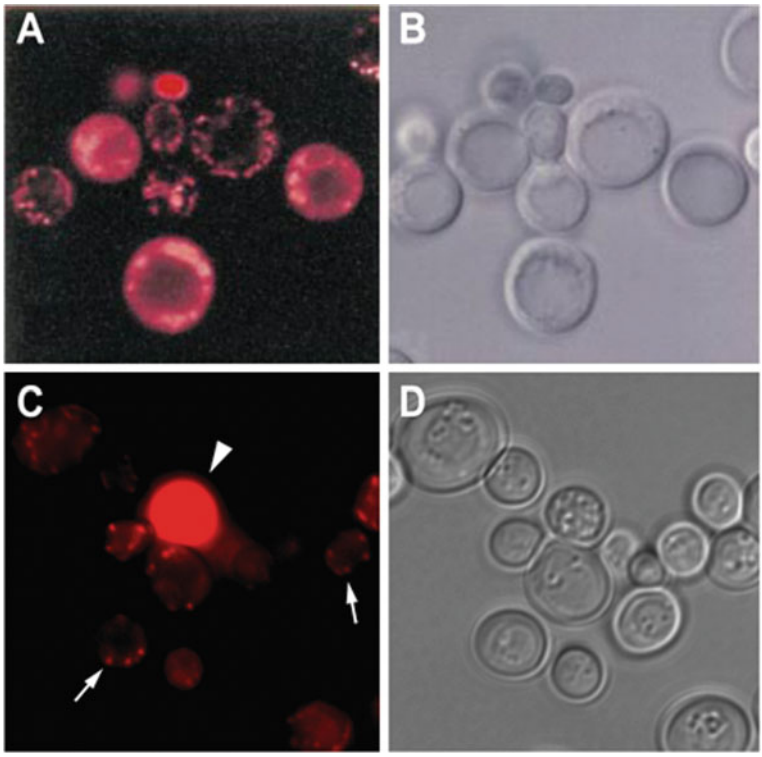

Fig. 3.3 **a** Old mother cells were stained with dihydrorhodamine (DHR, 5 mg ml^{-1}; stock solution 2.5 mg ml^{-1} in ethanol) and viewed and photographed under a confocal laser-scanning fluorescence microscope after 10 min using the rhodamine filter set. The stained cells show typical mitochondrial morphology. **b** The same sample in phase contrast (modified after Laun et al. 2001 with permission from John Wiley and Sons). **c** Chronologically aged yeast cells (day 5) were stained with dihydroethidium (DHE, 2.5 μg/ml in PBS, stock solution 2.5 mg/ml in DMSO) and analyzed using laser scanning confocal fluorescence microcopy. An exposure time of 4000 ms was used to visualize not only lethal doses of ROS (*arrowhead*, necrotic dead cell) but also the generation of sublethal doses of ROS at mitochondria (*arrows*, cells in early stages of apoptosis). **d** The same sample was analyzed using phase contrast

Accumulation of Mutations in the Mitochondrial Genome: Cause or Consequence of Aging?

Although the relevant measurements have not been made in yeast mitochondrial DNA, we should summarize briefly the current state of the situation concerning the role in aging of mitochondrial point mutations and large deletions in the mouse. In two laboratories homozygous mouse knock-in strains were constructed to contain exclusively a mitochondrial DNA polymerase with a defect in the proof-reading

domain (Trifunovic et al. 2004; Kujoth et al. 2005). These mice age prematurely as shown by a shorter lifespan and an increase of practically all diseases of aging of the mouse (for instance, cardiovascular diseases) at an age where the wild type does not show these disease phenotypes, while the heterozygous littermates are normal. However, the heterozygous mice have a normal lifespan and health, although in heart and brain they carry tenfold more mitochondrial point mutations than normally aged very old mice (Khrapko et al. 2006; Vermulst et al. 2007). This would clearly indicate that mitochondrial point mutations cannot be the cause for aging in these mice (see Fig. 3.4).

The matter depends very much on the ability to accurately measure spontaneous mitochondrial point mutations by sequencing. In a later paper (Vermulst et al. 2008) the Loeb group maintains that not point mutations but rather large deletions of mitochondrial DNA may be causative for aging. They argue that the heterozygous mice still have a low level of deletions, not higher than normally aged tissues, and that the formation of deletions (which are also very well researched in age-related pathology in humans) depends on mitochondrial homology-driven repair in the absence of proofreading. Trifunovic et al. contest this notion (Edgar et al. 2009, 2010) and maintain that mitochondrial point mutations are causative for aging. The question is open at present. The issue is complicated because mitochondrial genetic instability, including deletions and point mutations always start as being heteroplasmic, cell

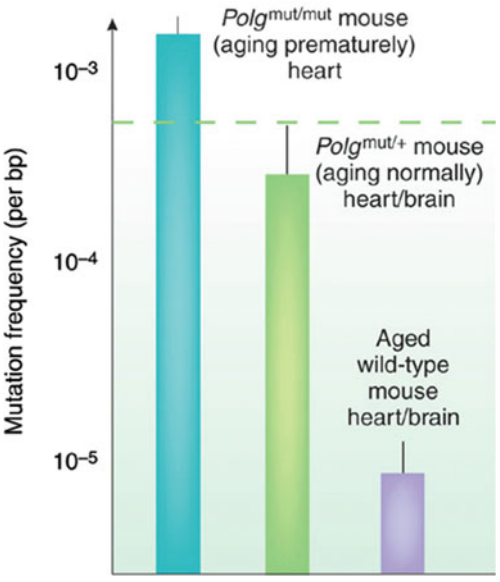

Fig. 3.4 Logic dictates that mtDNA mutations, when present at levels lower than in phenotypically normal *Polg*$^{mut/+}$ mice (*dashed green line*), are irrelevant for aging. Note that very old wild-type mice display a much lower frequency of mitochondrial mutations as compared to the normally aging heterozygotic mice. *Error bars* represent estimated variation of the data (adapted by permission from Macmillan Publishers Ltd: Aging Cell Khrapko and Vijg 2007, copyright 2007)

divisions then lead to segregation of mutant and wild-type mitochondria, and after a number of cell divisions a low threshold value of respiration is reached which causes loss of function of the particular cell concerned. This threshold value may be different in different organs of the mouse and of humans.

Mitochondrial Catalase

Although these very important experiments have been done in mice, and not in yeast, we want to introduce them briefly, because they are feasible and worth doing in yeast as well. In the wild type, mitochondria do not contain catalase. The superoxide produced at a low level in the respiratory chain is converted to hydrogen peroxide by mitochondrial MnSOD and can pass the mitochondrial membranes and can be further converted to ROS. Transgenic mice engineered to express human peroxisomal catalase in their mitochondria showed a significant increase in mean and maximum lifespan and a significant decrease in cardiac and cataract pathology. The authors also showed that a double transgenic mouse expressing human catalase and mouse SOD2 in their mitochondria had an even more enhanced median lifespan. Similar experiments with expression in the nucleus or in the peroxisome of mice did not significantly influence their aging process (Schriner et al. 2005). This constitutes one of the most convincing experiments showing that the combined antioxidative action removing ROS can increase fitness and lifespan and, because of the dependence on subcellular location, the experiment also shows that the mitochondrially generated oxygen radicals (and hydrogen peroxide) can limit the lifespan of the wild type.

A Decline in ATP Production

ATP production during the aging of yeast has never been measured in a systematic way, but the experiment would be worth doing. In higher cells, "AMP-kinase" is a signalling element responsive to the level of AMP/ATP in the cell, which plays an important role in aging. In yeast, the *sch9* deletion mutant is replicatively and chronologically long-lived (Fabrizio et al. 2001). The pathway represented by Sch9 is one of the partially redundant glucose-sensing pathways (together with the RAS/cAMP pathway and the TOR pathway) and it seems to be a general principle that down-regulation of glucose (or nutrient) sensing can improve longevity. This has been stressed in many review articles (for instance Kapahi et al. 2010) and is believed to constitute one of the "public mechanisms" of aging. It is not quite clear what is the gene corresponding to *SCH9* in higher organisms, but it could be S6 kinase, PKB or AMP kinase based on sequence comparisons. PKB (protein kinase B, AKT) of mice, flies and worms has been extensively researched and down-regulation of the IGF (insulin-like growth factor) signalling pathway in which PKB is involved seems to increase lifespan (Longo and Fabrizio, Chapter 5, this volume).

The Increase in Mitochondrial ROS and the Role of Apoptosis in Aging

This increase has been discussed above and has been observed both in RLS and CLS experiments. Problems inherent in the measuring methods are discussed in the chapter on oxidative stress (Aung-Htut et al. Chapter 2, this volume) and a general treatment of the role of apoptosis in aging is given by Laun et al. (Chapter 10, this volume). Increased ROS production is a marker of apoptotic and necrotic yeast cells (Madeo et al. 1997, 1999; Eisenberg et al. 2009). Mitochondria have been identified as a primary source of ROS in programmed cell death (Braun et al. 2006; Ruckenstuhl et al. 2009; Ludovico et al. 2002). Both apoptotic cells and necrotic cells are observed among replicatively as well as chronologically aged yeast cells (Eisenberg et al. 2010; see the Chapter 10 by Laun et al. this volume; Jungwirth et al. 2008; Herker et al. 2004; compare Fig. 3.3).

It is an interesting question whether apoptosis is just a phenomenon finalizing the aging process caused by other means, or if perhaps apoptotic genes and mechanisms are causally involved in the aging process itself. The first of these two possibilities seems to be true for RLS, based on observations with replicatively long-lived mutants. For instance, in the *afo1* mutant (Heeren et al. 2009), mitochondria staining positively for ROS and apoptotic phenotypes do occur in senescent mother cells, but they are seen at a time 50–60% later than in the wild type. This means that the longer RLS is not caused by a defect in the apoptotic process. On the other hand and pointing in the same direction, most of the genes and deletion mutants involved in yeast apoptosis are not replicatively long-lived. However, there may be some causal connection between apoptosis and CLS, because many apoptosis mutants are chronologically long-lived. An example is the yeast caspase deletion mutant, *yca1*Δ, which shows a normal RLS (Laun et al. unpublished data) and a longer than wild type CLS (Laun et al. 2008; Madeo et al. 2002).

Signalling by Mitochondria and Its Role in the Aging Process; Hypoxia and Aging

Mitochondria exchange information or "cross-talk" with the nucleo-cytoplasmic protein synthesis system in order to adapt the cell to a changing environment. A well-known example is the sensing of hypoxia in the environment and responding by degrading mitochondrial components on the one hand and synthesizing isoforms adapted to hypoxic conditions, on the other. This is achieved by the HIF-1 system in higher cells and by a different mechanism involving the *HAP* genes and the *ROX1* gene of yeast. The HIF-1 system is termed "positive regulation", because the transcription factor HIF-1 positively stimulates transcription of hypoxic genes. The HAP system is termed "negative regulation", because the HAP1 and HAP2/3/4/5 transcription factors stimulate mitochondrial biogenesis in high oxygen and repress hypoxic genes. In spite of these differences in transcriptional regulation, adaptation

of respiration to low oxygen works in a very similar way in the two types of cells, by introducing for the standard complex IV subunit cox4-1 (the ortholog is cox5a in yeast) a new isoform subunit cox4-2 (the yeast ortholog is cox5b), which can handle oxidative phosphorylation at low oxygen more efficiently without producing a high level of ROS. The new and exciting aspect is that activating this system (stabilizing HIF-1) under normoxic conditions where it is normally degraded by VHL (the Von Hippel-Lindau tumor suppressor, an E3 ubiquitin ligase), can substantially increase the lifespan of *C. elegans* (Mehta et al. 2009; Kaeberlein and Kapahi 2009; Muller et al. 2009). Similarly, mild inhibition of respiration can also activate HIF-1 and increase the lifespan (Lee et al. 2010) although this treatment actually increases ROS production. Conversely, other authors found that the HIF-1 deletion or RNAi-mediated downregulation of HIF-1 can also increase the worm's lifespan. This effect functionally interacts with CR, since it depends on nutrient availability and is not effective in calorically restricted worms (Chen et al. 2009). Actually lowering oxygen tension has been tested in *Drosophila*. Hypoxia can increase the lifespan of *Drosophila* (Vigne and Frelin 2007). The mechanism by which this works is independent of caloric restriction and IGF-1, but is under control by the TORC1 kinase. Taken together, the role of hypoxic regulation in lifespan determination and its interaction with dietary restriction must await further experimental results to fully explore the regulatory network that is active here.

The hypoxia response is one example of signalling from the nucleus to the mitochondrion leading to a major re-structuring of this organelle. Important aspects of this signalling mechanism remain to be determined, in yeast as well as in higher organisms, for instance the exact nature of the oxygen sensor (Lee et al. 2009). Multiple oxygen sensors seem to exist, one well researched example which, however, occurs only in higher cells, is the proline hydroxylase (Berra et al. 2006; Ratcliffe 2006) which, depending on oxygen tension, is functional in the degradation of HIF-1.

Another example for regulation of mitochondrial biogenesis is the regulation of mitochondrial mass and activity in response to a change in carbon source (Ohlmeier et al. 2004), which is necessary when the yeast cell switches from fermentative (on glucose) to respiratory metabolism on glycerol, ethanol, of lactate as carbon source. A third example is regulation of mitochondrial respiration and ROS production by the RAS/cAMP pathway (Pichova et al. 1997) under the selective influence of the catalytic subunit of protein kinase A, TPK3 (Hlavata et al. 2008; Leadsham et al. 2009; Gourlay and Ayscough 2006), leading to a very short replicative and chronological lifespan of yeast in mutants that over-express TPK3 or express the $RAS2^{ala18,val19}$ allele. The latter effect depends on the yeast-specific extension of the *RAS2* gene (Chen et al. 1990).

However, signalling by mitochondria to control nuclear gene expression, is also possible. The retrograde response is a gene regulation mechanism that controls among many other genes, also those controlling the synthesis of peroxisomal anaplerotic enzymes. These enzymes, for example *CIT2*, are needed for a fully functional amino acid metabolism in non-respiring yeast cells, which do not express

a full mitochondrial tricarboxylic acid cycle (Butow and Avadhani 2004; Liu and Butow 2006). As mentioned before, yeast respiratory-deficient cells (*petite* cells) are chronologically short-lived, but are replicatively long-lived in a nuclear genetic background that enables a strong retrograde response (Jazwinski 2004, 2005a, b, c; Kirchman et al. 1999). The nature of the signal emitted by the non-respiring mitochondria is unknown, but the role of the Rtg1/2 transcription factors downstream of the primary signal is well researched (Jazwinski 2005b; Liao and Butow 1993). The retardation of replicative aging by the retrograde response is independent of the CR effect on the lifespan (Jiang et al. 2000). An important but unanswered question is: does the retrograde response exist in human cells (Jazwinski 2005a), taking into account that during human aging respiratory-deficient cells do occur and may play a role in aging (Khrapko et al. 2006)?

Recent work from our laboratory (Heeren et al. 2009) showed that non-respiring mitochondria command yet another different way of back-signalling to the nucleus,

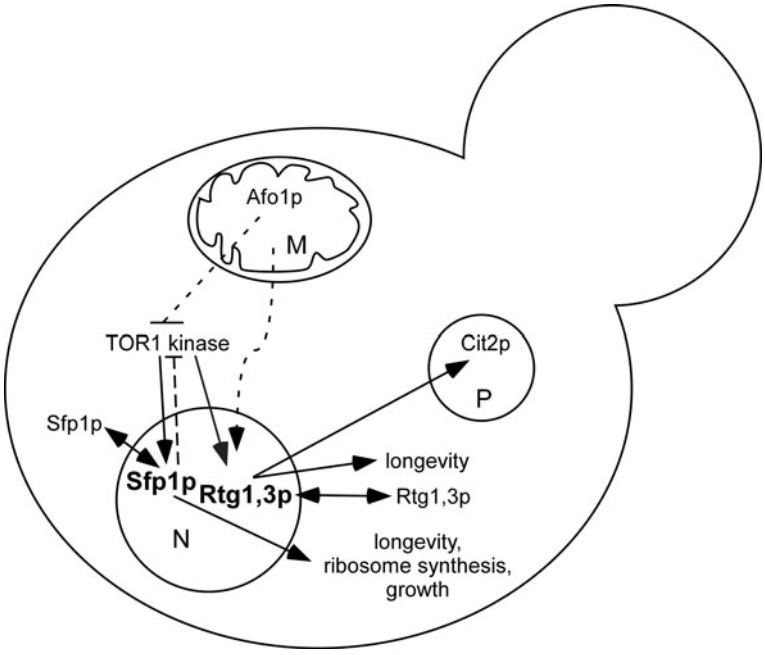

Fig. 3.5 Schematic diagram of back-signalling involving *AFO1* based on the results summarized. *Dashed arrows*: genetic interactions for which a molecular mechanism has not been determined. Both Sfp1p and Rtg1,3p shuttle to the cytoplasm when Tor1p is inhibited by rapamycin. They are indicated in **bold** in the nucleus, where they are active. An activating influence of the *TOR1* kinase complex on the transcription factor *Rtg1/Rtg3* has been postulated by Dann and Thomas (2006). Feedback inhibition of Tor1p by nuclearSfp1p is indicated. The RAS/cAMP and *SCH9* components are omitted for clarity. Their interaction with the TOR pathway is complex. *M*, mitochondrion; *N*, nucleus; *P*, peroxisome (adapted after Heeren et al. 2009, with permission from Impact Journals LLC)

which results in a long replicative lifespan as well as an unusually rapid rate of growth (comparable to the congenic rho-plus wild type) of these non-respiring cells. A deletion of the nuclear-encoded *AFO1* gene encoding a mitochondrial ribosomal protein displays these remarkable features together with resistance to three different oxidants. The *afo1* deletion leads to rapid loss of the mitochondrial genome, but on standard medium (YPD, 2% glucose) where the long lifespan is manifest, the retrograde response is not activated. However, the mutant is resistant to rapamycin and the transcription factor Sfp1 governing cytoplasmic ribosome synthesis remains active and nuclear in 100 nM rapamycin. The long lifespan of the mutant depends on the presence of a functional *TOR1* gene. We conclude that deletion of *AFO1* activates TORC1 in respiratory-deficient cells leading to the observed long replicative lifespan and the rapid growth on glucose. Since Afo1 is mitochondrial under all conditions tested, and since two other deletion mutants in genes coding for mitochondrial ribosomal proteins do not have any effect on lifespan and grow slowly like standard rho-zero yeast cells, we believe that Afo1 is involved in mitochondrial back-signalling, a function important for respiratory deficient yeast, but possibly independent of its primary function, which is mitochondrial translation (see Fig. 3.5).

The Mitochondrial Translation Complex Regulates RLS

Intriguingly, other mutations in components of the mitochondrial translation system can also substantially elongate the replicative lifespan of yeast cells. One example is *sov1Δ*, which has a strikingly similar phenotype to *afo1Δ*. It is respiratory deficient, does not trigger the retrograde response, is resistant to rapamycin (Xie et al. 2005), is resistant to certain kinds of oxidative stress, and replicatively (but not chronologically) long-lived. The mechanism that comes into play here could be similar to the one shown in Fig. 3.5, but a detailed comparison of the two mutants has yet to be done.

The synthesis of intricate mitochondrial structures, such as the respiratory complexes, is a well-coordinated process. In order to carry out the correct assembly of these complexes, proteins encoded by the nuclear genome have to match and be perfectly balanced with the ones that are directly synthesized in the mitochondria. This interplay between the nucleus and mitochondria demands a tight regulation of mitochondrial protein synthesis that, in yeast, requires more than 150 proteins regulating proper translation of mitochondrial genes encoding the respiratory complex subunits (Grivell et al. 1999). In particular, it has been shown that the translation of each of the mitochondrial-encoded genes requires at least one specific translation activator protein that in some cases also helps targeting the newly synthesized protein into the mitochondrial inner membrane (Fox 1996; Naithani et al. 2003; Perez-Martinez et al. 2003). This group of specific translation activators have been clustered and denominated the MTC (mitochondrial translation complex) module (Table 3.1; Perocchi et al. 2006).

Table 3.1 The 14 proteins of the mitochondrial translation complex with the mitochondrial target proteins for which they are specific

MTC subunit	Mitochondrial target proteins and function
Sov1p	Required for translation of *VAR1*
Mss51p Pet54p Pet111p Pet122p Pet309p Pet494p	Required for the translation of the mitochondrial *COX* genes (*COX1, COX2,* and *COX3)*
Cbs1p Cbs2p Cbp6p	Required for translational activation of *COB* mRNA
Aep1p Aep2p	Required for translation of *OLI1*
Suv3p Dss1p (Msu1p)	Form the mitochondrial degradosome, required for turnover of aberrant or unprocessed mtRNA

Interestingly, deletion mutations in at least 9 of the MTC genes results in an increased RLS (Caballero et al. 2011), and this enhanced longevity is totally independent of the lack of respiration, loss of mitochondrial DNA, altered ROS generation or, more interestingly, a global lack of mitochondrial translation, since a deletion in *IMF1,* which encodes a mitochondrial translation initiation factor does not extend life span by itself (Caballero et al. 2011). Furthermore, mutations in MTC module genes that increase RLS do so without activation of the retrograde response. Instead, knock-down mutations in *SOV1* or *CBS1* stimulate *SIR2*-dependent life span extension by elevating nuclear DNA silencing. Although the pathway through which MTC proteins affect nuclear silencing and life span is not fully understood, it is clear that life span extension achieved by deleting *SOV1* requires cAMP/PKA-dependent activation of the stress regulators *MSN2*/4. In addition, the presence of the cAMP/PKA target gene *PNC1* (Caballero et al. 2011), which encodes a scavenger of nicotinamide, a derivative molecule from NAD that inhibits Sir2p activity (Anderson et al. 2003; Gallo et al. 2004) is also required for Sov1p deficiency to extend RLS. Thus, the pathway involved in Sov1p-dependent life span control shares many features and players described previously for life span extension dependent on *TOR1* (Medvedik et al. 2007). At the present, it is not clear whether the MTC proteins have a role apart from translational control in the mitochondria that affects the efficacy of genomic silencing, or if defects in translational control trigger a compensatory response that activates *SIR2*-depedent silencing and life span extension. Nevertheless, it is interesting that a discrete, functionally related, group of mitochondrial proteins regulates yeast life span by affecting the efficacy of genome silencing and that this phenomenon may be independent of previously proposed RLS control circuits exerted by mitochondria in yeast (Ugidos et al. 2010).

Noticeably, some of the MTC proteins are also present in higher eukaryotic organisms; whether these proteins can also control life span is obviously something that remains to be tested.

Autophagy and Mitophagy and the Asymmetric Segregation of Mitochondria

As has been stated elsewhere in this book, during aging the unavoidable attrition and the effects of adverse chemical reactions originating from metabolism pertaining to all cellular components including proteins, lipids and nucleic acids makes degradation or repair of those components necessary for survival, just like the synthesis of those components itself. It appears that the most important of these degradation processes are proteasomal degradation and autophagy, of which several different pathways have been discovered (Deffieu et al. 2009; Bhatia-Kissova and Camougrand 2010). Conventional macroautophagy is believed to be essentially non-selective, meaning that portions of the cytoplasm of a cell are delivered into the lytic compartment (the yeast vacuole) on a random basis (Kissova et al. 2007). However, much more relevant for the present discussion are selective pathways of autophagy (Nair and Klionsky 2005) and in particular, mitophagy (Kim et al. 2007; Kanki and Klionsky 2008; Kanki et al. 2009; Bhatia-Kissova and Camougrand 2010). In the case of mitochondria, selectivity for mitochondria is achieved through the mitochondrial protein Atg32 together with Atg11 (Kanki et al. 2009). It is still not completely clear how non-functional mitochondria are discriminated from functional ones, but the existence of the correct membrane potential across the inner mitochondrial membrane could be essential for protection against mitophagy. This was shown by inducing mitophagy by genetically damaging mitochondria, for instance by depletion of Mdm38, which strongly induced mitophagy (Nowikovsky et al. 2007). However, this artificial situation does not reflect aging mother cells.

Autophagy/mitophagy and the other selective degradation processes do not work with 100% efficiency and they must be complemented by asymmetric segregation of damaged proteins and presumably also damaged mitochondria and other organelles in order to avoid clonal aging and death of the strain, as was first shown by Lai et al. (2002). The process is not specific to yeast, but is a universal one (Nystrom 2007) which, as we hypothesize, is generally necessary for survival of the species preventing clonal senescence. As shown by the Nyström group (Aguilaniu et al. 2003; Erjavec et al. 2008; Erjavec and Nystrom 2007; Liu et al. 2010), oxidized proteins are asymmetrically distributed between mother and daughter cells. The majority of the oxidized proteins were found to be cytoplasmic, but some were mitochondrial. Denatured proteins after heat shock were preferentially transported back to the mother cell in a process depending on the actin cytoskeleton, the polarisome, and on the heat shock protein, hsp104 (Liu et al. 2010).

In a recent paper (Klinger et al. 2010), we have shown that the protein of the mitochondrial matrix, aconitase, which is extremely sensitive to oxidative inactivation due to the 4Fe/4S centre in its active site, is inactivated during replicative aging without applying any external stress. The inactivated protein is preferentially retained in the mother cell, while the still active enzyme is preferentially transported to the daughter cell. This asymmetric segregation works in the direction to the daughter and, like the process just described (Liu et al. 2010), contributes to the rejuvenation process of the daughter cells, which in our view and according to a broad consensus is essential for the survival of the species. As aconitase in yeast remains a mitochondrial protein throughout the whole life cycle of the cells, it is clear from these results, that in the divisions of aging mother cells, whole mitochondria must be discriminated and segregated. The mechanism of this process is unknown at present. The old mother cells, due to internal oxidative stress contain small roundish mitochondria (not the familiar mitochondrial network) and it is quite conceivable that these structures are preferentially transported to the daughter, if they still have a near-normal membrane potential.

Closing Remarks

Overviewing the literature published in recent years, it becomes clear that the role of mitochondria in the aging processes of yeast is diverse and a unifying theme or mechanism has not yet been found. Of the three questions posed at the beginning of this chapter, only one can be (incompletely) answered at present time: Yes, biochemical changes can be found that look similar in aging yeast cells and in higher organisms. They could point to a basic but still unknown unity of the aging process. They may have to do with mitochondrial dysfunction. In the aging processes, any one of the possible metabolic and morphological changes of mitochondria which are listed below play a role for which some experimental evidence exists. These processes are not independent of each other and, probably, multiple simultaneous causes exist:

(i) Decline of ATP production
(ii) Increase in mitochondrial ROS production
(iii) Role of mitochondria in the apoptotic process
(iv) Mitochondrial signalling: the retrograde response; back-signalling
(v) Asymmetric segregation of damaged mitochondria, degradation of mitochondria
(vi) Defects in the fission and fusion processes of mitochondria.

What is rather well established for both replicative and chronological aging is an increase in internally generated cellular oxidative stress with age leading to small roundish mitochondria that stain positively for ROS production, as was described above in the main part of this chapter. Moreover these observations seem to be common also for the aging processes of higher cells. However, it is presently an open question, if mitochondrially generated ROS are a primary cause of aging (inducing,

among other things, the pathways of apoptosis), or, alternatively, the mitochondrial ROS observed in old cells are a by-product of aging.

The retrograde response and the mitochondrial back-signalling process can substantially increase the RLS despite respiratory deficiency, but present knowledge about the genetic mechanism of these effects is fragmentary.

We think that one of the most interesting questions to be further investigated in aging research generally, also relating to RLS only, is the asymmetric segregation of damage between mother and daughter cells in the aging process, and in particular the asymmetric segregation of whole mitochondria according to their functional state. The evidence is strong that processes of this kind are not yeast-specific but very general; however, many open questions remain, including the mechanism of discrimination, the sensing of damage (membrane potential?), the relation between mitophagy and asymmetric segregation, and the mechanism of transport of damaged and of still functional mitochondria. Mitophagy as well as asymmetric segregation are mechanistically largely unknown at present. Both processes could form a link to the aging of stem cell populations of higher organisms. Protein aggregates at the surface of mitochondria ("stress granules") could be involved in these processes (Rinnerthaler et al. 2006; Rinnerthaler et al. unpublished).

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Chapter 4
The Retrograde Response and Other Pathways of Interorganelle Communication in Yeast Replicative Aging

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Abstract A form of mitochondria-to-nucleus signaling is known to play a role in determining replicative life span in yeast. This retrograde response is triggered by experimentally-induced mitochondrial dysfunction, but it also is activated during the course of normal replicative aging, allowing yeast to have as long a replicative life span as they do. The components of the retrograde signaling pathway participate in diverse cellular processes such as mitophagy, which appear to be involved in mitochondrial quality control. This plethora of mitochondrial surveillance mechanisms points to the central importance of this organelle in yeast replicative aging. Additional pathways that monitor mitochondrial status that do not apparently involve the retrograde response machinery also play a role. A unifying theme is the involvement of the target of rapamycin (TOR) in both these additional pathways and in the retrograde response. The involvement of TOR brings another large family of signaling events into juxtaposition. Ceramide synthesis is regulated by TOR opening up the potential for coordination of mitochondrial status with a wide array of additional cellular processes. The retrograde response lies at the nexus of metabolic regulation, stress resistance, chromatin-dependent gene regulation, and genome stability. In its metabolic outputs, it is related to calorie restriction, which may be the result of the involvement of TOR. Retrograde response-like processes have been identified in systems other than yeast, including mammalian cells. The retrograde response is a prototypical pathway of interorganelle communication. Other such phenomena are emerging, such as the cross-talk between mitochondria and the vacuole, which involves components of the retrograde signaling pathway. The impact of these varied physiological responses on yeast replicative aging remains to be assessed.

Keywords Aging · Longevity · Yeast · Mitochondria · Retrograde response

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Introduction

The yeast *Saccharomyces cerevisiae* has been a useful model for the study of a wide variety of cellular processes. One of these processes is the replicative life span of individual cells. This feature of the yeast life cycle was first clearly described some 50 years ago (Mortimer and Johnston 1959). It entered into the purview of yeast genetics and molecular biology only 20 years ago with the finding that replicative life span can be genetically manipulated (Chen et al. 1990). The rich modern history of the study of yeast replicative aging has been recounted (Jazwinski 2003), and it is updated throughout this book. This chapter focuses on one of its aspects.

The notion of coordination of activities in various cellular compartments is both old and attractive. Most cellular proteins are translated in the cytoplasm from nuclear mRNA transcripts. Thus, it is not surprising that various intracellular and extracellular signals have nuclear gene regulatory effects that appear to be coordinated. The existence of a separate mitochondrial genome presents an additional challenge for gene regulation, because the mitochondrial DNA (mtDNA) in a separate compartment encodes only some of the subunits of the multi-subunit inner membrane complexes involved in oxidative phosphorylation, in addition to mitochondrial ribosomal RNA and transfer RNAs. This challenge is met differently in animal and in yeast cells. Defective transcription of the mtDNA in animal cells results in an apparent compensation that leads to the overproduction of the nuclear transcripts of mitochondrial proteins (Heddi et al. 1993). In yeast, there is a coordinated reduction in these nuclear mRNAs (Poyton and McEwen 1996). This coordinated gene expression has been termed intergenomic signaling (Poyton and McEwen 1996) in yeast.

In addition to the coordinated down-regulation of the genes encoding the oxidative phosphorylation complexes in the inner mitochondrial membrane, there are seemingly unrelated additional nuclear gene regulation changes that occur in the presence of mitochondrial dysfunction. These have been termed the retrograde response (Liu and Butow 2006). Rather than an effect that compensates for the lack of these inner membrane complexes, the retrograde response appears to adapt cells to a defect in the tricarboxylic acid (TCA) cycle (Liu and Butow 2006). This adaptation is not consequential for energy metabolism per se. Instead, it impacts gluconeogenesis and the provision of intermediates for biosynthetic reactions (Jazwinski 1999; Liu and Butow 2006).

Recently, another form of mitochondria-to-nucleus signaling has been described, called mitochondrial back-signaling (Heeren et al. 2009). This form of communication between the mitochondrion and the nucleus is clearly distinct from both the intergenomic signaling and the retrograde response, described above. There are, however, some intriguing commonalities among all three forms of mitochondria-to-nucleus signaling. In addition, cross-talk between the mitochondrion and the vacuole has also been shown (Chen et al. 2010) and again possesses features in common with mitochondria-to-nucleus signaling. Thus, it is appropriate to discuss the various pathways associated with cellular compartment coordination together as manifestations of interorganelle communication.

Mitochondrial Dysfunction and Nuclear Gene Expression

In 1987, an accumulation of unusual RNA species in rho^{-} or rho^{0} (mtDNA-deficient) cells of *S. cerevisiae* was described (Parikh et al. 1987). The identity of the mRNA component of these RNA was gradually enumerated, and the transcript of the *CIT2*, peroxisomal citrate synthase-encoding gene, became a diagnostic for this gene regulatory response called retrograde signaling. The nuclear gene expression changes were found to be extensive in a genome-wide survey (Epstein et al. 2001; Traven et al. 2001). The genes induced were metabolic and stress response genes. They encoded proteins destined for the cytoplasm, the mitochondrion, and the peroxisome. Certain electron transport chain inhibitors and uncouplers, such as antimycin A and oligomycin were also effective in activating retrograde signaling; however, the effects were nuanced (Epstein et al. 2001).

Three genes were shown to transduce the signal generated by the defective mitochondria, *RTG1*, *RTG2*, and *RTG3* (Liao and Butow 1993; Jia et al. 1997; Rothermel et al. 1997; Sekito et al. 2000; Liu et al. 2003). Rtg1 and Rtg3 were shown to constitute a heterodimeric, basic helix-loop-helix, leucine zipper transcription factor, whose translocation from the cytoplasm to the nucleus activated retrograde response target genes (Fig. 4.1), such as *CIT2* (Rothermel et al. 1995, 1997; Sekito et al. 2000). The Rtg1-Rtg3 transcription factor binds to the sequence GTCAC (R box) in the promoter of its target genes (Liao and Butow 1993; Jia et al. 1997). The translocation of Rtg1-Rtg3 required Rtg2, which altered the phosphorylation status of Rtg3 (Sekito et al. 2000). Rtg2 has an ATP-binding domain that is found in actin/Hsp70/sugar kinase superfamily members (Koonin 1994). Of the numerous gene expression changes found in rho^{0} yeast cells, only a portion were sensitive to deletion of *RTG2*, and of these *RTG2*-dependent genes only a portion were dependent on *RTG3* (Epstein et al. 2001). Thus, the gene expression changes seen in rho^{0} cells are not all the result of classic retrograde signaling, and Rtg2 may participate in a pathway(s) in which Rtg3 does not. Rtg2 could also regulate some of the same genes as Rtg3 but acting in parallel or independently of Rtg3. This and other observations raise the notion of more than one retrograde signaling pathway (Liu and Butow 2006). The retrograde response has been characterized as a gene-regulatory phenomenon. However, metabolic changes that are consistent with the gene-regulatory events have been identified in cells in which it is induced (Liao et al. 1991; Small et al. 1995).

A key element in retrograde regulation is the interaction between Rtg2 and Mks1 (Sekito et al. 2002; Liu et al. 2003; Dilova et al. 2004). *MKS1* is a negative regulator of the retrograde response (Dilova et al. 2002; Sekito et al. 2002; Tate et al. 2002). It promotes phosphorylation of Rtg3, preventing its translocation to the nucleus (Sekito et al. 2002; Dilova et al. 2004). Mks1 reversibly binds Rtg2, which is one of the means by which Rtg2 exerts a positive effect on the retrograde response (Liu et al. 2003). The action of Mks1 is also inhibited by its ubiquitin-mediated degradation, a process involving Grr1 which is a component of an ubiquitin ligase (Liu et al. 2005). Thus, Grr1 is a positive retrograde response regulator. The 14-3-3 proteins encoded by *BMH1* and *BMH2* are also negative regulators of the retrograde

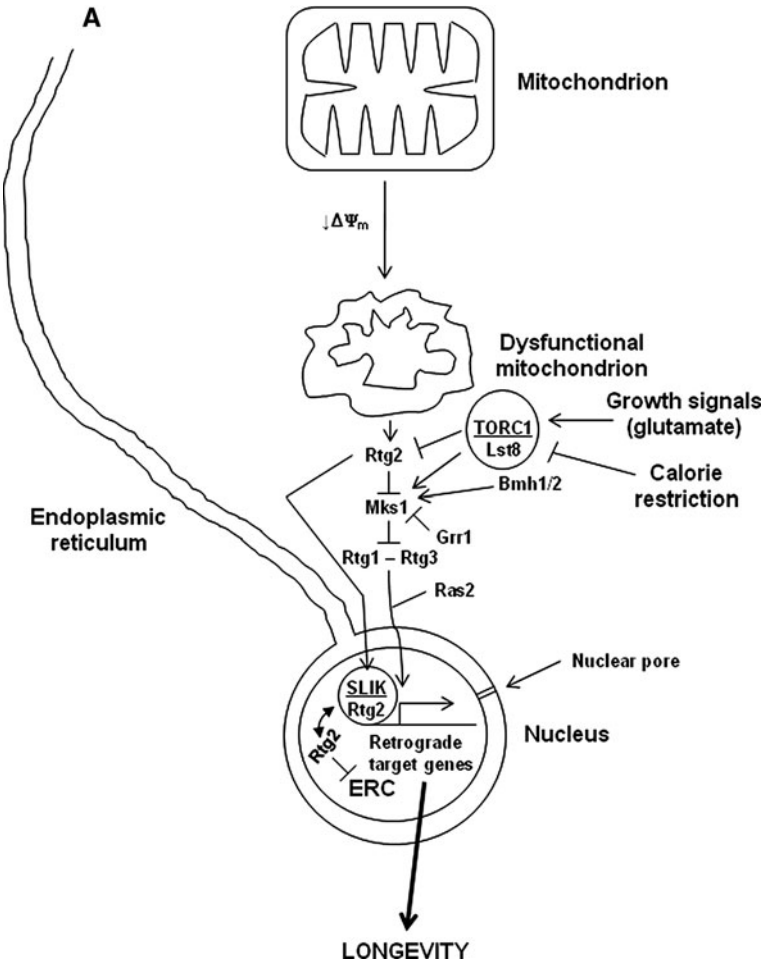

Fig. 4.1 Schematic of the retrograde response and many of the other pathways of interorganelle communication. **a** Retrograde response. **b** Pathways of mitochondrial quality control: cross-talk between mitochondria and the endoplasmic reticulum and the vacuole. TORC1/2, TOR (target of rapamycin) protein kinase complexes 1 and 2; SLIK, SAGA-like transcriptional activator complex; ERC, extrachromosomal ribosomal DNA circles; $\Delta\Psi_m$, mitochondrial membrane potential; Calcineurin, calcium/calmodulin-dependent, serine-threonine protein phosphatase; $M(IP)_2C$, mannosyldiinositolphosphoryl ceramides; Autophagy, degradation of the cell's components within the vacuole; Mitophagy, autophagy selective for degradation of mitochondria; *Arrow*, activation/stimulation; *Blunt-arrow*, inhibition/repression; *Two-headed arrow*, dichotomous functions. Atg1/Atg13, protein kinase complex in the autophagy pathway; Aup1, a protein phosphatase present in the intramembrane space in mitochondria; Bmh1/2, 14-3-3 protein isoforms; Grr1, an E3 ubiquitin ligase; Lag1/Lac1, ceramide synthases that form a complex in the endoplasmic reticulum membrane; Lst8, a WD-repeat protein that is a component of TORC1/2; Mks1, a negative regulator of the Ras2 pathway that forms a complex with either Rtg2 or Bmh1/2; Phb1/2, protein chaperones located in the inner mitochondrial membrane; Ras2, a small G-protein that stimulates adenyl cyclase when bound to GTP and also operates in an adenyl cyclase-independent pathway; Rtg1/3, subunits of a heterodimeric, helix-loop-helix, leucine zipper transcription factor that binds

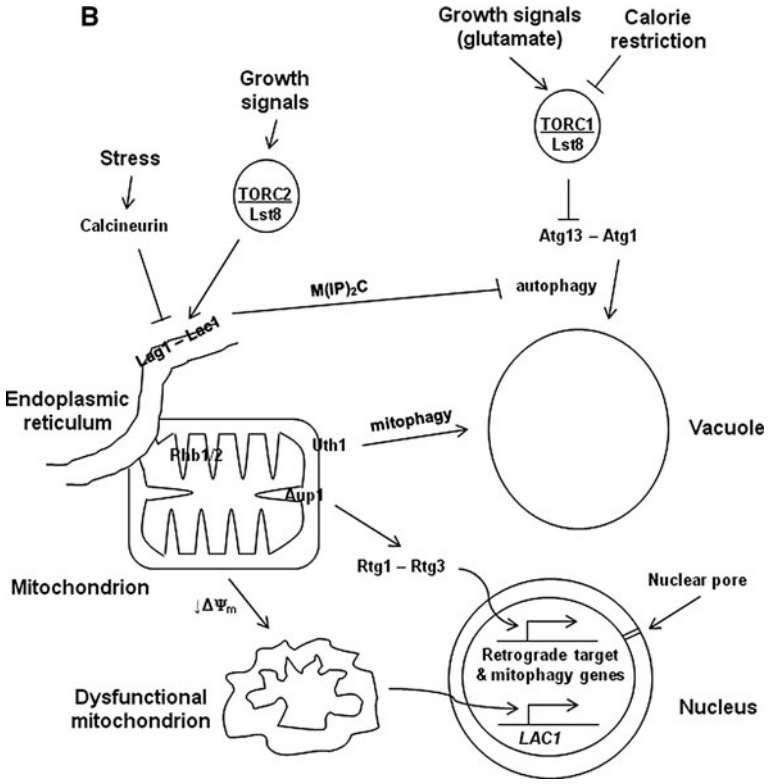

Fig. 4.1 (continued) to the R-box in the promoters of retrograde target genes; Rtg2, a component of SLIK that also plays a role in the translocation of Rtg1–Rtg3 to the nucleus, possesses an ATP-binding domain; Uth1, a mitochondrial outer membrane protein involved in mitochondrial biogenesis and mitophagy. For detailed discussion see text

response; they prevent the ubiquitination mediated by Grr1 that leads to Mks1 degradation (Liu et al. 2003, 2005). Bmh1 and Bmh2 also may bind Rtg3 maintaining it in an inactive state (van Heusden and Steensma 2001). Lst8, a WD-repeat protein, is yet another negative regulator of retrograde signaling, acting both upstream and downstream of Rtg2 (Liu et al. 2001).

The Retrograde Response and Yeast Replicative Aging

Strains entirely lacking or possessing defective mtDNA were shown to have an increased replicative life span in comparison with their rho$^+$ progenitors (Kirchman et al. 1999). This increase in life span was only evident under conditions in which the retrograde response was induced, as comparison of several different yeast strains revealed. In some strains, this induction was repressed by glucose, but growth on raffinose resulted in both de-repression of the retrograde response and extension

of life span. Similar results were obtained with so-called nuclear petites such as *cox4Δ*strains. The replacement of the deficient mitochondria with functioning organelles by cytoduction reversed the effects on life span (Kirchman et al. 1999). The requirement for retrograde signaling for life extension was demonstrated by its abrogation in *rtg2Δ* (Kirchman et al. 1999) and *rtg3Δ* (Borghouts et al. 2004) mutants. The retrograde response also postponed the manifestations of normal yeast aging (Kirchman et al. 1999).

The retrograde response does not function as a digital on-off switch. Instead, its action is more similar to that of a rheostat that titrates the extent of mitochondrial dysfunction with increasing levels of retrograde response activation and replicative life span extension (Jazwinski 2000). This feature suggests that the retrograde response compensates for increasing mitochondrial dysfunction during yeast aging. It has been postulated that the enhanced production of biosynthetic intermediates by the retrograde response is the means by which life span is extended, as the measure of yeast replicative life span is the production of daughter cells which requires biosynthetic precursors. In fact, mitochondria become dysfunctional during replicative aging, as measured by the gradual decline in mitochondrial membrane potential (Lai et al. 2002), and this is coupled to the commensurate activation of the retrograde response during aging (Borghouts et al. 2004). It should be stressed that this activation occurs both in rho^{+} and in rho^{0} cells, and that there is no increase in prevalence of petites during replicative aging. Mitochondria also produce increasing amounts of reactive oxygen species (ROS) during replicative aging (Laun et al. 2001), and they accumulate oxidative damage (Klinger et al. 2010), which may be a cause of accumulating dysfunction with replicative age.

The retrograde response possesses the features of a normal aging mechanism in yeast (Jazwinski 2004). These features are: (1) its activation extends replicative life span; (2) its activation postpones the normal manifestations of aging; (3) it impacts the normal decline in mitochondrial function experienced during yeast aging; and (4) it is progressively activated during aging. Indeed, the retrograde response appears to compensate for the decline in mitochondrial function during aging, allowing yeast to live as long as they do.

Recently, it has been reported that the yeast replicative life span is extended in rho^{0} cells independent of the retrograde response, ascribing this effect to intergenomic signaling (Woo and Poyton 2009). This report confirms earlier findings concerning the retrograde response and replicative life span, while providing evidence that decreased respiration per se does not result in life span extension. This had been concluded earlier based on the literature cited above. Curiously, this report cites the results of the *cox4Δ*, mentioned above, erroneously. The important result described in this paper (Woo and Poyton 2009) is the independence of the life extension in rho^{0} cells of *RTG2* and its dependence on *RTG3*, interpreted as independence of retrograde signaling. However, by-pass of *RTG2* for retrograde signaling has been shown for certain *LST8* and *MKS1* mutants before (Liu et al. 2001; Dilova et al. 2002; Sekito et al. 2002; Tate et al. 2002). Such by-pass mutations could be the source of the lack of requirement for *RTG2*. Clearly, the effects of mitochondrial dysfunction on replicative life span are complex and the pathways induced by

it overlap. Thus, it may not be useful to view them as incompatible but rather it may be more germane to consider them as pathways that constitute a continuum of responses. It is informative in this regard that Rtg2 also plays a role in determining chronological life span in yeast (Barros et al. 2004).

Another, recently described, pathway of signaling from the mitochondrion to the nucleus has been termed mitochondrial back-signaling (Heeren et al. 2009). It is activated upon deletion of the *AFO1/MRPL25* gene which encodes a mitochondrial ribosomal protein. It involves the Tor1 protein kinase and the transcription factor Sfp1. This pathway appears to be independent of the classic retrograde response, though it similarly engages TOR. This claim of independence derives from the fact that in the strain background in which this pathway operates the retrograde response is not activated when the cells are rho^0, as determined by lack of increase in *CIT2* expression and in replicative life span. The studies were carried out in medium containing glucose as the carbon source. The retrograde response was activated when the cells were grown on raffinose rather than glucose, resulting in life span extension, but the effect of *AFO1* deletion was not examined. This mitochondrial back-signaling is described in detail in another chapter.

Convergence of Multiple Signaling Pathways on the Retrograde Response

There is a remarkable degree of cross-talk between the retrograde response and other signaling pathways. *MKS1* was originally identified as a negative regulator of the Ras2-cAMP pathway (Matsuura and Anraku 1993). Given the requirement for *RAS2* for activation of the retrograde response (Kirchman et al. 1999), the negative regulation of this pathway by Mks1 is not surprising. It is not clear, however, that it is the cAMP-dependent pathway through which Ras2 has its permissive effect on the retrograde response, as discussed below.

Lst8 is a component of the TOR complexes TORC1 and TORC2 (Loewith et al. 2002; Wedaman et al. 2003; Reinke et al. 2004). TORC1 contains both Tor1 and Tor2, while TORC2 has only the latter protein (Wullschleger et al. 2006). TORC1 positively regulates anabolic processes, such as ribosome biogenesis and translation, while negatively regulating autophagy (Wullschleger et al. 2006). Rapamycin inhibition of TORC1 signaling activates retrograde response target genes in an Rtg2-dependent manner (Komeili et al. 2000). TOR signaling is also involved in nitrogen catabolite repression (Komeili et al. 2000). Mitochondrial dysfunction activates the retrograde response but not the nitrogen catabolite repression pathway (Giannattasio et al. 2005). Thus, it appears that TOR signaling converges with retrograde signaling at the level of Rtg1-Rtg3 (Giannattasio et al. 2005). On the other hand, Mks1 appears to be a target of TORC1 (Breitkreutz et al. 2010), suggesting that TOR signaling can act downstream of Rtg2. The 14-3-3 proteins Bmh1 and Bmh2 could facilitate or mediate this effect of TOR.

The involvement of Ras2 in the retrograde response deserves some comment. The location of the intersection between Ras signaling and retrograde signaling is

not yet known. Ras2 is a G (GTP-binding)-protein that stimulates adenylate cyclase to synthesize cAMP, which activates protein kinase A (Broek et al. 1985). The existence of a cAMP-independent Ras signaling pathway was adduced in studies of the yeast replicative life span (Sun et al. 1994) and later confirmed in studies of yeast filamentous growth (Mosch et al. 1996). This alternate pathway is a typical MAP kinase pathway. We do not know which of the two Ras pathways is involved in the retrograde response. However, it is known that activation of the cAMP-dependent pathway curtails yeast replicative life span (Sun et al. 1994). Indeed, hyperactivation of this pathway by an activated isoform of Ras2, val19, has a marked deleterious effect on mitochondrial function (Hlavata et al. 2003), and it curtails replicative life span (Pichova et al. 1997). There is an optimal level of Ras signaling that contributes to maximum life extension (Chen et al. 1990).

Retrograde signaling can be considered a response to metabolic stress. This interpretation acquires substance when one considers the dual regulation of TCA cycle genes by the Rtg proteins and the Hap complex (Liu and Butow 1999). The first four genes of the TCA cycle, *CIT1*, *ACO1*, *IDH1* and *IDH2* are under the control of the Hap complex under normal conditions. When mitochondrial function is compromised, control of these genes shifts to the Rtg proteins. The Hap complex controls a broad array of genes important for respiratory growth, among them the four genes listed. The activity of these four genes is vital for anabolic processes in contrast to energy production when mitochondria are otherwise compromised. This derives from the role of this portion of the TCA cycle in the production of biosynthetic intermediates, starting with glutamate. These anabolic processes must continue, and this is guaranteed by the activity of the front end of the TCA cycle and the glyoxylate cycle, both of which are activated in the retrograde response.

The anabolic role of the retrograde response for sustaining production of biosynthetic intermediates is further highlighted by the repressive effects of glutamate (and aspartate) on retrograde signaling (Liu and Butow 2006). Furthermore, mutants in all the Rtg genes are glutamate auxotrophs (Liao and Butow 1993; Jia et al. 1997). Glutamate and its metabolite glutamine are the source of all nitrogen in biosynthetic reactions. Production of cell mass depends on their availability. The retrograde response sustains the front end of the TCA cycle, providing α-ketoglutarate dehydrogenase with substrate to fuel production of glutamate. In fact, activation of the retrograde response results in a dramatic increase in cellular amino acid content (Chen and Kaiser 2002).

Glutamate and glutamine in the growth medium are sensed by the SPS amino acid sensor in the plasma membrane (Forsberg and Ljungdahl 2001). Certain mutations in *LST8* bypass *RTG2* in the retrograde pathway, while others are *RTG2*-dependent in activating the retrograde response (Chen and Kaiser 2003). Mutants in the SPS sensor resemble the latter (Roberg et al. 1997). Thus, the mechanism by which the retrograde response is repressed by glutamate seems clear in general outlines. It is also known that the retrograde response is repressed by glucose (Liao et al. 1991), but the precise mechanism in this case is not clear. This leaves the question of the signal proximal to the dysfunctional mitochondrion that is responsible for activating the retrograde response.

The Retrograde Response and Chromatin-Dependent Gene Regulation

The activation domain of Rtg3, AD1, possesses a motif which in other proteins has been found to activate transcription by recruiting the SAGA histone acetyltransferase complex (Massari et al. 1999). Interestingly, Rtg2 has been shown to be part of the SAGA-like, SLIK complex, which differs from SAGA by containing Rtg2 and only two other known differences in constituent proteins (Pray-Grant et al. 2002). SAGA and SLIK activate distinct, but overlapping sets of genes. SLIK has been shown to bind to the *CIT2* promoter where it helps activate transcription. These results suggest that modification of chromatin structure is important for the activation of at least some retrograde target genes and that SLIK is responsible for these chromatin modifications.

The histone acetyltransferase in both SAGA and SLIK complexes is Gcn5 (Pray-Grant et al. 2002). Deletion of *GCN5*, just like deletion of *RTG2*, prevents life span extension in rho^0 cells and prevents activation of the retrograde response (Kim et al. 2004). This firmly establishes the role of SLIK in retrograde response-mediated replicative life span extension in yeast. There is additional evidence in support of chromatin-dependent gene regulation as a determinant of longevity in yeast. SAGA, and presumably SLIK, largely acts at promoters of genes in response to stress, while TFIID is involved in the transcription of housekeeping genes, though there is overlap (Huisinga and Pugh 2004). The histone deacetylase complexes containing Rpd3 and Hda1 act preferentially at promoters of genes that respond to stress, overlapping with SAGA/SLIK (Huisinga and Pugh 2004). Consistent with this role, deletion of *RPD3* or *HDA1* extends replicative life span (Kim et al. 1999), lending support to the role of chromatin-dependent gene regulation in yeast aging.

Rtg2 and Genome Stability

Rtg2 plays a role in the cytoplasm in triggering translocation of the Rtg1-Rtg3 transcription complex to the nucleus. It also must enter the nucleus to be part of the SLIK complex and to aid in a more direct fashion in the activation of retrograde target genes. However, this is not its only role in the nucleus. Rtg2 is involved in the suppression of the expansion of trinucleotide repeats inserted into the yeast genome (Bhattacharyya et al. 2002). It has also been shown to suppress the production of extrachromosomal ribosomal DNA circles (ERC) (Borghouts et al. 2004). It is a limiting factor in this regard. However, it cannot suppress ERC production when the retrograde response is induced, because there is not enough of the protein or the complexes in which it participates to be able to do so. Thus, as mitochondrial dysfunction accumulates during yeast replicative aging and the retrograde response is concomitantly activated, ERC are expected to accumulate in the cell. This is exactly what happens (Borghouts et al. 2004). This accumulation of ERC is several-fold greater already in young rho^0 cells compared to old rho^+ cells, yet rho^0 cells have a considerably greater replicative life span than rho^+ cells (Borghouts et al. 2004).

Thus, there are activities induced in the retrograde response that must somehow compensate for ERC accumulation. This does not appear to be Rtg2.

Deletion of *GCN5* curtails life span and prevents activation of the retrograde response (Kim et al. 2004). However, it also suppresses ERC production. This has been interpreted to indicate the release of Rtg2 from the SLIK complex, making it available to suppress ERC production. This finding suggests that the intact SLIK complex, containing Rtg2, is not responsible for maintaining genome stability. Rtg2, alone or in a different complex, appears to be involved. The studies summarized thus far indicate that the retrograde response senses metabolic stress. It links this stress to the activation of metabolic and stress response genes, which involves chromatin-dependent gene regulation, and links metabolism with genome stability (Jazwinski 2005). The coordination of these processes plays a role in determining yeast replicative life span.

There is another link between mitochondria and genome stability in yeast. Recently, it has been shown that the inhibition of iron-sulfur cluster biogenesis results in loss of heterozygosity in diploid yeast cells, a form of genome instability (Veatch et al. 2009). This form of genomic instability develops as yeasts progress through their replicative life spans (McMurray and Gottschling 2003). Reduction in mitochondrial membrane potential, but not loss of respiration per se, correlates with this genomic instability. It is associated with defective iron-sulfur cluster formation in cells lacking mtDNA. The results suggest that mitochondrial dysfunction leads to nuclear genome instability by preventing the production of iron-sulfur cluster-containing proteins required for nuclear genome maintenance. This phenomenon appears to be related to a similar one in yeast and in animal cells whose etiology was known but whose precise mechanism has not been clear (Delsite et al. 2003; Rasmussen et al. 2003).

Relationship of Mitochondrial Quality Control to the Retrograde Response

Autophagy and Mitophagy

TOR signaling has surfaced repeatedly in the discussion of retrograde signaling thus far, but this is only part of the story. Inhibition of TORC1 with rapamycin triggers autophagy. It is now known that the effect of TORC1 on autophagy is mediated directly by phosphorylation of Atg13, an essential regulatory component of the Atg1 kinase complex that is necessary for autophagy (Kamada et al. 2010). This core autophagy machinery is involved in mitophagy as well (Bhatia-Kissova and Camougrand 2010). However, mitophagy requires certain protein components to make it selective, and there are mitochondrial proteins that play a role in this process.

UTH1 was initially identified as a gene whose deletion extends yeast replicative life span (Kennedy et al. 1995). Uth1 was subsequently found to be a mitochondrial outer membrane protein involved in mitophagy (Kissova et al. 2004). Lack of

Uth1 does not appear to eliminate mitophagy entirely, but it does alter its efficiency and selectivity (Kissova et al. 2007). Deletion of the *DNM1* gene also reduces but does not eliminate mitophagy (Kanki et al. 2009). Dnm1 is a protein that promotes mitochondrial fission, thus its elimination disturbs the balance in the mitochondrial fission-fusion equilibrium. It has been found that deletion of *DNM1* extends yeast replicative life span (Scheckhuber et al. 2007). These results suggest that elimination of mitochondria through mitophagy may be important at some level to maintain yeast replicative longevity; however, extensive mitophagy may not be conducive to a long life span. The role of the limited mitophagy under these circumstances may be in mitochondrial quality control in removing damaged and/or dysfunctional mitochondria rather than wholesale remodeling of the mitochondrial population as occurs during metabolic adaptations. Whether this is a process that is selective for damaged mitochondria or one that operates by mass action is open to discussion.

Aup1 is a mitochondrial protein phosphatase required for mitophagy in stationary phase in yeast (Journo et al. 2009). Retrograde signaling is defective in *aup1Δ* mutants and deletion of *RTG3* prevents stationary phase mitophagy. Deletion of *AUP1* in turn causes changes in the phosphorylation patterns on Rtg3. Conditions that induce mitophagy also induce the retrograde response in an *AUP1*-dependent fashion. Thus, there appears to be an involvement of retrograde signaling in mitophagy, although the importance of this association for replicative life span is not entirely clear. Spermidine extends yeast replicative life span and it also induces autophagy, but a requirement for autophagy in replicative life span extension in yeast has not been shown (Eisenberg et al. 2009). The same effects of spermidine hold true in the nematode *Caenorhabditis elegans*; however, in this case inhibition of autophagy prevents life span extension by spermidine (Eisenberg et al. 2009). Deletion of *TOR1* also extends yeast replicative life span (Kaeberlein et al. 2005), a situation in which autophagy would be induced. However, again it has not been shown that the induction of autophagy plays a role in the life extension.

Asymmetric Segregation of Mitochondria and Other Structures

Another mechanism of mitochondrial quality control during replicative aging is the maintenance of age asymmetry in the segregation of dysfunctional mitochondria between mother and daughter cells. During normal aging, dysfunctional mitochondria characterized by reduced mitochondrial membrane potential segregate to mother cells (Lai et al. 2002). However, as the mother cells become older, this age asymmetry begins to break down and daughters of older mother cells begin to receive dysfunctional mitochondria during cell division. This is a progressive process, and its acceleration by mutation of *ATP2* results in clonal senescence and the appearance of cells devoid of active mitochondria (Lai et al. 2002). The daughter cells no longer have a full replicative life span. Instead, they are born with the same replicative age as their mothers at the time of birth. As mentioned earlier, the accumulating mitochondrial dysfunction during replicative aging results in a commensurate activation of the retrograde response (Borghouts et al. 2004). Whether

this activation is limited to mother cells only, where the dysfunctional mitochondria tend to accumulate, remains to be determined. The dysfunctional mitochondria possess all of the features of the senescence factor whose action was deduced in earlier studies (Egilmez and Jazwinski 1989). As it turns out, the dysfunctional mitochondria also suffer from oxidative damage to aconitase, which has been used as a marker to confirm the asymmetric segregation of mitochondria between mother and daughter cells (Klinger et al. 2010).

The multi-drug resistance gene *PDR5* is activated in rho^0 cells (Hallstrom and Moye-Rowley 2000; Epstein et al. 2001). The regulation of this gene is complex. One of the pathways that induce *PDR5* expression in rho^0 cells involves Rtg1; however, other mitochondrial defects can activate *PDR5* in an Rtg-independent manner (Hallstrom and Moye-Rowley 2000). *PDR5* belongs to the group of plasma membrane-associated transporters that are in the multi-drug resistance family. These transporter genes are subject to retrograde regulation (Moye-Rowley 2005). During cell division, the daughter cell receives newly-synthesized multi-drug resistance proteins, while the old ones remain in the mother cell where they accumulate damage with replicative age (Eldakak et al. 2010). This is yet another example of age asymmetry. Deletion of multi-drug resistance genes shortens replicative life span, while one extra copy extends it. Multi-drug resistance proteins are important in metabolism, detoxification, and stress resistance. Thus, it is plausible that they have a role in determining replicative life span and are one of the factors contributing to age asymmetry in yeast.

Surveillance of Mitochondrial Quality by Prohibitins

Transcription of the *PHB1* and *PHB2* genes is activated in rho^0 cells (Traven et al. 2001), although this transcription may not be under the control of retrograde signaling. The protein products of these genes, called prohibitins, play a role in mitochondrial integrity, and they interact genetically with mitochondrial inheritance factors (Berger and Yaffe 1998). The prohibitins appear to perform a chaperone function for newly-synthesized mitochondrial membrane proteins protecting the cell from an imbalance in the assembly of inner mitochondrial membrane protein complexes (Nijtmans et al. 2000). It has been suggested that the prohibitins may deliver supernumerary mitochondrial membrane proteins to the inner membrane m-AAA protease for degradation (Kirchman et al. 2003). Imbalances in production of mitochondrial proteins may contribute to mitochondrial dysfunction during replicative aging. This possibility is suggested by the life span curtailment observed on deletion of *PHB1* and/or *PHB2* in rho^0 cells (Kirchman et al. 2003). In cells lacking prohibitins, mitochondria assume unusual morphologies and are not properly segregated to daughter cells, an effect that is accentuated in old mother cells (Piper et al. 2002; Kirchman et al. 2003). These phenotypes are reminiscent of those found in the *atp2* mutant, described above. The deleterious effects of deletion of the prohibitin genes are suppressed by deletion of *RAS2* (Kirchman et al. 2003). Ras2

plays a role in mitochondrial biogenesis by stimulating the synthesis of the respiratory component of the mitochondrial proteome (Dejean et al. 2002; Kirchman et al. 2003). Thus, deletion of *RAS2* would contribute to amelioration of the imbalance in production of mitochondrial proteins in the absence of prohibitins in rho^0 cells.

The importance of prohibitins in surveillance of mitochondrial quality has resurfaced recently (Wang et al. 2008). The deleterious effect of prohibitin mutants in rho^0 cells was confirmed. Interestingly, this phenotype was suppressed by deletion of *TOR1* or *SCH9*, among others, whose products normally stimulate mitochondrial biogenesis. The conclusion reached is identical to that based on the effect of the *RAS2* deletion on prohibitin mutants (Kirchman et al. 2003). A central role of mitochondrial membrane potential was adduced by examination of a mutant in the adenine nucleotide translocase Aac2 (Wang et al. 2008), which induced an aging-dependent mitochondrial degeneration similar to the *atp2* mutant (Lai et al. 2002) and the prohibitin mutants (Piper et al. 2002; Kirchman et al. 2003). As pointed out earlier (Lai et al. 2002), the loss of the respiratory chain is readily tolerated as long as the adenine nucleotide translocator and the F_1-ATPase cooperate to maintain mitochondrial membrane potential. Unlike the situation in the rho^0 cell, it is not clear exactly where the deficits appear during yeast replicative aging: the respiratory chain, the translocator, the ATPase, or combinations thereof.

The Mitochondrial Signal for the Retrograde Response

The nature of the signal generated by dysfunctional mitochondria that triggers the retrograde response is not known at present. Originally, it was thought that the only signal was the repressive effect of glutamate or aspartate in the growth medium. However, this effect only operates in the presence of mitochondrial dysfunction, suggesting an additional, positive signal to be in play. The mitochondrial dysfunction does not necessarily involve loss of or damage to mtDNA or the appearance of a petite phenotype more generally (Borghouts et al. 2004).

A priori there seem to be three possible signals of mitochondrial origin that could trigger the retrograde response. The first is a loss or reduction in the capacity to produce ATP by mitochondria. In this mechanism, Rtg2 functions as an ATP sensor, which is consistent with the requirement of its ATP-binding domain for its function (Liu and Butow 2006). In the presence of ATP, Rtg2 would be released from Mks1 which would inhibit retrograde signaling. This is a plausible mechanism; however, fermentative production of ATP sustains rapid growth of yeast cells and even more rapid division of *petite* mother cells than *grande* cells (Kirchman et al. 1999).

A second signal could be the ROS generated by dysfunctional mitochondria. This possibility has not been evaluated. ROS generation increases with replicative age (Laun et al. 2001), so this is a viable alternative. A possible ROS sensor in yeast mitochondria is the abundant protein aconitase, which has oxygen sensitive iron-sulfur complexes. In addition to its role in the TCA cycle, aconitase is an essential component of the mtDNA nucleoid (Chen et al. 2005). Its role in nucleoid maintenance is independent of its catalytic activity in the TCA cycle. Constitutively

expressed aconitase suppresses mtDNA instability even in the absence of Abf2, known for its essential role in mtDNA packaging and maintenance. The aconitase gene is activated in the retrograde response, explaining the stability of mtDNA during replicative aging during which the retrograde response is activated (Borghouts et al. 2004).

A third mechanism is related to the loss of mitochondrial membrane potential that accumulates with replicative age in yeast and is associated with activation of the retrograde response (Lai et al. 2002; Borghouts et al. 2004). Here, the mitochondrial membrane potential itself would be the signal. There is some evidence in support of this mechanism. It has been shown that a lowering of mitochondrial membrane potential with the uncoupler dinitrophenol (10 nM) extends replicative life span (Barros et al. 2004). On the other hand, carbonyl cyanide 3-chlorophenyl hydrazone (CCCP) shortens replicative life span on solid medium containing glucose at concentrations (5 μM) that have a moderate effect on growth on plates containing a non-fermentable carbon source (Stockl et al. 2007). Many of the transporters and other mitochondrial membrane proteins rely on the membrane potential for their activity, making mitochondrial membrane potential a suitable signal that can be relayed to the cytoplasm and the nucleus. A decrease in mitochondrial membrane potential, depending on its magnitude, could result in either a decrease or an increase in ROS production. Thus, ROS could be in the retrograde signaling pathway initiated by the decrease in mitochondrial membrane potential.

Ceramide Signaling and the Retrograde Response

The yeast *LAG1* gene was the first longevity determining gene cloned as such from any species (D'Mello et al. 1994). It encodes a ceramide synthase (Guillas et al. 2001; Schorling et al. 2001). In addition to being a precursor for (mannosyl)inositol phosphorylceramides which are the equivalent of mammalian sphingolipids, ceramide plays an important signaling function in cell cycle control and stress resistance, as well as other cellular processes (Dickson 2010). This makes ceramide mechanistically attractive as a longevity modulator. The finely-tuned nature of the response of replicative life span to *LAG1* expression levels suggests the importance of the mutual balance between growth and stress signals in yeast longevity (Jiang et al. 2004).

The TOR pathway intersects the retrograde response, autophagy, and other pathways discussed in this chapter. It also regulates ceramide synthase activity (Aronova et al. 2008), although it does so uniquely via the TORC2 complex in response to growth signals. This action is mediated by the Ypk2 protein kinase, a member of the AGC family of kinases that includes Ypk1 and Sch9. The phosphatidylinositol-dependent kinase orthologs Pkh1 and Pkh2 also activate Ypk2 (Kamada et al. 2005). Stress has the opposite effect to growth signals. The calcium/calmodulin-dependent protein phosphatase calcineurin counteracts the ceramide synthase stimulation by TORC2, mediating the effects of stress (Aronova et al. 2008). Interestingly, the downstream products of the ceramide-sphingolipid

biosynthetic pathway, the mannosylinositol phosphorylceramides negatively regulate autophagy in yeast (Thevissen et al. 2010). This is consistent with the role of TORC2, acting among others through ceramide synthase, in propagating growth signals.

Ceramide synthesis and multi-drug resistance are also coordinated in yeast, thrusting the former into closer juxtaposition to the retrograde response. In addition to the Lag1-ceramide synthase, yeasts possess another ceramide synthase encoded by the homologous gene *LAC1* (Jiang et al. 1998; Guillas et al. 2001; Schorling et al. 2001). The Lac1 ceramide synthase is under the same transcriptional regulation as *PDR5* (Kolaczkowski et al. 2004).

Metabolic-Regulation Overlap Between Calorie Restriction and the Retrograde Response

An analysis of the regulatory changes in genes of central metabolism during nutrient limitation has been carried out recently (Wang et al. 2010). Unlike earlier studies in yeast, this analysis utilized growth in a chemostat under conditions of metabolic equilibrium, eliminating the confusion of shifting metabolic requirements during batch culture. The conditions used resemble closely those encountered by individual yeast cells on agar plates during measurements of replicative life span. Both limitation of glucose and limitation of non-essential amino acids were compared in defined culture media. These conditions were shown previously to extend yeast replicative life span (Jiang et al. 2000). Extensive gene expression changes were detected, many of which were common to both nutrient limitation conditions.

Strikingly, the common changes in gene expression observed during both glucose and non-essential amino acid limitation included the induction of TCA cycle and retrograde response target genes (Wang et al. 2010). The former could be ascribed to the upregulation of *HAP4* expression. It is possible that the latter was due to inactivation of TOR pathway signaling, which could in turn result in activation of retrograde signaling. However, this was not due to decreased expression of *TOR1* or *TOR2* (Wang et al. 2010). If retrograde signaling was involved, it was not mediated by *RTG2* and/or *RTG3*, which are not needed for life span extension by these nutrient limitation regimes (Jiang et al. 2000). There is still much to be learned about the relationship of TOR signaling to the retrograde response. Rapamycin, an inhibitor of TORC1 that mimics nitrogen starvation, can cause repression of genes involved in cell growth. Cells that only possess the SLIK complex and no SAGA show increased resistance to these effects of rapamycin (Spedale et al. 2010). Because SLIK has Rtg2 as an obligatory component, a requirement for Rtg2 in the response to unavailability of non-essential amino acids would be indicated. The situation may be more complex than that because other nitrogen sources were available in the medium. In any case, it appears that TOR1 and nutrient limitation operate on the same pathway in determining yeast replicative life span (Kaeberlein et al. 2005).

ATO3 is a gene that encodes an ammonia outward transporter in the plasma membrane (Guaragnella and Butow 2003). This gene is markedly induced in rho^0 cells

(Epstein et al. 2001; Guaragnella and Butow 2003). Expression of *ATO3* is regulated by the transcription factor Gcn4, a transcriptional activator in the general amino acid control pathway (Guaragnella and Butow 2003). It has been shown that Gcn4 is required for replicative life span extension both by nutrient limitation and by deletion of *TOR1* (Steffen et al. 2008). This represents yet another intersection between retrograde signaling, TOR, and nutrient limitation. Ammonia export by Ato3 is an essential component of intercolony communication in yeast (Palkova et al. 2002), playing a role in colony morphology and differentiation. It will be of great interest to examine in more detail the interplay of the various physiological responses enumerated here and their significance for aging.

A Retrograde Response in Other Model Systems?

The existence of a retrograde response that is involved in life extension in *C. elegans* was proposed some time ago (Kirchman et al. 1999). This proposal was based on the upregulation of the glyoxylate cycle in old worms and in mutants that displayed extended longevity (Vanfleteren and De Vreese 1995). Subsequently, mitochondrial mutants were isolated that had an extended life span by targeting various nuclear genes encoding mitochondrial proteins with RNAi (Dillin et al. 2002; Lee et al. 2003). Recently, a systematic analysis has led to the conclusion that indeed a retrograde response functions in *C. elegans* to extend the worm's longevity (Cristina et al. 2009).

Normal human diploid fibroblasts in culture undergo a process of cell senescence that is characterized by the accumulation of oxidative damage to telomeric DNA and limitation of population doubling. It has been shown that treatment of these cells with the uncoupler dinitrophenol to lower mitochondrial membrane potential extends their replicative life span (Passos et al. 2007). This is accompanied by gene expression changes. In certain ways, this is very similar to the retrograde response in yeast.

The patterns of gene expression changes resulting from elimination of mtDNA or inhibition of the electron transport chain have been examined in a variety of mammalian tissue culture cells (Butow and Avadhani 2004). The results have not been very consistent from one cell type to another. A systematic comparison of the gene expression difference between respiration competent and functionally petite derivatives deficient in mtDNA from three different human cell types has been carried out (Miceli and Jazwinski 2005). Most gene expression changes were peculiar to an individual cell type. However, a limited set of the differences were common to all three cell types. The metabolic adjustments suggested by these common gene expression changes appear to be a compensation for the lack of respiration by stimulating glycolysis, enhanced protection from oxidative stress, and compensation for potential genome instability. As expected, glycolysis was stimulated in these cells (Miceli and Jazwinski 2005).

One of the genes induced in these human rho^0 cells was *MYC* (Miceli and Jazwinski 2005), which could function as the retrograde response transcription

factor, because it plays a role in the regulation of glycolytic genes (Collier et al. 2003). However, other studies have pointed to the potential role of NFκB as this retrograde transcription factor (Srinivasan et al. 2010). An extensive discussion of these issues has recently been published (Srinivasan et al. 2010).

Mitochondria-Vacuole and Mitochondria-Endoplasmic Reticulum Cross-Talk

The phospholipid cardiolipin is only found in the inner mitochondrial membrane. Mutation of the cardiolipin synthase gene *CRD1* paradoxically results in severe vacuolar phenotypes in yeast (Chen et al. 2008). These can be suppressed by deletion of *RTG2* but not *RTG3*. This suggests that some form of retrograde signaling from the mitochondrion has a drastic effect on vacuolar structure and function. The results suggest that Rtg2 as a component of the SLIK complex may activate nuclear genes that lead to the vacuolar phenotypes that are deleterious to the cell independently of the Rtg1-Rtg3 transcription factor. On the other hand, rho^0 *crd1Δ* cells show a typical retrograde response that requires both *RTG2* and *RTG3* (Chen et al. 2010). These cells exhibit slow growth and a division delay. One may conclude from these studies that the role of the vacuole may be more than simply a component of the autophagy apparatus.

Another form of interorganelle communication that has been recently discerned involves a physical junction between the mitochondrion and the endoplasmic reticulum (Kornmann and Walter 2010). Mutants in the components of this tether have a broad spectrum of phenotypic consequences. This has led to the proposal that these junctions play essential roles in many aspects of mitochondrial biogenesis and function. It is worth mentioning that the Lag1- and Lac1-ceramide synthases are located in the endoplasmic reticulum membrane. Thus, some of the functional outcomes of the association of mitochondria and endoplasmic reticulum could be elicited through ceramide signaling.

Final Thoughts

The retrograde response as a pathway of mitochondria-to-nucleus signaling is sometimes contrasted with other such pathways based on the requirement for the retrograde response (Rtg) genes. However, the complexity of retrograde signaling and the differential roles of these Rtg genes on nuclear gene expression had been noted very early, and the term RTG-independent retrograde response was coined to designate these other pathways (Epstein et al. 2001; Butow and Avadhani 2004). The retrograde response does not require a petite mutation or pharmacological inhibition of electron transport to be induced, as discussed above. Several cellular processes have been described in this chapter, which involve one or another of the Rtg genes. One way or the other, mitochondria seem to be involved in these processes even if

the activation of the classic retrograde target genes has not been demonstrated in each case. With the emergence of novel pathways, such as the cross-talk between mitochondria and vacuoles, it may be more relevant to recognize all of these processes in aggregate as interorganelle communication. One thread that remains in common almost without exception is the involvement of TOR signaling in some capacity. Given its role in the calorie restriction paradigm, TOR may be the most universal of the pathways that impinge on the replicative life span in yeast.

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Chapter 5
Chronological Aging in *Saccharomyces cerevisiae*

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Abstract The two paradigms to study aging in *Saccharomyces cerevisiae* are the chronological life span (CLS) and the replicative life span (RLS). The chronological life span is a measure of the mean and maximum survival time of non-dividing yeast populations while the replicative life span is based on the mean and maximum number of daughter cells generated by an individual mother cell before cell division stops irreversibly. Here we review the principal discoveries associated with yeast chronological aging and how they are contributing to the understanding of the aging process and of the molecular mechanisms that may lead to healthy aging in mammals. We will focus on the mechanisms of life span regulation by the Tor/Sch9 and the Ras/adenylate cyclase/PKA pathways with particular emphasis on those implicating age-dependent oxidative stress and DNA damage/repair.

Keywords Chronological aging · TOR (target of rapamycin) · RAS · Adaptive regrowth · Caloric restriction

Yeast as a Model for Aging Research: Two Aging Paradigms

Together with the roundworm *Caenorhabditis elegans*, yeast represents one of the simplest and most widely adopted model organisms to study aging. Although *S. cerevisiae* is by far the yeast most commonly used by gerontologists, the fission yeast *Schizosaccharomyces pombe* has recently joined the group of the simple model systems for aging research (Roux et al. 2006, 2009). While the life span of *S. cerevisiae* is measured either by monitoring the replicative potential of individual mother cells (replicative life span, RLS) or by determining the mean and maximum

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survival time of populations of non-dividing cells (chronological life span, CLS), the life span of *S. pombe* is mostly measured chronologically (Roux et al. 2006, 2009). This is due to the fact that, although subtle visual differences can be observed between two cells generated by fission, it is challenging to base a survival assay on them (Barker and Walmsley 1999). Conversely, the obvious size difference between mother and daughter cells in the budding yeast *S. cerevisiae* allows the counting of the total number of daughter cells generated by individual mother cells before cell division halts (RLS) (Steinkraus et al. 2008).

Replicative Life Span

A method to measure RLS was originally set up by R Mortimer and JR Johnston 50 years ago (Mortimer 1959) but it was not until 30 years later that the RLS became widely studied for aging research (Egilmez and Jazwinski 1989; Kennedy et al. 1994). Among the principal outcomes associated with the RLS is the identification of the extrachromosomal ribosomal DNA circles (ERCs) as toxic species, whose accumulation causes yeast replicative senescence (Sinclair and Guarente 1997) and of several genes implicated in life span regulation such as *RTG2, LAG1*, and *SIR2* (Steinkraus et al. 2008; D'Mello et al. 1994; Borghouts et al. 2004). The latter, *SIR2*, which encodes for a NAD-dependent histone deacetylase highly conserved in higher eukaryotes, has become one of the most studied gerontogenes (Kaeberlein et al. 1999; Longo and Kennedy 2006; Longo 2009). In yeast, the activity of Sir2 is known to prevent replicative senescence by reducing recombination between rDNA repeats and consequently the formation of ERCs (Kaeberlein et al. 1999). However, recent evidence has demonstrated that Sir2 functions to extend the RLS also independently of ERCs formation by maintaining low levels of histone 4 lysine 16 acetylation and consequently inducing silencing at the telomeres and subtelomeric regions (Dang et al. 2009). The activity of the closest Sir2 homologues has been shown to promote life span extension in both *C. elegans* and *Drosophila melanogaster* (Tissenbaum and Guarente 2001; Rogina and Helfand 2004). Since ERCs accumulation occurs only in *S. cerevisiae*, it will be of interest to establish whether the link between Sir2, telomeric and subtelomeric DNA silencing, and life span is also found in higher eukaryotes.

Chronological Life Span

As an alternative to the replicative life span measurement, a method to monitor the chronological life span was developed in the nineties and is currently used by several laboratories worldwide (Fabrizio and Longo 2003; Longo et al. 1996). One of the advantages of the chronological life span is that it measures survival of non-dividing cells, and it can serve as both a system to model aging in post-mitotic mammalian cells but also as a very simple model for organismal aging. From a technical point

of view, monitoring the CLS is extremely simple, does not require micromanipulation, and is generally based on the use of cultures of millions of yeast, which facilitates the screen of longevity mutants and allows the performance of a wide range of genetics, genomics, and biochemistry assays (Fabrizio and Longo 2007; Fabrizio et al. 2005b). In a standard CLS experiment yeast are grown in synthetic complete medium (SDC) until nutrient depletion promotes cell cycle arrest. The majority of cells stops dividing within 2–3 days from the starting of the culture and viability is usually assayed by colony forming units (CFUs) measurement beginning on day 3 until survival reaches 1–5% of the day 3 CFUs (Fabrizio and Longo 2007). In analogy with post-diauxic phase cultures (see section "The TOR/Sch9 Pathway"), chronologically aging populations are characterized by the constant presence of a small fraction of budded cells (~3–8% depending on the genetic backgrounds). Several lines of evidence suggest that these budded cells may be improperly arrested in S/G2 (starvation normally induces G1-arrest) (Weinberger et al. 2007; Allen et al. 2006). However, it is possible that a very small percentage of them may be dividing although, under the standard conditions described above, the low pH (3.5) would cause a very slow growth (Fabrizio et al. 2004a). Therefore, under these conditions, cell division is very unlikely to affect the CLS measurements. By contrast, cell division can occur and it is easily detected (population size raises up to 100 times) in chronologically aging cultures after the majority of the cells has died (Fabrizio et al. 2004a). This growth of the population, defined as "adaptive regrowth", appears to be due to mutations or possibly epigenetic changes that allow yeast to reenter the cell cycle by consuming the nutrients released by dead cells and it shares some similarities with the bacterial Growth Advantage in Stationary Phase (GASP) phenotype (Zinser and Kolter 2004; Zambrano et al. 1993; Zambrano and Kolter 1996). The latter was originally described in stationary phase cultures of *Escherichia coli* grown in rich medium (LB), whose viability, after a rapid decline, remains stable for extended periods of time, reflecting cycles of death and regrowth in the populations rather than extended survival (Zambrano et al. 1993). In analogy with adaptive regrowth, the GASP phenotype also arises after the acquisition of mutations that trigger cell division by promoting the catabolism of nutrients released by dead microorganisms (Zinser and Kolter 2004).

For aging studies the period in which no cell division occurs represents the life span. In yeast this phase is characterized by a gradual increase of mortality rates and it can last up to a few weeks depending on the yeast strain (Fabrizio and Longo 2003). On the contrary, in *E. coli* ~99% of the culture loses viability within 2–3 days, thus providing a more limited time window to observe age-related changes.

Links Between Replicative and Chronological Life Span

The degree of overlap between the mechanisms that control CLS and RLS is only partially understood. We have known for several years that certain genes that increase CLS can actually reduce RLS, possibly by affecting growth and not aging

(Fabrizio et al. 2004b) but we have also known that chronological aging can reduce the RLS of mother cells (Ashrafi et al. 1999), indicating that distinct but overlapping mechanisms are regulating the two aging paradigms. In fact, the two major yeast pro-aging pathways, TOR/Sch9 and Ras/adenylate cyclase/PKA (see next section), promote aging and early cell death in both CLS and RLS paradigms. CLS extension induced by lowering the activity of either of the two pro-aging pathways requires the activity of protein kinase Rim15 and stress resistance transcription factors Msn2/4 and Gis1 (Fabrizio et al. 2001, 2003; Wei et al. 2008). Msn2/4 and Rim15, however, limit the RLS extension of a mutant with reduced Ras/PKA activity and overexpression of Msn2 shortens the RLS of wild type yeast (Fabrizio et al. 2004b). Analogously, while mitochondrial superoxide dismutase (Sod2) is required for CLS extension, its overexpression shortens RLS (Fabrizio et al. 2003, 2004b). Intriguingly, Msn2/4 were shown to mediate the RLS extension associated with decreased TOR signaling (Medvedik et al. 2007). The key players in prolonging the RLS in TOR-deficient yeast were reported to be members of the Sir2 family (sirtuins) (Medvedik et al. 2007). Consistently, an additional copy of *SIR2* prolongs RLS in wild type yeast (Kaeberlein et al. 1999; Medvedik et al. 2007). The activity of Sir2, nevertheless, reduces significantly the CLS of yeast lacking the serine/threonine kinase Sch9, which live 3-fold longer than wild type but 5-fold longer in a *sir2Δ* context (Fabrizio et al. 2005a). Taken together, our current knowledge suggests that the relationship between CLS and RLS is complex and that several life span determinants such as Sir2 and Msn2/4 may play opposite roles in controlling the two life span paradigms. The most likely possibility is that the pro-aging TOR/Sch9 and Ras/adenylate cyclase/PKA pathways and the downstream stress resistance transcription factors affect aging by similar mechanisms in both the RLS and CLS. However, because RLS is based on cell division and since protective enzymes and stress-resistance transcription factors can negatively affect cell division, replicatively aging cells in which protective systems are activated can stop dividing before they are severely damaged or dead (Fabrizio et al. 2004b). A comprehensive analysis of the genes regulating either CLS, RLS, or both based on a comparison between partial genome-wide datasets relative to screens for CLS- and RLS-regulatory genes has been published by Laun et al. According to this study, only a handful of genes prolongs both RLS and CLS (Laun et al. 2006). These results may depend in part on the negative role of anti-chronological aging genes on cells division discussed above but also on the threshold selected to determine whether a mutant is long-lived or not. Notably, genome-wide life span studies often yield false positive/negative results (Hansen et al. 2005; Powers et al. 2006) and the long/short-lived phenotype of mutants identified by genome-wide screen must be confirmed by measuring the life span of each mutant individually. Thus, the results of the analyses based on genome-wide data might change substantially once all the individual validation experiments are performed. Nevertheless, these analyses can be informative. For example, the consistent reduction of both life spans caused by the deletion of numerous mitochondrial genes reported by Laun et al underscores the importance of functional mitochondria for normal life span (Laun et al. 2006).

Hereafter we will review the major findings associated with yeast chronological aging with particular emphasis on those that contributed to discover the evolutionary conserved longevity pathways. We will describe the similarities between these pathways in different species. We will also discuss how CLS is contributing not only to the understanding of the genetics of aging but also to elucidate the key modifications occurring in senescent cells that might be relevant to the onset of age-related diseases such as cancer.

Conserved Life Span-Regulatory Pathways

Simple model organisms and *S. cerevisiae* in particular have been instrumental to the study of fundamental cellular processes, e.g. cell cycle regulation and DNA transcription, which have not diverged dramatically throughout evolution. Aging appears to be the new process that this unicellular eukaryote helps us understand. In fact, several conserved proteins/pathways were found to mediate longevity in phylogenetically distant organisms and work done in the last 15 years supports the theory that life span regulation emerged early in evolution, most likely in ancestral microorganisms to overcome periods of starvation (see section "The TOR/Sch9 Pathway") (Longo et al. 2005; Kenyon 2005). The CLS of *S. cerevisiae* seems to be particularly well suited to identify conserved life span mediators because life span measurements are performed in conditions similar to those under which the longevity regulatory pathways have evolved (Longo et al. 2005). In fact, in the wild, microorganisms alternate rare periods in which they grow rapidly on glucose and other nutrients (e.g. on grapes) to others, usually lengthy ones, in which they survive under starvation conditions (Fabrizio and Longo 2003). To mimic this natural environment, CLS is monitored in populations of yeast grown in SDC medium containing a limited amount of nutrients (section "Chronological Life Span"). Once glucose and other nutrients are exhausted, cell division stops and yeast can survive for extended periods of time in different metabolic states (Fabrizio and Longo 2003).

The TOR/Sch9 Pathway

Conclusive evidence for the conservation of life span regulation throughout evolution was obtained in 2001. Prior to then, the insulin/insulin-like growth factor-like signaling (IIS) pathway had been demonstrated to control life span in *C. elegans*. In fact, lowering the activity of DAF-2, homolog of both human insulin and IGF-I receptors, was known to double worm life span and numerous downstream mediators activated or inhibited by DAF-2 activity had been identified as part of the IIS cascade (Kimura et al. 1997). Among these, the phosphoinositide-3-kinase AGE-1, the serine/threonine kinases AKT-1/2, PDK-1, SGK-1, and the fork-head transcription factor DAF-16 (Friedman and Johnson 1988; Morris et al. 1996; Paradis and Ruvkun 1998; Paradis et al. 1999; Hertweck et al. 2004). The latter had

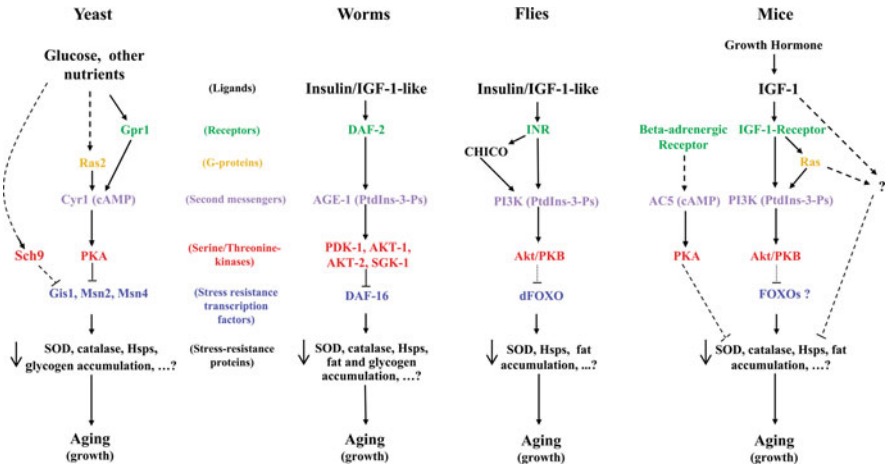

Fig. 5.1 Conserved regulation of the life span-regulatory pathways. In yeast, worms, and flies the activation of the pro-growth nutrient-sensing/IIS pathways inhibits the activity of stress resistance transcription factors (Gis1, Msn2/4, DAF-16, dFOXO), reduces cell protection and accumulation of fat and/or glycogen, and promotes aging. Mutations that reduce the activity of these pathways prolong life span. In yeast and worms this longevity extension requires the activation of a stress response dependent on Gis1/Msn2/4 and Daf-16, which lead to the activation of anti-oxidant enzymes and heat-shock proteins. In flies overexpression of dFOXO extends lifespan most likely via the activation of stress response analogous to that of yeast and worms. In mice, reduction on the IIS by mutation of the IGF-1 receptor gene or by lowering growth hormone (GH) synthesis promotes longevity and stress resistance possibly by activating the transcription factors FoxOs. GH might also function to promote aging in part independently of IGF-1 signaling. Dampening the beta-adrenergic signaling by deleting the adenylate cyclase 5 gene (*AC5*) or reducing the activity of PKA also extends mice life span

been shown to play a key role in antagonizing DAF-2 activity and mediating the longevity extension observed in the *daf-2* mutants (Ogg et al. 1997; Lin et al. 1997) (Fig. 5.1).

In 2001 three articles demonstrated that the role of IIS in life span regulation was evolutionary conserved. In fact, on the one hand, it was found that lowering the activity of Sch9, a protein kinase involved in cell growth and cell cycle regulation in response to nutrients, extended CLS up to 3-fold (Fabrizio et al. 2001) and, on the other hand, it was shown how reduced IIS promoted longevity in *Drosophila* (Clancy et al. 2001; Tatar et al. 2001). Sch9 shares a high degree of homology with Akt and S6 kinase (S6K) in higher eukaryotes and numerous proteins activated in Sch9-deficient yeast are also activated in the *daf-2* mutants and in long-lived flies, e.g. superoxide dismutase and heat-shock proteins (Longo and Finch 2003) (Fig. 5.1). Taken together, the novel results obtained in yeast combined with those from worms and flies, indicated that life span extension can be obtained through the inactivation of nutrient signaling and pro-growth pathways and the consequent activation of a "survival program" characterized by increased cellular protection (Longo and Finch 2003).

In most long-lived mutants, longevity is also associated with the accumulation of either reserve carbohydrate (glycogen), fat, or both (Longo and Fabrizio 2002) (Fig. 5.1). Some of the phenotypes observed in the long-lived mutants, e.g. fat accumulation, are shared by both worms in a state of developmental arrest (dauer larva) and flies in adult reproductive diapauses (McElwee et al. 2006; Tatar and Yin 2001). Both the dauer larva stage and the *Drosophila* diapause are characterized by prolonged life span and triggered by nutrient shortage or temperature shifts. Entry into dauer larva or diapause depends on IIS suggesting a significant overlap between the molecular mechanisms that promote longevity in adult worms and flies and those regulating life span in developmentally arrested worms and diapause flies (Larsen et al. 1995; Williams et al. 2006). *S. cerevisiae* has also evolved to enter "resting states" to prolong its life span when nutrients are limited. Depending on its ploidy yeast can enter stationary phase (haploid or diploid) or undergo spore-formation (diploid) both characterized by extended life span. Several of the phenotypes of the chronologically long-lived mutants are shared by stationary phase yeast, e.g. high resistance to cellular stress, and the same pathways can control both CLS and entry into stationary phase (Fabrizio and Longo 2003; Werner-Washburne et al. 1993). Taken together, the similarities between the life span regulatory pathways in model organisms and their link to alternative developmental programs/resting states that allow long-term survival in hostile environments has led to the hypothesis that the molecular mechanisms underlying longevity extension have evolved in ancestral microorganisms in order to overcome periods of starvation by arresting growth and allocating energy into cell maintenance and stress resistance (Longo et al. 2005).

Is life span regulation conserved in mammals? At the time when yeast, worms, and flies were shown to "grow old together" (Strauss 2001), there was no conclusive evidence for a role of IIS in mammalian life span regulation. However, it was known that dwarf mice carrying mutations affecting the development of their pituitary gland (*Prop-1* and *Pit-1*) had low levels of plasma growth hormone (GH), insulin, and IGF-I as well as thyroid hormones and prolactin (Longo and Finch 2003). Notably, these mice live 25–65% longer than wild type (Brown-Borg et al. 1996; Flurkey et al. 2002) and, as the long-lived worms and flies, accumulate fat. Successive work showed that lowering IGF-I signaling is important for extending the life span of mice (Fig. 5.1). In fact, mice carrying only one IGF-I receptor (IGF-IR) functional allele were shown to be long-lived (26% mean life span extension) and resistant to oxidative stress as the IIS mutants of other species (Holzenberger et al. 2003). Interestingly, this effect was not confirmed in a different genetic background (Ladiges et al. 2009), suggesting that part of the phenotypes caused by GH deficiency are independent of IGF-I signaling or that it is difficult to reach a level of IGF-I signaling that is sufficient for normal function but also for life span extension. Further evidence that IIS signaling modulate mice life span came from the discovery that overexpressing *Klotho*, a hormone that represses the intracellular response triggered by both insulin and IGF-I, leads to longevity extension (Kurosu et al. 2005). Notably, *Klotho* transgenic mice were reported to have higher levels of mitochondrial superoxide dismutase activity and to be more resistant to oxidative stress at both the cellular and organismal levels (Yamamoto et al. 2005). These

effects were shown to depend on the activation and nuclear translocation of the FoxO fork-head transcription factors, which is triggered by lowering IIS but may also depend on the activity of other signaling pathways (Yamamoto et al. 2005) (Fig. 5.1).

The yeast model system has been remarkably successful and perhaps the most successful at identifying genes later shown to regulate life span in mammals. In fact, mice lacking the Sch9 homologue S6 kinase 1 (S6K1), are long-lived and are protected against several age-dependent defects (Selman et al. 2009). Furthermore, mice lacking adenylate cyclase 5 (AC5) or with a reduced activity of PKA, AC5 and PKA being orthologues of the yeast pro-aging adenylate cyclase and PKA, are long-lived and display protection against age-dependent diseases or damage/loss of function (Yan et al. 2007; Enns et al. 2009) (see section "The Ras/Adenylate Cyclase/PKA Pathway"). The additional major gene implicated in yeast life span regulation is TOR, which is believed to function upstream of Sch9 to control growth in response to nutrients (Polak and Hall 2009). TOR is a serine-threonine protein kinase conserved in organisms ranging from yeast to humans originally implicated in the regulation of life span in worms and flies. In fact, knocking down CeTOR from the first day of adulthood approximately doubles the life span of *C. elegans* and a similar effect is obtained by reducing the activity of Daf-15, the worm ortholog of the mammalian TOR-interacting protein raptor (Vellai et al. 2003; Jia et al. 2004). Analogously, in *Drosophila* the overexpression of either dominant-negative dTOR or of TOR-inhibiting dTsc1/2 prolongs life span (Kapahi et al. 2004). Recently the TOR inhibitor rapamycin has been shown to extend longevity in mice (Harrison et al. 2009). In all organisms so far investigated except yeast there is one TOR isoform that functions in two different multiprotein complexes, TORC1 and TORC2. In yeast two TOR kinases are present, Tor1 and Tor2. Either one of them can be found in the yeast TORC1 complex but only Tor2 is found in association with the TORC2 complex (Rohde et al. 2008). Down-regulation of TORC1 activity extends both yeast life spans (Wei et al. 2008; Powers et al. 2006; Kaeberlein et al. 2005). With respect to the CLS, genetic data suggest that TORC1 and Sch9 function in the same molecular pathway to control longevity (Wei et al. 2009; Pan and Shadel 2009 and P. Fabrizio, unpublished results) in agreement with the recently demonstrated role of TORC1 in the direct activation of Sch9 via the phosphorylation of several of its C-terminal amino acid residues (Urban et al. 2007). Notably, in higher eukaryotes the IIS cascade can activate TORC1, which in turn activates S6K, known to negatively affect longevity not only in mice (see above) but also in worms and flies (Kapahi et al. 2004; Hansen et al. 2007). Interestingly, both S6K and Sch9 play important roles in the activation of translation in response to nutrients and longevity regulation and are both dependent on the TORC1 complex for their activation. Mammalian S6K shares the same degree of homology with Sch9 as Akt. Since no closer homologue to S6K or Akt has been identified on the yeast genome, it is plausible that Sch9 originated from an ancestral protein that underwent duplication throughout evolution leading to the presence of S6K and Akt in metazoans. Importantly, both of them have maintained roles in controlling life span.

The Ras/Adenylate Cyclase/PKA Pathway

The first chronological long-lived mutant identified was the *ras2Δ* mutant (Longo 1997). *RAS2* codes for one of two highly conserved G-proteins known to activate PKA via adenylate cyclase (Cyr1). Its deletion double CLS and promotes a marked increased of heat and oxidative stress resistance (Fabrizio et al. 2003). Ras proteins directly activate Cyr1 and a mutation causing a partial loss of Cyr1 function was shown to extend CLS as well (Fabrizio et al. 2001). In presence of glucose the Ras/PKA pathway mediates the activation of a pro-growth transcriptional program which depends in part on the inhibition of the stress resistance transcription factors Msn2/4 (Zaman et al. 2009). The function of these factors is key for the CLS extension of both *ras2* and *cyr1* mutants (Fabrizio et al. 2001, 2003) although another transcription factor, Gis1, has been recently shown to contribute to the effect of lowering the activity of the Ras/PKA pathway on CLS and resistance to stress (Wei et al. 2008).

For a long time the role of the Ras/Cyr1/PKA pathway in the control of life span appeared to be limited to yeast, although mice lacking p66Shc, a signal transducer that might trigger mitosis via Ras activation, were shown to be resistant to oxidants and live 30% longer than wild type littermates (Migliaccio et al. 1999). However, more recently mammalian aging has been shown to depend on the adenylate cyclase/PKA signaling. In fact, Yan et al. reported the implication of one of the mammalian adenylate cyclase isoforms, *AC5*, in the regulation of mice longevity (Yan et al. 2007). According to these authors, *AC5* knock-out mice's median life span is approximately 30% longer than that of control littermates. *AC5* is expressed mostly in brain and heart and is activated by the β-adrenergic receptor signaling (Fig. 5.1). *AC5 KO* mice are protected against age-dependent cardiomyopathy. Notably, in agreement with results in yeast (Fabrizio et al. 2001, 2003), both myocytes and fibroblasts isolated from *AC5 KO* mice are more resistant to oxidative stress than control cells. Accordingly, MnSOD levels in heart, brain, and kidney were higher in *AC5 KO* mice than in controls confirming the association between protection against superoxide and life span extension (Yan et al. 2007). Further evidence to support a role for the adenylate cyclase/PKA cascade in mice aging has been provided by Enns et al. who have reported that the disruption of RIIβ, which codes for one of the mammalian PKA regulatory subunits, promotes median and maximum life span extension in male mice (Enns et al. 2009).

Molecular Mechanisms Responsible for Life Span Extension

While the field of the genetics of aging is progressing rapidly, we are still lacking a comprehensive view of how aging occurs at the cellular and organismal level and how it can be delayed. The yeast CLS is providing an important tool to address this issue.

Oxidative Stress Response

With few exceptions, life span extension is associated with an increased ability to withstand different types of stress (Longo and Fabrizio 2002). Oxidative stress resistance in particular has been the object of intensive study in the context of aging since the "Free radical theory of aging" was first proposed (Harman 1956). According to this theory aging is caused by the cellular damage produced by highly reactive oxygen species with unpaired electrons, which are generated mostly in the mitochondrion at complexes I and III of the electron transport chain (Longo 1997). As mentioned in the previous sections, mutations that prolong life span in different model organisms are associated with high levels of antioxidant enzymes. All the yeast mutants with prolonged CLS are highly resistant to the superoxide-generating agents menadione and paraquat and to hydrogen peroxide and mitochondrial superoxide dismutase (MnSOD) activity is required for long term survival and for the longevity extension promoted by lack of Sch9 or Ras2 (Longo et al. 1996; Fabrizio et al. 2003; Longo and Fabrizio 2002). However, the overexpression of individual antioxidant enzymes or combination of them leads to a maximum of 30% mean CLS extension, which is relatively modest when compared to the 3-fold life span extension obtained by deleting *SCH9* (Fabrizio et al. 2001, 2003). Similar results were obtained in *Drosophila* and mice (Sun et al. 2002; Orr and Sohal 1994; Hu et al. 2007; Schriner et al. 2005) suggesting that, while MnSOD activity provides a fundamental anti-oxidant defense to mitochondria, its role in longevity extension is significant but limited. Nevertheless, work done using the yeast CLS paradigm strongly supports a causative role for oxidative-damage in aging. In fact, firstly, exploiting the ability of yeast to grow on glucose as a carbon source in absence of functional mitochondria (fermentative growth), it was possible to show that loss of mitochondrial function precedes death in both mutants lacking MnSOD activity and wild type (Longo et al. 1999). Secondly, it was reported that the mitochondrial enzymes aconitase and succinate dehydrogenase, which contain iron-sulfur clusters particularly sensitive to superoxide-dependent oxidation, are primary targets of age-dependent mitochondrial damage in yeast (Fabrizio et al. 2001, 2003; Longo et al. 1999). Thirdly, an age-dependent accumulation of oxidation-induced DNA damage/mutations has been shown in chronologically aging yeast (Madia et al. 2009) (see section "Age-Dependent Genomic Instability in Yeast").

Metabolic Switches

In order to further understand the mechanisms that lead to life span extension, we have recently obtained the gene expression profiles of chronologically aging wild type and long-lived mutants (Wei et al. 2009). In our analysis we compared the transcriptomes of the *sch9Δ, ras2Δ*, and *tor1Δ* mutants to those of wild type yeast at day 2.5. This age was chosen to avoid both the noise that could originate from residual cell growth at younger ages and the transcriptional changes associated with reduction of metabolic rates that normally occurs at day 4–5 (Fabrizio

and Longo 2003; Fabrizio et al. 2003). Our results have shown a significant degree of overlap between the genes either up- or down-regulated in the different mutants in comparison to the wild type underlying how the different life span regulatory pathways may impinge on a set of common downstream effectors controlled by the same transcriptional activators to modulate longevity (Wei et al. 2008, 2009). Importantly, yeast long-lived mutants exhibit transcriptional changes consistent with a wide range of metabolic changes. In fact, glycolytic/fermentative genes are up-regulated in all the mutants, while a vast set of mitochondrial genes, which include those coding for electron transport proteins, TCA cycle enzymes, and mitochondrial ribosomal proteins among others, are down-regulated (Wei et al. 2009). Our data also suggest that a part of dihydroxy-acetone-phosphate (DHAP), a glycolysis intermediate, is metabolized to produce glycerol. In fact, all the long-lived mutants showed a general activation of glycerol biosynthetic genes, above all *GPD1* and *GPD2*, which code for the key enzymes required for glycerol biosynthesis from DHAP. Consistently, our in depth analysis of the chronologically aging *sch9Δ* mutant revealed a significant accumulation of glycerol both intracellularly and extracellularly, which was not observed in the wild type (Wei et al. 2009). The role of *GPD1/2* and other glycerol biosynthetic genes in CLS extension in a *sch9Δ* context was confirmed by epistatis analysis, which underscored the importance of glycerol biosynthesis in promoting both life span and resistance to stress (Wei et al. 2009). Notably, in aging *sch9Δ* mutants the metabolic switch to glycerol biosynthesis is associated with ethanol catabolism (Wei et al. 2009). We have previously demonstrated that ethanol is accumulated in aging wild type yeast and negatively affects their CLS in spite of being a carbon source that yeast can utilize by mitochondrial respiration (Fabrizio et al. 2005a). Acetic acid is also accumulated in chronologically aging cultures and it has been proposed to play a pro-aging role (Burtner et al. 2009). However, it should be underlined that at the level generated during chronological aging (approximately 6 mM) acetic acid is a carbon source that promotes acidification, cell cycle arrest, and high respiratory rates (Fabrizio et al. 2003, 2004a, 2005a) and not a toxin, as it can be at high concentrations (Ludovico et al. 2001; Madeo et al. 2004). Thus, acetic acid, may generate the appropriate conditions (no division, high metabolism, etc.) to obtain a short and high metabolism life span that models cells from higher eukaryotes. The remarkable number of genes later shown to promote aging in higher eukaryotes identified using this CLS paradigm supports this notion. Under certain incubation conditions, the ethanol generated during log phase appears to be converted to acetic acid. Mutants lacking Sch9 but not wild type cells, appear to redirect acetic acid to glycerol production (V Longo unpublished results). We now know that ethanol as glucose represents a pro-aging carbon source. In fact, when yeast are exposed to a constant concentration of either of them under experimental conditions that do not allow cell growth (lack of an essential amino acid), their CLS is dramatically reduced when compared to that of yeast exposed to glycerol (Wei et al. 2009). In this respect, exposure to glycerol has the same CLS extending effect as carbon source removal, which is a method to promote caloric restriction (CR) in yeast (Wei et al. 2009). This is remarkable because it suggests that the molecular anti-aging strategies activated by CR, the only non

genetic intervention known to prolong life span in all species so far tested (Mair and Dillin 2008), are not repressed by glycerol. Thus, the CLS extension of the *sch9Δ* mutant is due, in part, to key metabolic changes that mimic CR by triggering ethanol consumption and glycerol accumulation (Wei et al. 2009). Very recent evidence has implemented our data by showing that phosphoenolpyruvate carboxykinase (Pck1), a key enzyme for gluconeogenesis, is required for ethanol consumption and for both normal life span and CR-dependent CLS extension (Lin et al. 2009). Pck1 was shown to be dependent on the histone acetylase complex NuA4 for activation and on Sir2 for inactivation (Lin et al. 2009), which is consistent with our findings demonstrating that Sir2 activity limits the longevity extension promoted by CR (Fabrizio et al. 2005a). Intriguingly, our gene expression profile data did not show transcriptional activation of genes involved in gluconeogenesis in the early phase of chronological aging. By contrast, as mentioned above, they pointed to an activation of the glycolytic function in long-lived yeast (Wei et al. 2009). Further studies are needed to clarify this point and establish whether glycolysis or gluconeogenesis is predominant in yeast under CR and which enzymatic activities are key for glycerol accumulation and ethanol catabolism.

How do the metabolic switches observed in long-lived yeast translate to other long-lived organisms? Do they represent a conserved anti-aging strategy? We do not have an answer yet. However, gene expression data suggest that both glyceroneogenesis and gluconeogenesis are up-regulated in calorie restricted *C. elegans* (Castelein et al. 2008). Furthermore, an anti-aging role for a metabolic switch to gluconeogenesis was originally hypothesized based on gene expression profile data obtained from calorie restricted mice (Lee et al. 1999) and is supported by preliminary results showing that transgenic mice overexpressing the cytosolic form of phosphoenolpyruvate carboxykinase in the skeletal muscle are long-lived (Hakimi et al. 2007).

Conserved Pro-aging Genes, Genomic Instability, and Cancer

Age-Dependent Genomic Instability in Yeast

Yeast CLS extension is associated with a reduction in genomic instability (Fabrizio et al. 2004a; Madia et al. 2008, 2009). By performing simple mutation assays on chronologically aging yeast, it is possible to monitor the age-dependent accumulation of different types of DNA mutations including base substitutions, small DNA insertions/deletions, and gross chromosomal rearrangements (GCRs) (Madia et al. 2007). The frequency of all these mutations increases in an age-dependent manner (Fabrizio et al. 2004a; Madia et al. 2009). A reduction of this effect is observed in mutants lacking Sch9 (Fabrizio et al. 2004a; Madia et al. 2009) and it was shown to depend on their reduced sensitivity to the superoxide-dependent DNA damage and the inactivation of the error-prone Rev1-Polζ DNA polymerase complex, which is involved in DNA-repair by translesion synthesis (TLS) (Madia et al. 2009). As discussed in the section “Oxidative Stress Response”, oxidative damage is believed to

play a key role in aging and high levels of protection against oxidants are detected in yeast long-lived mutants (Longo and Fabrizio 2002). In a *sch9*$^{-}$ context both *SOD2* transcription and Sod2 activity are up-regulated during chronological aging (Fabrizio et al. 2003, 2004a) and the levels of 8-hydroxy-2'-deoxiguanidine (8-OHdG), one of the most frequent oxidative DNA lesions, are reduced in comparison with wild type (Madia et al. 2009). Importantly, the overexpression of either cytosolic or mitochondrial Sod reduces the age-dependent accumulation of mutations suggesting that superoxide is a principal mediator of DNA-damage and mutagenesis observed in aging yeast or that superoxide or Sods are regulating other poorly understood processes that lead to hypermutability states (Madia et al. 2009). By studying the mutational spectra obtained by sequencing *CAN1*, a gene coding for an arginine permease, in wild type and *sch9*$^{-}$ clones originated from 7-day old cells previously screened for the presence of *can1* mutations, the deoxycytidyl-transferase Rev1 was identified as key for age-dependent mutagenesis (Madia et al. 2009). Rev1 is found in a complex with polymerase zeta (Polζ) and it is implicated in the repair of abasic sites in damaged DNA by TLS. The activity of Rev1-Polζ is error-prone and compatible with the mutational spectra observed in old wild type cells. Furthermore, Sch9 activity and oxidative stress were shown to promote Rev1 function, which, although crucial to prevent DNA double strand breaks, causes the generation of point mutations when aging non-dividing cells undergo the first round of replication (Madia et al. 2009). Interestingly, in *sch9*$^{-}$ mutants TLS is completely abolished suggesting that lack of Sch9 not only represses *REV1* expression, which is not absolutely required for TLS in yeast (Pages et al. 2008), but it may also reduce the activity of further components of this error-prone repair system (Madia et al. 2009).

In addition to its role in regulating TLS, Sch9 was shown to contribute to the regulation of mitotic recombination, which is also important for age-dependent genomic instability (Madia et al. 2008). In fact, in yeast lacking Sgs1, the homolog of the human BLM and WRN RecQ helicases, which are mutated in the human progeroid Werner and Bloom syndromes (see next section), a dramatic increase of the age-dependent point mutations and GCRs is dampened by the deletion of *SCH9*. This effect is due mostly to the down-regulation of the error-prone recombination between sister chromatids (Madia et al. 2008).

In summary, our current knowledge suggests that Sch9 controls genomic instability in aging yeast by: (1) down-regulating the expression of anti-oxidant genes such as Sod2, which limits DNA oxidation, (2) activating error-prone repair systems such as Rev1-Polζ , which generates mutations to repair oxidative DNA lesions when old cells resume growth, and error-prone mitotic recombination and, (3) regulating the production and catabolism of ethanol and acetic acid and the generation of carbon sources that reduce the aging rate. The discovery of the mechanisms behind the enhanced genomic stability of the long-lived *sch9Δ* mutant is important not only because of its possible conservation and implication in the regulation of aging in mammals but also because, if conserved, these mechanisms might be central for tumorigenesis (see next sections).

Oxidative DNA-Damage and Cancer

Besides yeast, an age-dependent accumulation of DNA mutations has been observed in other model organisms and in human cells and tissues (Vijg 2007; Longo et al. 2008). A causative role for DNA damage/mutations in aging is supported by a number of human segmental progeroid syndromes characterized each by the premature appearance of a few aging features. Notably, many of these syndromes are caused by mutations affecting the activity of either DNA-repair or DNA-damage sensing genes and promote cancer (Hasty et al. 2003). Although to what extent genomic instability contributes to aging is still uncertain, its role in tumorigenesis is well established and several lines of evidence suggest that oxidative DNA damage plays a pivotal role in causing an age-dependent accumulation of DNA mutations and cancer. In fact, (1) a vast group of mutations detected in the anti-oncogene p53, which is mutated in ~50% of the human cancers, is generated in an attempt to repair the damage caused by reactive oxygen species (ROS) (Pfeifer 2000), (2) high levels of DNA damage/mutations and cancer have been found in mice with reduced activity of either cytosolic or mitochondrial SOD (Busuttil et al. 2005; Van Remmen et al. 2003), (3) in *S. cerevisiae* the lack of cytosolic Sod causes an increase of mutations and a high frequency of adaptive regrowth, a phenotype described as "cancer-like" because, as discussed in the section "Chronological Life Span", it is characterized by the ability of aging cells to resume cell division under conditions that normally do not promote growth (Fabrizio et al. 2004a; Longo et al. 1999; Madia et al. 2007, 2009). Importantly, the adaptive regrowth phenotype is extremely rare in cultures of the long-lived *ras2Δ* or *sch9Δ* mutants consistently with their high degree of protection against superoxide (Fabrizio et al. 2004a) and the low mutation frequency detected in aging sch9*Δ* discussed in the previous section.

Pro-aging Genes and Cancer in Higher Eukaryotes

As discussed broadly in the section "Conserved Life Span-Regulatory Pathways", the nutrient-sensing/insulin-IGF-like pathways promote aging in several model organisms. The expression of conserved pro-aging genes such as Sch9 causes aging, genomic instability, and a "cancer-like" phenotype in yeast. Is there a similar association between pro-aging genes/pathways and cancer in other organisms? It certainly seems to be the case. In fact, life-extending mutations that decrease IIS in worms block germ cells proliferation and induce apoptosis in a *C. elegans* mutant that forms germ-line tumors (Pinkston et al. 2006). Analogously, in flies reducing IIS not only extends life span but also delays the growth of germline cysts (LaFever and Drummond-Barbosa 2005).

Most interestingly, long-lived GH/IGF-I-deficient mice are long lived and show lower levels of lifetime tumor incidence (Vergara et al. 2004; Ikeno et al. 2003). A similar reduction of tumors is observed in Akt-deficient mice (Skeen et al.

2006). Human studies indicate that dampening IGF-I signaling may reduce cancer incidence likely because of the pro-growth activity of IGF-I (Longo et al. 2008). However, we hypothesize that the IIS-dependent regulation of genomic instability is also important for tumorigenesis and cancer progression (see next section).

The Role of Pro-aging Genes in Cancer

Yeast pro-aging Sch9 and Ras2 are homologues of mammalian proto-oncogenes Akt and Ras, which are activated in many human cancers (Rodriguez-Viciana et al. 1994; Yoeli-Lerner and Toker 2006). In both yeast and mammals Ras and Sch9/Akt signal through pathways that regulate cell growth and promote aging (see section "Conserved Life Span-Regulatory Pathways"). In humans oncogenic mutations that lead to the activation of Ras, Akt, or in the up-stream IGF-I-receptor are commonly believed to promote cancer by allowing the survival and growth of damaged cells, which are normally removed by apoptosis. They have also been proposed to increase genomic instability because the high proliferation rates they promote may increase the occurrence of errors during replication across sites of unrepaired damage (Pollak et al. 2004). The accumulation of further mutations in cells already hit by an initial oncogenic mutation is thought to be responsible for tumor growth and metastasis.

Our opinion, based on studies on the conserved role of oncogene homologues in life span regulation, is that the tumorigenesis scenario might be more complex. In fact, in analogy with yeast, the activity of Ras and Akt might reduce cellular protection and in particular oxidative stress resistance in human cells leading to increased mutational rates, which in turn may cause oncogenic mutations activating Ras and Akt themselves and/or the additional mutations required for cancer development. This hypothesis is supported by data showing that cells isolated from long-lived mice are resistant to several different DNA-damaging agents (Longo et al. 2008).

Conclusions and Perspectives

The study of the pathways and mechanisms regulating longevity has benefited greatly from the use of *S. cerevisiae*. This simple unicellular organism has been instrumental in the discovery of the conservation of the principal life span-regulatory pathways and, thanks to the amenability of the CLS to genetic, genomic, and biochemistry assays, it is providing an excellent system to gain further insights into the metabolic changes occurring in senescent cells. Recently, yeast CLS has been used successfully to study the mechanisms behind age-dependent genomic instability leading to the identification of oxidative DNA damage and error-prone DNA repair systems as key for the mutation accumulation observed during chronological aging. This is an important finding because of the possibility that

these molecular mechanisms may be conserved through evolution and involved in enhancing genomic instability and promoting cancer in mammals.

We expect that in the near future additional yeast genetic determinants of aging and novel mechanisms to explain how life span can be prolonged will be discovered. These are expected to contribute to further understand aging and diseases in a wide range of species including humans.

Appendix

While this book was in production we published two articles relevant to the topic of this chapter. For the sake of completeness, we believe it is appropriate to discuss them briefly here. The first article has reported the results of a screen of the yeast deletion collection aimed at identifying novel life span determinants (Fabrizio et al. 2010). Besides confirming the importance of the mitochondrial function and the autophagic process in long-term survival, our screen has uncovered numerous novel genes involved in the process of determining yeast longevity. Among others *ACB1, CKA2*, and *TRM9*. The deletion of each of these three genes prolongs life span and increases heat resistance. *ACB1* codes an acyl-coA binding protein involved in lipid biosynthesis and vesicle formation. Cka2 is the catalytic subunit of a serine-threonine kinase, CK2, which controls several cellular functions including cell growth and proliferation. Trm9 is a tRNA methylase that targets the uridine residues at the wobble position in tRNA(Glu) and tRNA(Arg3). Currently, the mechanisms by which these proteins regulate longevity have not been described. It will be important to elucidate them given the high degree of conservation of these novel life span determinants and the possibility that their role in aging extends to other organisms.

The second article concerns the role of the conserved pro-aging pathways in the regulation of genomic instability and cancer. In the section "Conserved Pro-aging Genes, Genomic Instability, and Cancer" we have discussed how the activity of the Sch9 and GH/IGF-I pathways promotes DNA damage in yeast and mice, respectively. We have also mentioned that GH/IGF-I-deficient mice show decreased rates of cancer incidence. Recently this observation has been extended to humans with growth hormone receptor deficiency who display a major reduction in cancer and diabetes, which is associated with reduced levels of several orthologs of the key yeast pro-aging genes (Guevara-Aguirre et al. 2011). Importantly, serum from GH/IGF-I signaling-deficient individuals protects cells in culture from H_2O_2-dependent DNA damage and down-regulates the expression of N-Ras, PKA, and TOR while activating SOD2 transcription. This suggests that a reduction of GH/IGF-I signaling may lead to cellular protection and reduced DNA damage in vivo via the inactivation of the pro-aging Ras, PKA, and TOR pathways, which in turn may contribute to lower incidence of cancer and other diseases. Thus, this new evidence from a human study further supports a causative link between the activity of the conserved pro-aging pathways, genomic instability, and diseases.

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Chapter 6
Aging and the Survival of Quiescent and Non-quiescent Cells in Yeast Stationary-Phase Cultures

M. Werner-Washburne, Sushmita Roy, and George S. Davidson

Abstract In this chapter, we argue that with careful attention to cell types in stationary-phase cultures of the yeast, *S. cerevisiae* provide an excellent model system for aging studies and hold much promise in pinpointing the set of causal genes and mechanisms driving aging. Importantly, a more detailed understanding of aging in this single celled organism will also shed light on aging in tissue-complex model organisms such as *C. elegans* and *D. melanogaster.* We feel strongly that the relationship between aging in yeast and tissue-complex organisms has been obscured by failure to notice the heterogeneity of stationary-phase cultures and the processes by which distinct cell types arise in these cultures. Although several studies have used yeast stationary-phase cultures for chronological aging, the majority of these studies have assumed that cultures in stationary phase are homogeneously composed of a single cell type. However, genome-scale analyses of yeast stationary-phase cultures have identified two major cell fractions: quiescent and non-quiescent, which we discuss in detail in this chapter. We review evidence that cell populations isolated from these cultures exhibit population-specific phenotypes spanning a range of metabolic and physiological processes including reproductive capacity, apoptosis, differences in metabolic activities, genetic hyper-mutability, and stress responses. The identification, in *S. cerevisiae*, of multiple sub-populations having differentiated physiological attributes relevant to aging offers an unprecedented opportunity. This opportunity to deeply understand yeast cellular (and population) aging programs will, also, give insight into genomic and metabolic processes in tissue-complex organism, as well as stem cell biology and the origins of differentiation.

Keywords Stationary phase · quiescent · Starvation · Non-quiescent · Respiration · Reactive oxygen species · Nomenclature · Post-diauxic phase

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Introduction

Everyone *knows* what aging is even though there is no single, operational definition for this ubiquitous process. As one moves from a focus on single cells, to more tissue-complex organisms, to populations, and beyond, the phenotypic details and complexity of the process increase dramatically. These phenotypes are probably additive but do not tell us, at a systems level, what are the specific changes at different levels that cause or constitute aging. For example, the morphological and physiological signs of aging in humans include wrinkled skin, graying hair, slower gait, decreased short term memory, and, ultimately, death from "natural causes", among others. Aging in tissue-complex model organisms, such as *Drosophila melanogaster* or *Caenorhabditis elegans*, is often evaluated by the length of time the organism survives (Paaby and Schmidt 2009). Rarely do aging studies in tissue-complex eukaryotes investigate exactly what fails, where the system breaks, or what is the exact cause of every "natural" death. This system complexity and the difficulty in identifying the root causes of early or delayed "aging" as measured by death is the reason that simpler, model organisms provide powerful resources in the study of aging.

Because of the complexity of the process and the relative sparseness of the data, connecting the dots to understand the causes and mechanisms of aging has been incredibly challenging. With the advent of genomics, the number of dots has increased exponentially. Model Organism Databases, such as WormBase, FlyBase, etc., and other databases, e.g., "Human Ageing Genome Resources" http://genomics.senescence.info and "An Age", a database of animal aging and longevity (de Magalhaes et al. 2009), have begun to put aging information into one, searchable space. However, even having searchable data does not necessarily increase knowledge that leads to pinpointing *THE* gene or the sets of genes, proteins, or other macromolecules required for youth or aging or even to the "aha" that allows us to incontrovertibly build a model of the process of aging. A significant outcome, when data is assembled, is that additional experiments, in previously unexamined experimental space, are rapidly identified. However, in the end, while a definition of aging, the process that we are all so familiar with, is being narrowed, it is still elusive.

Because aging is assumed to be an evolutionarily conserved process, model organisms, including the yeast *Saccharomyces cerevisiae,* have proven useful. Studies in these organisms have led to the identification of genes required for both chronological and replicative aging, which are described fully in other chapters in this volume. The beauty of studying aging in a simple eukaryotic organism like the yeast *S. cerevisiae* is that cells are easy to grow and study and a wide range of mutants are available, allowing even genome-wide screenings. Studies of model organisms, including *S. cerevisiae* and *C. elegans*, have led to the recognition that conserved pathways that affect aging are also involved in glucose signaling through insulin (Bartke 2008; Longo and Fabrizio 2002; Shimokawa et al. 2008; Wolkow 2002), cAMP-dependent protein kinase (Cheng

et al. 2007), silencing (Ashrafi et al. 1999; Kaeberlein et al. 1999; Kennedy et al. 1995; Sinclair et al. 1998) and the TOR pathway (Fabrizio and Longo 2003; Longo 2009; Paaby and Schmidt 2009; Parrella and Longo 2008; Wei et al. 2008). However, the simplicity and availability of tools can sometimes lead to the false assumption that the organism and its life cycle are simple. If my laboratory has learned anything from two decades of studying yeast stationary phase, a time when nutrients are limited and cells appear to be dormant, it is that still waters run deep and that even single-celled organisms may have complex life cycles.

Two types of aging or life span have been studied in yeast: replicative life span (RLS) and chronological life span (CLS) (Bitterman et al. 2003; Jazwinski 1990; Laun et al. 2006). RLS has been studied using single mother cells isolated from daughters by micromanipulation. In contrast, CLS was identified in cells from stationary-phase cultures, in which cultures are nutrient limited and cell division is extremely slow (Ashrafi et al. 1999). CLS is often studied simply by following single cells or letting cultures, typically of deletion mutants, "age" in stationary phase (Fabrizio and Longo 2003, 2008; Powers et al. 2006). It has been argued that CLS is more similar to aging in tissue-complex eukaryotes. Both types of aging studies have measured or assumed they were measuring aging in single, independent cells. But what if stationary-phase cultures are structured so that cells have different aging characteristics, like cells in tissues of more complex organisms? What if there are many ways to affect longevity or re-growth of a culture? How might this inform our thoughts about studies of aging in more tissue-complex organisms?

This chapter will cover several elements of these studies. First, we will provide an introduction to stationary phase and the differentiation of quiescent and non-quiescent cells in stationary-phase cultures. Second, we will present the rationale for the need for precise nomenclature to avoid confusion and aid in experimental design. Third, we will describe how studies of stationary-phase yeast cultures provide novel insights into aging and approaches for studying aging. Finally, based on our work with yeast, we will present a hypothesis about the structure of stationary phase communities, relating it to cellular organization and aging in higher eukaryotes.

Yeast Stationary Phase

Yeast cells, grown in liquid culture, go through reproducible stages of growth until the carbon is exhausted and the cultures enter stationary phase (Fig. 6.1) (Gray et al. 2004; Werner-Washburne et al. 1993).

Although yeast can grow using a variety of carbon sources, our studies have been primarily in glucose-based rich medium (YPD). In this medium, cultures increase in density exponentially until glucose is exhausted at the diauxic shift. During the post-diauxic phase, after glucose exhaustion, only non-fermentable carbon sources

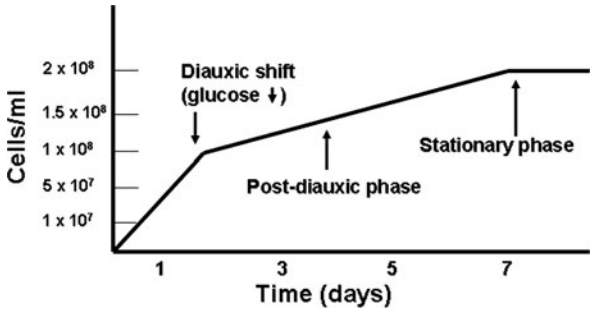

Fig. 6.1 Growth curve for yeast grown in rich, glucose-based medium. Glucose is exhausted at the diauxic shift, typically about 1 day after inoculation. During the post-diauxic phase, cells survive with only non-fermentable carbon sources. Cultures reach stationary phase 5–7 days after inoculation

are available to support growth. When carbon is exhausted, the cultures enter stationary phase, at which time there is little change in growth as measured by cells/ml or colony-forming units.

The Black Box of Stationary Phase

Surprisingly, in 1988, when our laboratory started this work, many researchers assumed that: (1) cells that weren't growing were simply "shut down" and, therefore, uninteresting and (2) died after a few days in culture, i.e. when humans were no longer watching them (numerous personal communications). However, because most cells on earth are in a non-dividing state and because yeast survived for millennia without human intervention, both assumptions seemed counterintuitive.

Our work has led to several conceptual and experimental "breakthroughs" that have allowed us to look very differently at stationary-phase cultures and the cell populations that comprise them. When we looked more carefully at the yeast growth curve, our first breakthrough was to realize there was one more doubling after glucose exhaustion at the diauxic shift (Fig. 6.1). Knowing that growth did not arrest, we then defined three parts to this process, starting at the diauxic shift: entry into, survival during, and exit from stationary phase, which allowed us to begin to identify characteristics of each phase. The second breakthrough was finding mRNAs and proteins that accumulated or were induced late in this process, i.e., as cultures approached stationary phase (Fuge et al. 1994; Werner-Washburne et al. 1987, 1993). This finding provided confirmation that cells did not just shut down after glucose exhaustion and entry into stationary phase is a dynamic process. We then discovered that thousands of mRNAs were sequestered in quiescent cells in stationary-phase cultures and that these mRNAs were released into the cytoplasm in a stress-specific manner (Aragon et al. 2006, 2008).

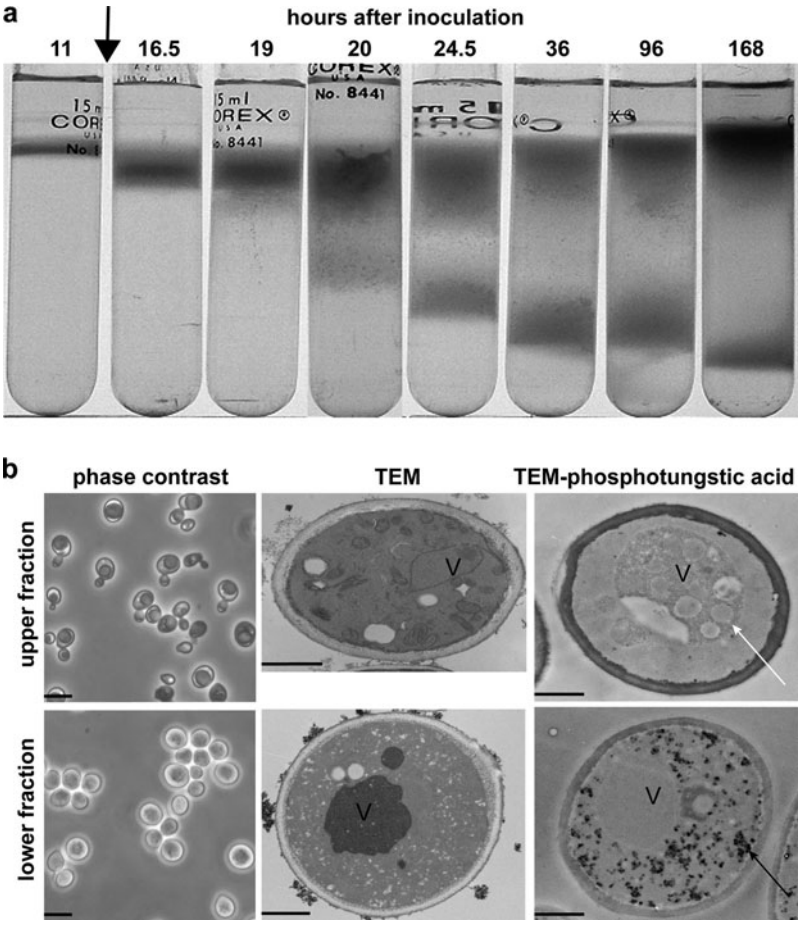

Fig. 6.2 Two distinct cell populations are formed in yeast cultures entering SP. **a** Density gradient separation of two, distinct cell fractions in S288c cultures as a function of time after inoculation. Glucose exhaustion (*black arrow*) occurred 12 h post inoculation. **b** Phase contrast, TEM, and phosphotungstic acid-stained TEM micrographs of upper- and lower-fraction cells from SP cultures (7-days post inoculation): *white arrow*, autophagosomes; *black arrow*, accumulated glycogen; and V, *vacuole Scale bars* represent 10 and 1 μm, for light and TEM micrographs, respectively. © Allen et al. (2006). Originally published in J Cell Biol 174:89–100

These observations were followed by the discovery of two major cell fractions in stationary-phase cultures, composed of quiescent and non-quiescent cells (Fig. 6.2) (Allen et al. 2006; Aragon et al. 2008).

Quiescent cells, in contrast to non-quiescent cells, contain the sequestered mRNAs, survive for longer times, and retain a high level of reproductive competence, genome stability, and low reactive oxygen species (ROS). The discovery and characterization of these cell types in stationary-phase cultures provide the basis for important contributions to the study of aging, especially CLS, in yeast. Recently, we found two more intriguing aspects of this differentiation: first, that NQ cells

are essentially unable to respire, even though they are in media containing only non-fermentable carbon sources (Davidson et al. 2011).

The Importance of Precise Nomenclature for Thinking About Complex Processes Like Stationary Phase and Aging

Although stationary phase (which relates to cultures) contains cells that are aging, stationary phase is not the same as aging, which is a process that can be detected in cells, tissues, cultures, and organisms. However, there are many ways in which the study of stationary phase parallels the studies of aging. One of these is the complexity of the process. For example, in studying stationary-phase in cultures, it is easy to confuse the phase of the culture, which shows little increase or decrease in cells/ml, with the state of the cells within this cultures. This confusion can easily lead to the assumption that the cells in stationary-phase cultures are homogeneous, which they aren't. They are often referred to as stationary-phase cells, which they also aren't. Because of this potential for confusion and mistaken assumptions, the words used in describing elements of this process are critical to understanding this process. Although most researchers don't spend a lot of time thinking about nomenclature (and would probably prefer to avoid it), we believe that part of the problem of understanding many critically important, complex processes is that we and our research communities are careless in our selection or use of words.

Linguists have thought about this situation for many years. For example, the Whorf hypothesis (SWH) (also known as the "linguistic relativity hypothesis") postulates that a particular language's nature influences the habitual thought of its speakers, such that different language patterns yield different patterns of thought (Casasanto 2008). While we don't believe researchers are *imprisoned* by their language systems we do believe that, "it is not the *language system*, but the (group-specific or commonly established) *use of language* for which one of Wittgenstein's most famous claims is truly valid: 'the limits of my language are the limits of my world.'" Thus, it is important in presenting complex processes, like entry into stationary phase and aging, that we and our community are extremely careful in our choice of, and consistent in our use of words. Processes and systems that are "black boxes" can be made more transparent by careful choice and application of conceptualizations and words.

If You Can't Reproduce, Are You Dead?

One of the most serious errors in nomenclature usage that permeates yeast research, including research on aging and stationary phase, is the consistent use of "inviable" or "dead" when referring to cells that cannot form colonies on plates.[1] Cells that

[1] Please note that I have been as guilty as any in the misuse of the terms "inviability" and "survival", dating back to the years when I studied the HSP70 genes (Werner-Washburne et al. 1987).

cannot produce colonies may be dead or they may also be living but unable to re-proliferate or senescent (Campisi 2005; Campisi and di Fagagna 2007). The difference between these two states is significant and informative (Allen et al. 2006; Minois et al. 2005). Cells that are alive but cannot reproduce or form colonies, as all geneticists *know*, may still accumulate suppressor mutations that could allow a cell to regain reproductive competence. Because colony formation is not a direct test of death, the phenotype, when cells cannot form colonies, should be referred to as "fitness" or "loss of reproductive capacity", which would include dead cells and cells that cannot divide. A variety of vital stains and fluorescent dyes are available to directly quantify dead cells and should be the standard when using the term "inviable" as a microbial phenotype.

There are many fields in which researchers agree that there is a difference between death and the inability to reproduce. Studies of viable but unculturable microbes, which represent 99.9% of the microbes on earth (Lewis and Gattie 1991), underscore the significance of the difference between dead cells and cells that cannot divide. Studies of stem cells and cancer incorporate the concept that cells that are not currently dividing may well resume division at some time in the future. The increase in human cancers with age is likely an example of the accumulation of mutations in non-dividing cells that induce re-proliferation. However, "inviable" is such a broadly misused term, that no one has thought to re-examine the 1063 yeast genes annotated as "essential" based on the inability of haploid spores to produce colonies on rich medium (Winzeler et al. 1999). These "essential" genes are very likely to be a mixture of genes required for survival, growth on rich medium, and aging genes required for maintenance of reproductive capacity or fitness. We raise this issue, not because we are innocent of misusing these terms, but because of its profound impact on the models we develop and the questions we subsequently fail to ask.[2]

Stationary Phase Is a Property of Cultures Not Cells

We use the term stationary phase to describe a culture (not a cell) in which the number of reproductively competent cells as measured by colony-forming units (CFUs), is relatively stable. The term "stationary phase" does not imply that the culture is homogenous or lacks inter-cellular dynamics, although this has typically been the assumption in studies of chronological aging and other work, including our own. Stationary phase is a term that relates only to the steady state property of a culture, i.e., a culture that is not significantly increasing or decreasing in cell number.

[2] It is also important, as senior scientists, that we communicate this to our students: (1) so they make a mental note to check methods every time they read "survival", "death", or "inviability" and (2) as a commitment to improving scientific discourse.

Introducing New Terms

We have identified two major cell fractions in stationary-phase cultures, separable by density, named *quiescent* and *non-quiescent* (see breakthrough discussion above) (Allen et al. 2006). We typically refer to these as "cell fractions" because we assume they also are heterogeneous. The non-quiescent cell fraction is clearly heterogeneous, containing several cell types, including: viable replicative and non-replicative cells; the quiescent fraction is less so. We also refer to quiescent and non-quiescent cells in these fractions, while realizing that, as we learn more, these names may have to be modified to reflect different types of quiescent or non-quiescent cells with specific trajectories.

The Differentiation of Quiescent and Non-quiescent Cells in Cultures After Glucose Exhaustion

When yeast cells grown on glucose-based, rich medium exhaust glucose at the diauxic shift, they begin to differentiate into quiescent and non-quiescent cell fractions (Figs. 6.2 and 6.3) (Allen et al. 2006).

As cultures enter stationary phase, the quiescent cell fraction becomes more dense, refractile by phase contrast microscopy; and thermotolerant. They are also

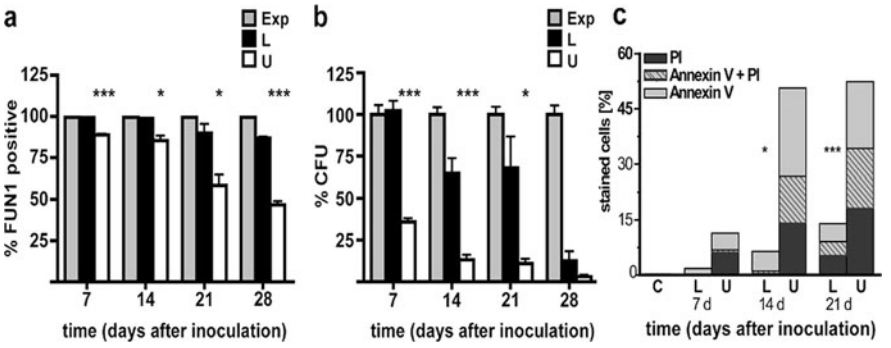

Fig. 6.3 FUN-1 metabolic activity (viability), colony-forming capacity; and apoptosis/necrosis of cells from quiescent (*L*) and non-quiescent (*U*) fractions from SP cultures. **a** Viability of cells from quiescent lower (*L*) and non-quiescent upper (*U*) fractions from 7-, 14-, 21-, and 28-days-old S288c SP cultures as determined by FUN-1 uptake measured by flow cytometry. Exponentially growing cells (*Exp*) were positive controls. **b** Colony-forming capacity of cells from S288c SP cultures determined by plating assay. **c** Flow cytometric quantification of 7-, 14-, and 21-days-old S289 lower (*L*) and upper (*U*) fraction cells or exponentially growing (*C*) cells co-stained with AnnV and PI, to detect apoptotic and necrotic markers, respectively. Values are expressed as the percentage of colony forming units (CFU) of exponentially growing cultures plated in parallel on each day. At T = 0, the number of colonies produced by each population was normalized to 100%. Error bars indicate SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. © Allen et al. (2006). Modified from a figure originally published in J Cell Biol 174:89–100

exquisitely synchronous, a property that makes them exceptionally useful for cell-cycle and aging studies (Laun et al. 2006; Moore and Miller 2007). Quiescent cells also: (1) retain a high level of viability and reproductive capacity, i.e., the ability to form colonies, for at least 21 days (Fig. 6.3a, b); (2) exhibit low levels of reactive oxygen species (ROS); and (3) are genomically stable (Allen et al. 2006; Aragon et al. 2008). Ninety one percent of these cells are daughters and the remaining 9% are mothers who have budded only once (Allen et al. 2006).

Extensive microarray analysis of soluble mRNAs in quiescent cells from stationary-phase cultures suggests that these cells are prepared to survive long periods of stress (Aragon et al. 2008). These mRNAs encode proteins involved in vesicle-mediated transport and other membrane-related functions, oxygen and reactive oxygen metabolism as well as response to oxidative stress, fatty acid oxidation, and signal transduction (Allen et al. 2006; Aragon et al. 2008). Other studies demonstrating the selective release of over 2000 mRNAs sequestered in protein-mRNA complexes *only* in quiescent cells, indicated that these cells are also poised to respond to a variety of environmental signals (Aragon et al. 2006, 2008).

In quiescent cells, the accumulation of mRNAs encoding key proteins in signaling pathways is particularly noteworthy. mRNAs encoding key growth-regulating kinases, including Tor1p and Tor2p and Tpk1p and Tpk2p (the catalytic subunits of cAMP-dependent protein kinase A), as well as the proteins that inhibit these kinases are abundant in quiescent cells (Aragon et al. 2008). Because down-regulation of both Tor- and Pka-regulated pathways is required for cells to survive in stationary phase (Gray et al. 2004), quiescent cells appear to be poised to respond to a variety of environmental signals and stress through rapid degradation or modification of inhibiting proteins and subsequent kinase activation. The observation that mRNAs encoding kinases and their inhibitors accumulate in quiescent cells is consistent with early biochemical work from our laboratory in which we demonstrated that, at the protein level, both Tpk1p and the cAMP-dependent, Tpk1p-inhibitor Bcy1p increase approximately 8-fold in cells in stationary-phase cultures (Werner-Washburne et al. 1991). Quiescent cells also contain fully functional mitochondria and exhibit relatively high rates of respiration (Davidson et al. 2011).

Non-quiescent Cells

After cultures exhaust glucose, a second, less-dense fraction is observed. This fraction retains viability, as determined by the accumulation of FUN1, indicating metabolic activity, and 60% of these cells remain viable up to 21 days post-inoculation (Fig. 6.3a). Despite their viability, non-quiescent cells rapidly lose the ability to reproduce (Fig. 6.3b) (Allen et al. 2006). Mothers and daughters in the non-quiescent fraction show similar losses of reproductive capacity, indicating the loss of reproductive capacity is essentially independent of replicative age. By day 7, about 45% of these cells are ROS positive and by day 14, half are apoptotic or

senescent (Fig. 6.3c). At day 7, the non-quiescent cell fraction consists of about 50% daughters, suggesting cells continue to divide during the post-diauxic phase.

Microarray analysis of abundant, soluble mRNAs in the non-quiescent fraction identified those encoding proteins involved in Ty element transposition, DNA transposition and recombination, and DNA metabolism (Allen et al. 2006; Aragon et al. 2008). In contrast to our finding in quiescent cells, there are few mRNAs present in protein-mRNA complexes in the non-quiescent cell fraction. Non-quiescent cells also exhibit a loss of mitochondrial GFP-fusion proteins and essentially no respiration at 7 days post inoculation (Davidson et al. 2011). The abundance of mRNAs encoding proteins involved with DNA repair, recombination, and rearrangement is consistent with our finding that by 14-days post inoculation, 50% of non-quiescent cells are apoptotic or necrotic (Fig. 6.3c). These results indicate that the physiology of quiescent and non-quiescent cells is distinct and that non-quiescent cells appear to be in a transition that would keep them from re-entering the mitotic cell cycle much less responding to environmental changes.

The identification of apoptotic, non-quiescent cells relates directly to the extensive work done by Frank Madeo, Valter Longo, Michael Breitenbach, and others in the area of apoptosis and aging (Breitenbach et al. 2003; Buttner et al. 2006; Fabrizio and Longo 2008; Frohlich and Madeo 2000; Laun et al. 2001; Longo et al. 1997; Madeo et al. 1997, 2002; Rockenfeller and Madeo 2008). While these studies of aging have focused primarily on cells in minimal medium, experiencing heat or oxidative stress, or aged mother cells, the same characteristics are also observed in cells in stationary-phase cultures grown in rich, glucose-based medium. In fact, a new paper shows that in the minimal medium used for aging studies, quiescent cells are dying and undergoing hypermutation (Madia et al. 2009), a process we predicted would happen under extreme stress, based on sequestered mRNAs (Aragon et al. 2006). Deletion of *SCH9,* an Akt homologue, restores survival of quiescent cells (Madia et al. 2009), suggesting that cells are better able to down-regulate this kinase in rich than in minimal medium.

These connections make it is clear that research into aging and stationary phase can provide important, reciprocal insights. Our work in stationary phase provides a developmental story to the induction of apoptosis. It identifies specific cell populations that become apoptotic, strengthening the hypothesis that induction of apoptosis is not a completely uncontrolled event, but part of a multi-pronged effort for species survival. It also provides insight into CLS and perhaps identifies a novel type of aging, not yet studied.

Mutability and Loss of Mitochondrial Function in NQ Cells

At 7-days post inoculation, the high proportion (40%) of reproductively competent non-quiescent cells produce petite colonies (Fig. 6.4), indicating a high rate of mutation affecting mitochondrial function.

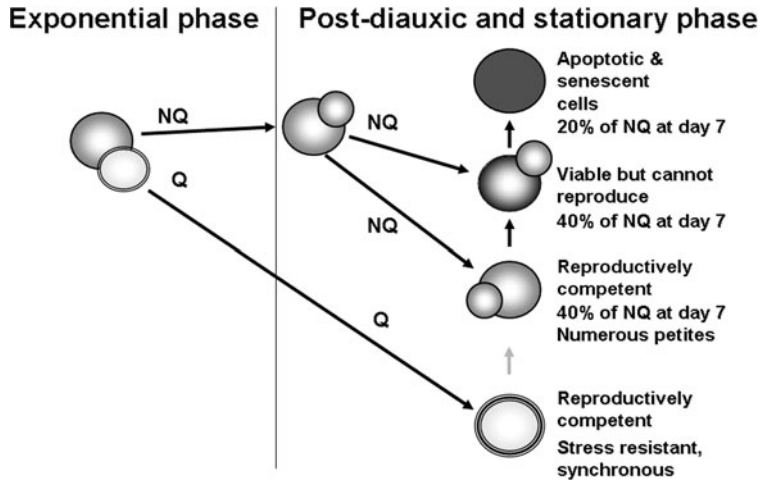

Fig. 6.4 Model of quiescent (Q) and non-quiescent (NQ) cell differentiation during growth to stationary phase in rich glucose-based medium. Daughter cells and mother cells in exponential phase cultures have distinct trajectories at or about the time glucose exhaustion. After the diauxic shift, cell division is symmetric with respect to protein abundance (Davidson et al. 2011). The lighter arrow from Q cells indicates that Q cells have the capacity to become NQ cells but it probably occurs at a slow rate over time under normal conditions. © Davidson et al. (2011). Modified from a figure originally published in Mol Biol Cell 22:988–998

Many reports have indicated that decreased mitochondrial function leads to decreased modulation of chronological lifespan and, depending on the mutation, an increase or decrease in apoptosis (Aerts et al. 2009; Breitenbach et al. 2003; Buttner et al. 2008; Eisenberg et al. 2007). Thus, cells in the non-quiescent cell fraction producing petite colonies may represent intermediates that have started to mutate but not advanced to a full apoptotic state. RLS studies have shown that older mother cells exhibit an increase in production of daughters that form petite colonies (Laun et al. 2007). However, the production of petite colonies from non-quiescent cells from stationary-phase cultures is relatively independent of age (Allen, unpublished).

We hypothesize that the mutable population of non-quiescent cells is also the source of previously observed genomic rearrangements (Coyle and Kroll 2008) and movement of transposable elements (Dunham et al. 2002) in stationary-phase cultures or yeast cells grown in glucose- or carbon-limited medium. Our observations are also consistent with recent work identifying genomic instability in mother cells (Qin et al. 2008). We hypothesize that reproductively competent, non-quiescent cells may represent the widely debated "hypermutable" fraction reported in microbial literature (Gonzalez et al. 2008; Sniegowski 1995; Sundin and Weigand 2007) and that, because this mutability is predictable and occurs in response to a common stress, this subpopulation may contribute significantly to yeast evolution.

Sudden-Onset, Environmentally Stimulated Aging?

By 7-days post inoculation, approximately 50% of viable non-quiescent cells have lost the ability to reproduce. This loss of reproductive capacity is independent of age (Allen et al. 2006). This phenotype does not seem to fit either reproductive or chronological aging and occurs to all mothers present during exponential growth and daughters that are produced after glucose exhaustion (Fig. 6.4). Genes have also been identified that affect this loss of reproductive capacity, indicating that this process is genetically programmed (Aragon et al. 2008). Because non-quiescent and quiescent cells are present in the same culture, we believe that the sudden loss of reproductive capacity could represent a cell-type specific aging event and not simply chronological aging.

The Community of Cells in Stationary-Phase Cultures

At7-days post inoculation, the community structure of cells in stationary phase culture is complex (Fig. 6.4). It includes quiescent cells, mostly daughters, with the potential to respond to environmental stimuli in a variety of ways, including becoming apoptotic (Aragon et al. 2008). Non-quiescent cells consist of at least two cell types: those that can divide and those that cannot. Those cells that can divide appear to be "hypermutable" suggesting that they may be the "Hail Mary play" for the species, with a chance of survival in a completely novel environment. The numbers of reproductively competent non-quiescent cells decrease over time and, we assume (but have not tested) that, eventually all the non-quiescent cells become apoptotic or necrotic. It is easy to imagine a scenario in which the non-reproductive, non-quiescent cells serve as food stores for quiescent cells and it is extremely interesting to speculate that they may actually signal quiescent cells not to divide (Buttner et al. 2006; Herker et al. 2004). It also seems possible that quiescent cells may signal non-quiescent cells, regulating the progression of non-quiescent cells into senescence and apoptosis.

Whatever the community interactions in these cultures, it is clear that stationary-phase cultures are more complex than previously assumed. It is also clear that this community structure is predictable and genetically controlled, based on the identification of mutants that affect the phenotypes of each cell type (Aragon et al. 2008). It should also be clear to the reader that both the structure and heterogeneity have important implications on a variety of levels for aging studies, especially studies of CLS.

Significance of This Differentiation for Aging Studies

To summarize, there are several important contributions that work on stationary-phase cultures makes to aging studies. These include:

- Demonstrating that: (1) stationary-phase cultures are not homogeneous and (2) loss of reproductive capacity of non-quiescent cells is not the same as death.
- Providing easy methods for isolating quiescent cells, which appear to be the best, most homogenous starting material for large-scale CLS studies.
- Identifying a new cell type, quiescent cells, that appear to be ideal for large-scale, RLS studies, also. These cells may continue to divide synchronously and, if daughter cells are removed, they could provide larger samples of same-aged cells, increasing the breadth of analyses that are possible.
- Providing a new aging paradigm: the environmentally regulated, sudden loss of reproductive capacity independent of reproductive age, observed in the non-quiescent cell fraction (Allen et al. 2006).
- Identifying cell types that should allow a more detailed analysis of the regulation of induction of apoptosis in yeast. If markers can be identified that allow early differentiation of subpopulations of the non-quiescent cell fraction, it would lead to greater ability to study the induction of apoptosis and its relationship to observed hypermutability in non-quiescent cells.
- Identifying a new model system to study the roles of cellular differentiation and community structure in aging. Because of the tractability of yeast, this approach may lead to important insights into aging in more tissue-complex model organisms, from flies and worms to humans (Kuningas et al. 2008).
- Pushing back the "black box" and providing a foundation for studies of cellular communities that are even older, i.e. weeks, months, or years. Several laboratories have observed a "regrowth" phenomenon in very old yeast cultures from a variety of strains (Martinez et al. 2004; Minois et al. 2009). In this situation, colony-forming units per ml decrease and then, after a week or more, begin to increase. The origin of the reproductively competent cells is not known, but could represent outgrowth of a mutant arising in stationary phase. The significance of this phenomenon and its relationship to the long-term survival of quiescent and non-quiescent cells has not been explored.

CLS Studies and the Complexity of Stationary-Phase Cultures

One of the major assays for CLS is to measure the ability of cultures to increase in cell density after re-feeding a stationary-phase culture. Even if we allow that it is not death but the ability to reproduce that is indicative of chronological aging, the assay is still unable to detect the important phenotypic differences of cells in mixed populations. The complexity of these cultures means that by measuring simply reproduction, genes could be identified that increase or decrease: (1) the loss of reproductive capacity of non-quiescent cells by a variety of effects on apoptosis, mutation, etc.; (2) the life span of quiescent cells (which would probably be the most accurate, CLS phenotype); and (3) the reproductive competence of quiescent or non-quiescent cells by changing the community dynamics through effects on inter-cellular communication, release of nutrients from non-quiescent cells, or other physiological states of any of the subpopulations.

VxInsight Clustering of "Aging" Genes in Quiescent/Non-quiescent Cell Dataset

VxInsight is a clustering and topology program developed at Sandia National Laboratories (Aragon et al. 2008; Davidson et al. 1998, 2007). We have used this program in the past to analyze microarray/gene expression data and overlay other types of data, such as protein-protein interaction data (Werner-Washburne et al. 2002). In VxInsight, data from ≥15 arrays are imported and the correlation of expression (Pearson's R or other correlation) of each mRNA/gene is calculated in an all-against-all manner. Clustering based on Pearson's R or other correlation statistic is done by force-directed placement, an unsupervised method that clusters genes into hills representing gene clusters (Fig. 6.5).

The height of the hill is related to the number of genes in that cluster. VxInsight topographies can be shown with the skin on (Fig. 6.5) or off (Werner-Washburne et al. 2002), in which each dot represents a gene. It is possible to show gene names, degree of correlation, or other attributes through addition of gene lists to the underlying database. Although VxInsight is not commercially available now, research licenses can be requested through Sandia National Laboratories.

To carry out a preliminary analysis the potential for previously identified aging genes to affect different cell types in stationary phase cultures, we used the top 200 genes identified from a classical chronological aging study (Powers et al. 2006). In this study, genes essential for chronological aging were identified by growing individual yeast deletion mutants in 96-well plates for various lengths of time. After incubation, aliquots were tested for their ability to grow when re-fed rich medium. "Aging genes" were identified as those genes, which when deleted, resulted in increased culture density after 24 h. We asked whether the top 200 aging genes

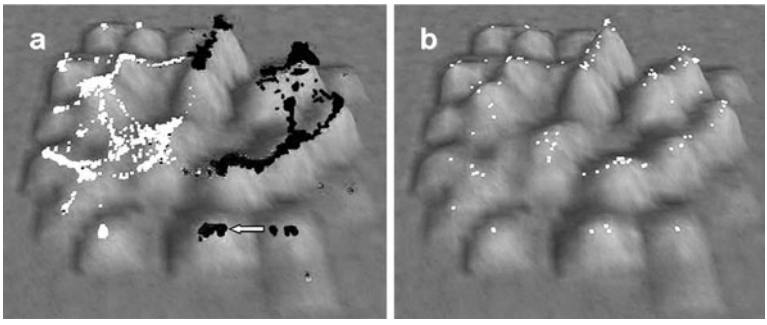

Fig. 6.5 Localization of Q, NQ, and aging-related genes on VxInsight cluster map of Q/NQ compendium data. **a** *White dots* on the *left half* of the cluster map identify the cluster location of genes that encode NQ-abundant mRNAs; *black dots* on the *right hand side* identify the cluster location of genes encoding Q-abundant mRNAs. The *black* and *white arrow* points to the cluster of cytoplasmic, ribosomal genes. **b** *White dots* identify the cluster location in the same cluster map as in (**a**) of the top 200 aging-related genes from (Powers et al. 2006). © Aragon et al. (2008). Modified from a figure originally published in Mol Biol Cell 19:1271–1280

clustered with mRNAs from quiescent or non-quiescent cells, assuming that genes whose mRNAs accumulated in different populations might differentially affect these populations. The gene expression topology was generated by VxInsight (Davidson et al. 1998; Werner-Washburne et al. 2002) using data from approximately 200 microarrays of mRNA isolated from quiescent and non-quiescent cells (Fig. 6.5) (Aragon et al. 2008).

We have shown previously that mRNAs that accumulate in quiescent or non-quiescent cells define two distinct halves of the topology (Fig. 6.5a) (Aragon et al. 2008). There are more genes on the right side (Q) of the topology than the left (NQ). The white arrow marks the location of most ribosomal genes. It can be seen that the 200 aging genes are distributed relatively randomly in both halves of the topology (Fig. 6.5b), suggesting that these genes might affect different cell subpopulations.

Recent data from a study of alleles of *ssd1*, a gene known to regulate several genes involved in CLS, provided the first evidence of differential effects on quiescent and non-quiescent cells (Li et al. 2009). *ssd1* alleles affected quiescent cell reproductive capacity but not viability while it affected both loss of viability and reproductive capacity of cells in unseparated stationary-phase cultures. This evidence suggests that *SSD1* affects non-quiescent cell viability independent of its effect on quiescent cells.

Multi-Cellularity, Communities in Stationary-Phase Cultures, Stem Cell Niches, and Aging

The surprising discovery of differentiation of cells in stationary-phase yeast cultures led us to ask whether the community structure in these cultures is analogous to cellular organization in other organisms, including multi-cellular organisms and how this might relate to aging. Quiescent yeast cells exhibit many characteristics seen in other types of quiescent cells, including eggs, neurons, and stem cells. They are daughter cells, for the most part, they have abundant localized mRNAs associated with protein-mRNA complexes, low ROS and apoptosis, and, like eggs and stem cells, retain the ability to reproduce (Allen et al. 2006; Aragon et al. 2006, 2008). Although yeast cells divide asymmetrically prior to glucose exhaustion and exhibit lineage-specific inheritance from spores (Thorpe et al. 2009), preliminary results suggest that cells in the post-diauxic phase divide symmetrically, with respect to protein expression (Werner-Washburne, unpublished). Like other quiescent cells, yeast quiescent cells (based on mRNA sequestration and accumulation of signal transduction proteins and mRNAs) are poised to respond in different ways to a variety of environmental signals (Aragon et al. 2006, 2008; Gray et al. 2004).

Fairly recently, researchers working with bacteria have begun to accept earlier models of bacterial heterogeneity in clonal populations and have started to study the extent and predictability of this heterogeneity (Rosenberg 2009). These heterogeneous populations, found in starved cultures, have been termed "multicellular" because the observed differentiation appears to provide a community

benefit. The cell types observed in *E. coli* cultures appear to be very similar to cell types observed in stationary-phase yeast cultures. In bacteria, these subpopulations include a small population of mutating cells, persister cells that can live for years; and cells that undergo programmed cell death (Amitai et al. 2009; Lewis 2000, 2007). Unlike yeast, the specific cells from which these cell types arise is not yet known. However, *Vibrio* sp. and other types of bacteria, when starved, have been shown to differentiate into at least two populations of cells separable by density gradient centrifugation (Nayak et al. 2005; Nishino et al. 2003), suggesting some similarities with yeast stationary-phase cultures. Bacterial multi-cellularity is clearly a compelling problem because of the high level of conservation of these cell types and the ability to use these systems to understand the role of cell-cell signaling in the formation of complex communities (Lopez et al. 2009).

In higher eukaryotes, one of the major areas of aging research related to quiescent cells is the examination of aging in stem cell niches from *Drosophila* to humans (Drummond-Barbosa 2008; Rossi et al. 2008; Wallenfang 2007). One of the hypotheses is that stem-cell aging actually is the ultimate cause of age-related death because of the inability of tissues to repair themselves. Although stem cell function declines with age, it is not clear whether aging causes stem cell decline or stem cell decline causes aging (Drummond-Barbosa 2008).

Both extrinsic factors, such as signaling from niche cells adjacent to stem cells, as well as intrinsic factors, such as DNA repair; and protection from oxidative stress, play a role in stem cell survival and function (Drummond-Barbosa 2008). Extrinsic factors have been shown to play a significant role in maintenance of function of mouse spermatogonial stem cells and satellite stem cells in mouse muscle because these cells are fully functional when positioned in niches or provided with fluids from cells of younger mice (Wallenfang 2007). Recently, it was shown that apoptosis of *C. elegans* oocytes extends the life span of the remaining oocytes. This observation is consistent with the idea that quiescent cells are also prepared for apoptosis, because the most significant group of sequestered mRNAs in quiescent cells encode the Ty-element transposition, DNA repair and recombination, identical to the abundant, soluble mRNAs in non-quiescent yeast cells (Aragon et al. 2006, 2008).

With respect to intrinsic factors, conserved pathways related to nutrient status and/or insulin signaling are involved in regulating stem cell maintenance and proliferation in both *C. elegans* and *D. melanogaster* (Bartke 2008; Narbonne and Roy 2006; Shimokawa et al. 2008; Waskar et al. 2005), including orthologs of *SNF1, TOR1*, and *PHO*85 (Zaman et al. 2008). mRNAs encoding these proteins are not only conserved in yeast but accumulate in quiescent cells (Aragon et al. 2008). Stem-cell life span is also known to be extended by overproduction of superoxide dismutase (Drummond-Barbosa 2008). In hematopoietic systems, it is not clear whether depletion in the stem cell pool as mice age is caused by loss of reproductive capacity, but ROS management, control of DNA repair, and telomere maintenance have been shown to be important (Warren and Rossi 2009). Telomere maintenance in quiescent yeast cells has not been studied, but DNA repair and decreased ROS are

clearly associated with survival of quiescent cells and loss of reproductive capacity of non-quiescent cells.

It is not within the scope of this chapter to dig more deeply into the similarities between yeast quiescent cells and stem cells or the complexity of the community structure in yeast stationary-phase cultures and stem cell niches. However, there are striking similarities and yeast has already proven to be an exceptional model system for other highly conserved processes; thus pursuing this line of thought is compelling. The kinds of questions being asked about stem cells and aging could easily be addressed using the community of cells present in stationary-phase yeast cultures. The only element of this relationship in yeast that still needs to be demonstrated is cell-cell communication – beyond production of ethanol by non-quiescent cells that would keep quiescent cells stable longer. Earlier work has suggested that a chemical exists in the medium that does this signaling (Herker et al. 2004), but it has not been further characterized or the effect shown specifically for quiescent cells. Finally, while adhesion molecules may be unique to tissue-complex eukaryotes, in yeast strains in the wild, lectin-mediated flocculation is typical and, if strains do not flocculate during growth on glucose, they clump as soon as glucose is exhausted (Mota and Soares 1994; Soares and Mota 1996; Stratford 1993) – exactly the time at which the differentiation into the complex community observed in stationary-phase cultures begins.

As we learn more about differentiation of yeast cells in stationary-phase cultures and the role of different cell types in aging, we are likely to find that the process by which microorganisms survive starvation is a primitive model of the process that leads to the development and survival of the most complex organisms on earth. We are also likely to find that aging is even more complex than we imagined, that it is a multi-cellular process, and that all cells on earth are more connected than we know. It is heartening, in this era of translational medicine that yeast, which has proven to be an astounding organism for biological research for well over 100 years, still has the power to surprise us and open our eyes to new worlds.

Addendum

Since this chapter was written, several relevant papers have been published. First, acetic acid poisoning was shown to be responsible for the death of cells in minimal medium (SC) (Burtner et al. 2009). This is consistent with our early observations that cells die in this medium but retain viability in rich, glucose-based medium (Werner-Washburne, unpublished) and consistent with the observation that dying Q cells are the source of mutation in SC medium (Madia et al. 2009). We determined that NQ cells are mutating and Q cells are stable for at least 14 days in YPD. Breeden's laboratory has taken Q cells in water for 11 weeks, showing they retain high viability (Li et al. 2009). However, we also noted that the largest group of sequestered mRNAs in Q cells, like the largest groups of soluble mRNAs in NQ cells, encoded proteins involved in DNA transposition, recombination, and repair,

and suggested that under some conditions, Q cells were capable of exhibiting the high mutation rates of NQ cells (Aragon et al. 2006).

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Chapter 7
Maximising the Yeast Chronological Lifespan

Peter W. Piper

Abstract When investigating aging it is important to focus on the factors that are needed to attain, and which can be manipulated to extend, the longest lifespans. This has long been appreciated by those workers who use *Drosophila* or *Caenorhabditis elegans* as model experimental systems to study aging. Often though it seems it is not a consideration in many studies of yeast chronological aging. In this chapter I summarise how recent work has revealed the preconditioning that is needed for yeast to survive for long periods in stationary phase, therefore for it to exhibit a long chronological life span (CLS). Of critical importance in this regard is the nature of the nutrient limitation that, during the earlier growth phase, had forced the cells to undergo growth arrest. I have attempted to highlight those studies that have focussed on the longest CLSs, as this helps to identify investigations that may be addressing – not just factors that can influence chronological longevity – but those factors that are correlated with the authentic processes of chronological aging. Attempting to maximize long-term stationary survival in yeast should also enhance the potential relevance of this organism as an aging model to those who wrestle with the problems of aging in more complex systems. Finally I also give a personal perspective of how studies on the yeast CLS may still yet provide some important new insights into events that are correlated with aging.

Keywords Chronological lifespan · Survival · Starvation · Somatic maintenance · High throughput screen

Abbreviations

CR	calorie restriction
Cu,Zn-Sod	copper zinc superoxide dismutase
Mn-Sod	manganese superoxide dismutase
ROS	reactive oxygen species

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The "Survival/Fecundity Balance": How Does It Impact on Yeast?

According to the disposable soma theory of aging (reviewed in Kirkwood 2008) processes associated with the age-related degeneration of cells and tissues over time (e.g. oxidative damage to DNA and proteins) can generally be slowed by the diversion of extra resources to somatic maintenance (e.g. by increasing DNA repair or protein turnover capacity). Survival and reproduction are two competing priorities, such that the allocation of extra resources to somatic maintenance in order to improve survival (longevity) will generally operate to the detriment of reproduction (fecundity). Many species, including the *Drosophila* and *C. elegans* models of aging, rapidly readjust this "survival/fecundity balance" in response to nutritional availability or environmental stress. Furthermore, it would seem that evolution has conserved much of the mechanism for this switching between basic maintenance and reproduction. Thus diverse organisms – from yeast through to mammals – are now known to achieve a balance between either growth/proliferation or longevity by the gain or loss of a conserved activity, the conserved TOR ("*T*arget *O*f *R*apamycin") protein kinase (Anisimov et al. 2010; Blagosklonny 2008; Powers et al. 2006).

In yeast this survival/fecundity balance is reflected in the capacity to switch between a stationary, nonreproductive cell (G_0-arrest, low metabolic activity and high stress resistance) when starved and an actively-dividing cell (high metabolic activity but lower stress resistance) when nutrients are abundant. Survival will depend on its ability to switch efficiently between these two cell states in response to nutrient availability. Furthermore, as long-term starvation is the condition most commonly encountered by microbes in the wild, one must expect that yeast can survive for months, or even years in the starved state. But how long can it survive? CLS is temperature-dependent (MacLean et al. 2001; Qin et al. 2008). Therefore the *maximal* yeast CLS might be strongly dependent upon the ambient temperature. The longest-lived organism identified to date is a mollusc which can live for up to 400 years on the seabed at 4°C (Abele et al. 2008). It is quite possible that senescence is completely arrested under such conditions. Nevertheless it would be very interesting to know if the psychrophilic yeasts found in Antarctica (Deegenaars and Watson 1998) are also extremely long lived. Recently it was shown that the long-term chronological survivals of yeast deletion mutants at 4°C do not correlate with their oxidative stress resistance or their lifespans as measured at higher temperatures (Postma et al. 2009). However this was a study of *S. cerevisiae*, a species that is not a psychrophile.

Nutritional Status During the Earlier Growth Phase, Genetic Background of the Strain and Heterogeneity in the Population of Aging Cells All Affect CLS

There have been a number of studies of yeast stationary phase (reviewed in Herman (2002), Werner-Washburne et al. (1996)) and, nowadays, all such investigations make the claim that they are addressing "chronological aging". Unfortunately, most

have not attempted to maximize chronological longevity. As described below, it is now apparent that CLS is heavily impacted by the precise conditions that the cells had experienced during the early stages of nutrient depletion (i.e. whether their earlier growth arrest was caused by a starvation for nitrogen, carbon, sulphur or phosphate) and the true nature of the "post-mitotic growth arrest" that this preconditioning had induced. The problem that now needs to be addressed is not the practical measurement of CLS (readily amenable to automation, see below) but whether – in each study – the preconditioning of the cells had enabled them to survive for long periods in stationary phase. If this latter condition is not met, the work might have identified factors that influence chronological longevity under the experimental conditions that were employed, but such factors might not be identical to those that delimit the maximal possible CLS.

This preconditioning of the cells is not the only problem in CLS analysis. CLS studies tend to use laboratory *S. cerevisiae* strains of different genetic origins, strains that exhibit dissimilar CLSs even under identical conditions of stationary maintenance (MacLean et al. 2001; Qin et al. 2008). Some of these strains have been quite extensively mutagenised in their history. They therefore carry poorly-characterised mutations that may reduce overall fitness and shorten life span. As this is less of a problem with the S288c-derived strains of the deletion mutant collection (Winzeler et al. 1999), it is generally now considered prudent to conduct most CLS analyses using these S288c-based strains, especially as this also facilitates comparisons between different studies (Piper 2006). However, increasingly secondary mutations are being discovered in this deletion mutant collection, raising some not insignificant concerns that unknown secondary mutations might have influenced some of the results of genomic screens for altered CLS. As such mutations will mostly be recessive, genomic screens for altered CLS should ideally use the library of diploid stains homozygous for single gene deletions. Despite this some genomic studies (Matecic et al. 2010) still use of the libraries of haploid deletion mutants.

As an additional complexity in CLS analysis, cultures of stationary yeast are not homogeneous. Instead they generally comprise at least two cell populations; truly quiescent (Go) cells, the daughters formed in the final cell division, and larger cells with higher ROS levels that more rapidly display the markers of apoptosis as they die in stationary phase (Fabrizio et al. 2004; Herker et al. 2004). Apoptosis of the latter, shorter-lived cells leads to the release of cell contents which – in dense cell cultures – "cross-feed" the longer-lived cells, thereby enhancing the survival of these longest survivors (Burtner et al. 2009; Herker et al. 2004).

The Strong Influence of Cell Preconditioning on CLS

After cultures have been grown to stationary phase in liquid media, CLS is generally measured as the percentage of the cells remaining viable over time. However, it is clear from the literature that there is no general consensus as to exactly how this should be done. Different laboratories grow their yeast to saturation using different growth media (Table 7.1). Some, after the cells reach stationary phase, leave

Table 7.1 Influences of growth medium and the subsequent cell maintenance conditions on longevity in stationary phase (data for cells maintained at 30°C, taken from several notable studies of chronological survival)

Initial culture and subsequent maintenance	Time for cell viability to decline to less than 50%; or below 1% in stationary cultures maintained at 30°C
SC[a], subsequent maintenance in "spent" medium	<50% ~5–7 d; <1% ~11–14 d (strain DBY746) (Fabrizio and Longo 2003; Wei et al. 2008) <50% ~8 d; <1% ~16 d, extended to ~17–20 d; <1% >30 d with lowering of initial glucose level to 0.5% (strain BY4741)(Smith et al. 2007) <50% extended to ~23 d with lowering of initial glucose level to 0.05%, or buffering the medium to pH6 (strains W303 and BY4743) (Burtner et al. 2009)
SC[a], subsequent maintenance in water	<50% ~11 d; <1% ~27 d (strain DBY746); <1% >40 d (strain BY4741) (Fabrizio et al. 2005; Fabrizio and Longo 2003)
SC – Hopkins mix[b], subsequent maintenance in "spent" medium	<50% ~15–18 d; <1% ~23 d if pregrown 2% glucose; <50% >30 d; <1% ~33 d if pregrown 0.5% glucose (strain BY4741) (Matecic et al. 2010)
SD[c] with essential growth supplements; subsequent maintenance in "spent" medium	<50% ~2–5 d; <1% ~10 d (strain DBY2006) (Bonawitz et al. 2006; Pan and Shadel 2009)
YPGlycerol[d], subsequent maintenance in water	<50% ~24 d; <1% ~50 d (strain FY1679-28c) (Harris et al. 2005)

[a]SC – Glucose Synthetic Complete medium (Ausubel et al. 2003) with 4x the recommended supplementation of auxotrophic requirements
[b]SC – Hopkins mix (Matecic et al. 2010)
[c]SD – Synthetic Defined Medium (Sherman 1991)
[d]YPGlycerol – 2% bactopeptone, 1% yeast extract, 3% glycerol.

the cells in the original ("expired", "spent") medium, determining CLS as the subsequent survival in this spent medium. Others, following the initial growth, will transfer the cells to sterile water, measuring CLS as the survival over time of these cells maintained in aqueous suspension. While this transfer to water is a simple manipulation that extends the CLS quite considerably, it can be viewed as imposing an extreme form of caloric restriction (Fabrizio et al. 2005).

Table 7.1, data compiled from a number of studies, shows how these different pretreatments can have a marked affect over the CLS. Cultures grown on glucose yeast nitrogen base (SD) medium exhibit a relatively poor stationary survival. One must therefore question why certain laboratories that claim to be studying chronological aging are still using this medium for growing their cultures. In contrast, glucose synthetic complete medium (SC) – especially when supplemented with excess levels of auxotrophic requirements and used in conjunction with lowered glucose levels so that the cells are calorically restricted during their growth – yields cells of a much longer CLS. SC Hopkins mix – richer in amino acids compared

Table 7.2 Treatments that increase longevity in stationary phase

Treatment	Probable reason for the increase in chronological survival
Stationary maintenance at lower temperature (MacLean et al. 2001)	True for diverse species, probably a reflection of slower metabolism
Stationary maintenance in water, not the original "spent" culture medium (Burtner et al. 2009; Granot and Snyder 1993; MacLean et al. 2001)	When cultures are pregrown on glucose the medium accumulates millimolar levels of acetic acid which act to shorten CLS (Burtner et al. 2009); also the medium pH often declines to ~2–3, a factor that contributes to the leakage of glutathione from the cells (Perrone et al. 2005). Buffering cultures at pH 6 extends CLS (Burtner et al. 2009; Matecic et al. 2010)
Increasing the levels of respiration during the initial growth period, achieved either genetically (e.g. by Hap4 overexpression (Piper et al. 2006)); by preculturing the cells in medium containing 0.5% rather than 2% glucose ("caloric restriction"); or by growth on a respiratory carbon source (Barros et al. 2004; Burtner et al. 2009; MacLean et al. 2001; Matecic et al. 2010; Oliveira et al. 2008)	Now validated in numerous studies. It is probably a reflection of efficient survival in stationary phase requiring respiratory metabolism (Werner-Washburne et al. 1996). The increase in respiration with caloric restriction leads to increases in NAD+/NADH ratio; reduced oxidative stress (Barros et al. 2004); increased mitochondrial biogenesis; slower apoptotic DNA degradation (Weinberger et al. 2007); and reduced medium acetic acid levels (Matecic et al. 2010)
Increasing the osmolarity of the medium during stationary maintenance (Smith et al. 2007; Weinberger et al. 2007)	Suggested to act similarly to caloric restriction (Kaeberlein et al. 2002; Weinberger et al. 2007)
Ensuring the cells do not enter stationary phase as the result of starvation for an auxotrophic requirement (Boer et al. 2008; Gomes et al. 2007)	Starvation caused by limitation of auxotrophic requirements results in a failure to achieve a prompt cell cycle arrest and entry to Go, unlike starvation for either carbon, phosphate or sulphate (Boer et al. 2008)
Removal of the preferred nitrogen sources asparagine and glutamate from SDC media (the total nitrogen content being held constant) (Powers et al. 2006)	Thought to act by causing a diminished TORC1 signalling in response to deficits in levels of intracellular amino acids (especially glutamine) (Powers et al. 2006)

to the standard SC, appears to be increasing survival still further (Matecic et al. 2010). Caloric restriction serves to enhance the levels of mitochondrial respiration, the latter a key determinant of an extended CLS (Table 7.2). Our laboratory actually pregrows cultures on a respiratory carbon source (glycerol) in order to ensure that they have a long CLS (MacLean et al. 2001; Piper 2006). It should be noted that YEPD (Sherman 1991) – the "workhorse" rich glucose medium of the yeast laboratory – is not usually considered ideal for CLS studies, as it is difficult to determine

when a true stationary phase starts, the initial phase of rapid growth on glucose being followed by postdiauxic phase of slow respiratory growth on ethanol that can last for up to 4–8 days (Barford 1990). YEPD-grown cells are still though employed in certain studies of stationary phase (Pawar et al. 2009).

The synthetic media are commercially available from several companies, including Difco, Clontech, and QBioGene Inc.

How Do These Pretreatments Impact on the Determinants of Longevity?

The different pregrowth conditions in Table 7.1 are now known to impact on a number of the factors which influence how long cells will survive in stationary phase (see Table 7.2 for summary). Only recently has the importance of some of these factors been fully appreciated, for example the highly detrimental effect of a growth arrest caused by starvation for an essential auxotrophic requirement on subsequent survival (Boer et al. 2008; Gomes et al. 2007).

The Practical Measurement of CLS

CLS is measured by determining the percentage of cells that remain viable over time when cultures, initially pregrown in liquid media (Table 7.1), have entered stationary phase. Generally this is done by the traditional colony counting approach – periodically removing an aliquot of the aging culture so as to determine what fraction of the cells are still capable of forming a colony when plated onto rich medium agar plates (either by "spreading" or by "pinning" serial dilutions onto the agar plates). This method of determining cell viability has certain advantages. It is simple. It also provides an ability to monitor the CLS of a large number of individual mutants or growth conditions at the same time. Several strains can be pregrown in 96 microtitre well format. They can then be left in their original "spent" medium for CLS determination. Alternatively, the cells can be spun down, washed (resuspended in water then repelleted) and finally resuspended in water for long term stationary maintenance – all in the same microtitre plate. To make the procedure less labor-intensive, a simple robot system can be used to routinely pin the cells onto rich media as they age, providing an assay that is about as consistent as the traditional colony counting approach to determining maximal CLS (Fig. 7.1). A disadvantage of these agar plating/colony counting techniques, though, is that they can miss relatively small differences in CLS.

More high-throughput methods of determining the CLS of large numbers of strains have now been developed, mainly to facilitate genome-wide investigations designed to identify mutants of increased chronological longevity. Two such approaches to analyzing CLS of collections of gene deletion strains have been sued to date:

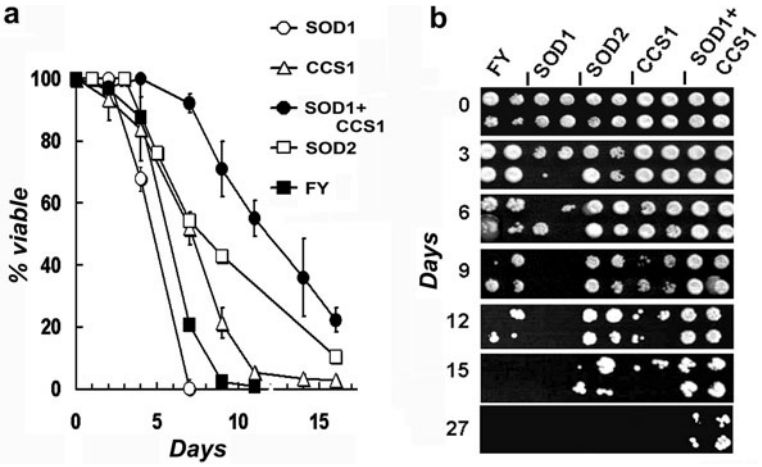

Fig. 7.1 Automated measurements of 37°C CLS in microtitre plate format for a wild type strain (FY) and its derivatives engineered for overexpression of the genes for either mitochondrial Mn-Sod (SOD2), the Sod1 apoprotein of cytosolic Cu,Zn-Sod (SOD1), Ccs1 the chaperone that inserts copper ions into the Sod1 apoprotein (CCS1), or a combined Sod1 and Ccs1 overexpression (SOD1+CCS1). **a** Life spans as determined by manual dilution, replica plating and colony counting, showing how these strains have varying lifespans, either shorter (SOD1) or longer (SOD2, CCS1, and SOD1+CCS1) than that of the FY parental strain (data from Harris et al. 2005). **b** Viabilities of the same liquid cultures as determined by automated replica platings at 0, 3, 6, 9, 12, 15, 27 days. At each of these times 4 replica platings from each culture were performed (cultures arrayed in 96 well plate format, replica platings performed using the 96-pin replicator of a Biomek® 2000 Laboratory Automation Workstation, with movements programmed using the BioWorks™ Version software (Beckman)), each of these replica platings being programmed to 4 adjacent positions of a YPD agar plate). Plates were photographed after 3 days growth at 30°C. The data from the automated replica platings in (**b**) closely correlates with that obtained by manual dilution and replica plating in (**a**)

- The mutants are initially grown (each mutant as an individual culture so as to avoid any effects of the mutation affecting growth rate), these individual cultures then being mixed and *chronologically aged as a pool* in liquid culture. Aliquots of this pool of aging mutants are then selected, so that the mutants that are still viable can be detected, at sequential time points. One such study (Matecic et al. 2010) used the collection of haploid deletion strains, each tagged with a unique, identifying DNA "bar-code" sequence, the mutants remaining viable in the pool being grown, then detected, using microarray DNA hybridization to quantify the abundance of the barcode tags. There is also now the possibility to apply recently developed, very high throughput sequencing techniques (http://www.dnavision.com/nextgen-sequencing.php) in order to rapidly identify the long-term survivors in such a mixed culture population.
- As an alternative approach, the *chronological survival of each mutant can be analysed individually*, the gene deletion mutants arrayed in small aging cultures. The Kaeberlein lab has pioneered this latter approach, their procedure involving the robotic transferral of a small aliquot of each of the arrayed deletion mutant

cultures – at different time points as they age – to rich medium, then a monitoring of the kinetics of outgrowth of cells in these rich medium cultures (Burtner et al. 2009; Murakami et al. 2008; Olsen et al. 2010; Powers et al. 2006). Such outgrowth is readily measured by any apparatus (e.g. a Bioscreen (www.bioscreen.fi/bioscreen.html)) that can record increases in optical density over time.

These two alternative ways of screening the deletion mutant collections both have their merits. Whilst they readily identify large numbers of mutants with a shortened CLS, it is a more demanding challenge for them to comprehensively identify all the mutants in a strain collection that have an extended CLS. Notably some of the long-lived mutants identified by the mixed population approach were not detected when mutants were grown individually (Matecic et al. 2010). There are a number of possible reasons for this, for example the use of haploid (Matecic et al. 2010) versus diploid (Powers et al. 2006) deletant strain collections, or differences in the levels of "cross-feeding" of cells by the nutrients released by cells undergoing apoptosis. In the initial screen (Powers et al. 2006) mutants were individually pregrown on 2% glucose SDC, then maintained in the expired medium. Subsequent mixed population screening was able to demonstrate that most long-lived mutants have their lifespan increased by a pregrowth in 0.5% compared to 2% glucose SC ("caloric restriction") (Matecic et al. 2010). It should be noted that all the genome-wide screens for mutants of long CLS to date appear to have been conducted on cultures maintained in the original growth medium, not cultures aged in water. Such a regimen has probably resulted in long, but not maximal, CLS (Table 7.1). Therefore, while these studies have clearly identified a number of the key determinants of chronological longevity, it is still debatable whether they have identified all of the factors needed to ensure and extend the maximal CLS.

A major finding to emerge from these screens is that decreased activity of the TORC1 signalling pathway increases CLS (Powers et al. 2006). Structurally and functionally conserved from yeast to man, TOR complex 1 (TORC1) and TORC2 are large, essential multiprotein assemblies (Urban et al. 2007; Wang and Proud 2009; Wullschleger et al. 2006). TORC1 is active when sufficient nutrients are present and noxious stressors are absent, mediating the signals that promote cell growth through the stimulation of anabolic processes, such as protein synthesis and ribosome biogenesis, and the inhibition of catabolic processes, such as autophagy.

The autophagy promoted by decreased TORC1 signalling is critically important both for a long CLS (Alvers et al. 2009; Matecic et al. 2010) and for any extension to this CLS induced by low concentrations of rapamycin (Powers et al. 2006). Clearly it is vital to have an ability to recycle resources in starvation, probably not just because of the need to regenerate cellular components, but also to eliminate the damaged proteins that arise as the cells age. In vertebrate species, mTOR protein kinase is absolutely essential in development, but in nondividing cells and tissues it appears to drive the events of senescence and ageing (Blagosklonny 2008). mTOR would seem therefore to be a classic example of a function which exhibits antagonistic pleiotropy – beneficial early in life but causing aging later (Kirkwood 2008).

Some Future Challenges for Studies on the Yeast CLS

Investigating Which Aspects of TORC1 Signalling Are Fundamental for Longevity

Structurally and functionally conserved from yeast to man, TORC1 is subject to much more diverse and complex regulation in the latter (Urban et al. 2007; Wang and Proud 2009; Wullschleger et al. 2006). Even in yeast, TORC1 has so far only been shown to phosphorylate two activities *directly*, Tap42 and Sch9 (Huber et al. 2009; Urban et al. 2007). Sch9 is a protein kinase, the orthologue of the ribosomal S6 kinase that was recently shown regulate lifespan in (female, but not male) mice (Selman et al. 2009). A downregulation of Sch9 appears to be a major factor in the CLS increase caused by loss of TORC1 activity, since lack of Sch9 extends the yeast CLS (Fabrizio and Longo 2003). One of the major downstream targets of Sch9 is Rim15, the latter a protein kinase that activates the gene expression needed in order to reprogramme cells for efficient maintenance in G_0. With TORC1 active, Rim15 is phosphorylated by Sch9, causing it to become associated with 14-3-3 proteins and retained in the cytoplasm. With the inactivation of TORC1 signalling, Rim15 becomes dephosphorylated and enters the nucleus, whereupon it induces the transcriptional programme for entry to G_0 (Wang and Proud 2009; Wullschleger et al. 2006). This series of events – critical for the downregulation of TORC1 to establish the physiological state that will ensure a long CLS – would appear to be occurring during the *entry* to stationary phase, since the CLS increase induced by the TORC1 inhibitor rapamycin is only apparent if this drug is administered *before* the cells enter stationary phase (Powers et al. 2006). Most rapamycin-dependent changes to the yeast phosphoproteome appear to be directed through Sch9 (Huber et al. 2009).

Even in yeast the events upstream of TORC1 that regulate TORC1 activity; as well as events downstream of TORC1 that couple this activity, also Tap42 and Sch9, to their diverse range of readouts are only partially characterized (Huber et al. 2009; Wang and Proud 2009). Over the next few years we can expect a much clearer picture to emerge of how individual regulatory proteins participate in this signaling and influence CLS. Some of these aspects (e.g. TORC1 effects on maintenance repair of DNA, see below) are still relatively unstudied. Yeast, with its amenable genetics, will undoubtedly provide a number of unique insights. There is also the possibility to exploit strains of yeast in which TORC1 function is completely bypassed genetically (Huber et al. 2009).

Investigating Why Increased ROS Scavenging Has Markedly Effects on CLS in Yeast, But Not It Seems in Other Model Systems of Aging

CLS is strongly influenced by the levels of respiratory activity, these in turn affecting both NAD+/NADH ratios (Barros et al. 2004; Jimenez-Hidalgo et al. 2009) and

the resistance of the cells to oxidative stress (Harris et al. 2003, 2005; Longo et al. 1996; Sturtz et al. 2001). So interrelated are the redox status and oxidative stress resistance of cells, that it is practically impossible to separate their roles in the determination of CLS. Remarkably the yeast CLS is not markedly shortened with loss of the transcriptional regulators of the oxidative stress response, some of which are redox-regulated (Piper 2006). It seems doubtful therefore that chronologically-aging yeast mounts any effective transcriptional response to its increased prooxidant status over time (Piper 2006). Instead it appears more important that the cells are preprogrammed for an efficient maintenance in G_0 through the aforementioned actions of Rim15.

We found CLS to be extended only slightly by catalase overexpression, rather more by an overexpression of the mitochondrial Mn-Sod, and up to 2-fold by a 7- to 8-fold overexpression of active Cu,Zn-Sod (Harris et al. 2005). These dramatic effects of an increased Cu,Zn-Sod activity on the CLS in yeast seem counterintuitive to the findings in other model systems of aging. The initial reports of the *Drosophila* lifespan being extended by Cu,Zn-Sod overexpression could not be confirmed in longer lived fly lines (Orr et al. 2003); neither did Sod overexpression extend lifespan in *C. elegans* or in mice (Doonan et al. 2008; Perez et al. 2009). While overexpression of MnSOD increased lifespan by 20% in adult *Drosophila*, it appears this may be an effect exerted through a reduced insulin/IGF-1 signalling (Curtis et al. 2007). One possible explanation for this abnormally strong effect of Cu,Zn-Sod overexpression on the yeast CLS is the tendency of stationary yeast to lose its glutathione to the medium (Perrone et al. 2005). Loss of glutathione will severely compromise those antioxidant defences that rely on the reduced forms of glutathione and thioredoxins, with the result that the activities that scavenge superoxide and peroxides will then become much more critical in the prevention of oxidative damage. Whether this is indeed a major factor, or whether stationary yeast is unable to repair some critical target of oxidative damage (see below), remains an unresolved issue.

Investigating the Nature of the Critical Oxidative Damage Targets

What is the nature of the critical oxidative damage in chronologically aging yeast? Is it DNA damage? If so, to what extent are the cells able to counteract this through maintenance DNA repair and is TORC1 – itself a target of oxidative stress (Neklesa and Davis 2008) – involved?

ROS cause base damage, as well as double-strand breaks (DSBs) with non-complementary ends of complex structure, the latter similar to those produced by ionizing radiation (Pawar et al. 2009). In haploid yeast DSBs are repaired primarily by homologous recombination between sister chromatids in the S or G2 phase of the cell cycle; while in diploid cells, such recombinational repair can also occur between homologous chromosomes throughout the cell cycle. DSBs are also repaired by the nonhomologous end-joining repair (NHEJ) pathway but this, while responsible for

the repair of most DSBs induced in mammalian cells, is a relatively minor pathway for the repair of radiation-induced DSBs in vegetative yeast.

Remarkably many of the details of how stationary yeast performs DNA repair are still lacking. Isogenic haploid and diploid cells exhibit an essentially similar CLS (MacLean et al. 2001), suggesting that the capacity for recombinational repair between homologous chromosomes in chronologically-aging cells is not a major determinant of CLS. Moreover if resected DSBs were allowed to persist for any length of time in such cells they should cause a checkpoint activation that will prevent any reentry to the cell cycle – thus shortening the apparent CLS. Checkpoint activation is normally monitored in yeast as the phosphorylation of Rad53 checkpoint kinase (the functional orthologue of human Chk2) (Pawar et al. 2009). In S phase yeast cells, the Rad53 that is activated in response to DNA-damage (as induced by ROS or methyl methanesulfonate) promotes the activation of TORC1 signaling (Shen et al. 2007). This acts as a survival pathway, the increase in TORC1 signaling being required for S-phase progression and viability in response to such genotoxic stress. One of its best-characterised effects is to increase the activity of ribonucleotide reductase (RNR), thereby providing the increased levels of deoxynucleotide triphosphates (dNTPs) necessary for the action of error-prone translesion DNA polymerases. In this way TORC1 activation promotes cell survival. However this comes at the cost of an increased rate of mutation, as higher levels of dNTPs promote the bypass of DNA lesions during translesion DNA synthesis (polymerases generally have a higher K_m for binding nucleotides opposite a damaged base (Chabes and Stillman 2007)). In S phase yeast cells rapamycin, by inhibiting TORC1 and thereby suppressing the RNR induction in response to DNA damage, enhances cell lethality and abrogates mutagenesis (Shen et al. 2007).

Maintenance DNA repair is important for survival of chronologically-aging yeast, as underlined by studies with DNA repair mutants, which display a shortened CLS and enhanced rates of spontaneous mutagenesis to canavanine resistance during stationary maintenance (MacLean et al. 2003; Ringvoll et al. 2007). Increases in DNA nicking are also revealed by the TUNEL assay (Madeo et al. 1999); while overexpression of active Cu,Zn-Sod lowers rates of spontaneous mutagenesis (Harris et al. 2005). That CLS is shortened when cells are aged in the presence of hydroxyurea, a highly selective inhibitor of RNR (our unpublished observations), suggests that the RNR-catalysed synthesis of dNTPs is of importance for a long CLS. However it is completely unknown whether this RNR activity in chronologically-aging cells reflects DNA damage and/or oxidative stress activation of TORC1, such as is seen in vegetative cells (Neklesa and Davis 2008; Shen et al. 2007). If TORC1 is indeed activated in chronologically-aging cells, the studies on S-phase cells (Shen et al. 2007) indicate that the resultant increases in dNTPs might both promote both DNA repair (beneficial) and mutagenesis (detrimental). Endogenous ROS might also be a key factor, not only as a determinant of the levels of DNA damage and TORC1 activity, but also because RNR can itself be inactivated by oxidative stress. One of the protein components of RNR, Rnr2p, contains a oxidoferric centre for the generation of the diferric-tyrosyl radical [(Fe)2-Y·] – a radical

essential for the reduction of ribonucleotides, therefore dNTP synthesis. A major benefit of increasing the resistance to oxidative stress in aging cells may therefore be the protection of this capacity for [(Fe)2-Y·] radical generation. Preventing the oxidation of the two Rnr2p-liganded ferric ions as cells age and become steadily more prooxidant may be critical, as it will allow these cells to retain their capacity for [(Fe)2-Y·] radical generation, therefore their competence in dNTP synthesis and maintenance DNA repair.

Yeast has a tremendous potential to reveal whether there is indeed a significant degree of interplay between the levels of oxidative stress and DNA damage experienced by aging cells and their associated effects on altered TORC1 signalling. These are issues that have major implications for cancer and aging in man, where – both in nondividing cells and in stem cells – any such interplay might have an important impact both on damage accumulation and the events of senescence.

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Chapter 8
Amino Acid Homeostasis and Chronological Longevity in *Saccharomyces cerevisiae*

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Abstract Understanding how non-dividing cells remain viable over long periods of time, which may be decades in humans, is of central importance in understanding mechanisms of aging and longevity. The long-term viability of non-dividing cells, known as chronological longevity, relies on cellular processes that degrade old components and replace them with new ones. Key among these processes is amino acid homeostasis. Amino acid homeostasis requires three principal functions: amino acid uptake, de novo synthesis, and recycling. Autophagy plays a key role in recycling amino acids and other metabolic building blocks, while at the same time removing damaged cellular components such as mitochondria and other organelles. Regulation of amino acid homeostasis and autophagy is accomplished by a complex web of pathways that interact because of the functional overlap at the level of recycling. It is becoming increasingly clear that amino acid homeostasis and autophagy play important roles in chronological longevity in yeast and higher organisms. Our goal in this chapter is to focus on mechanisms and pathways that link amino acid homeostasis, autophagy, and chronological longevity in yeast, and explore their relevance to aging and longevity in higher eukaryotes.

Keywords Amino acid · Homeostasis · Chronological longevity · Caloric restriction · Autophagy

Abbreviations

BCAA	branched side-chain amino acids
CLS	chronological life span
CR	calorie restriction
GAAC	general amino acid control
ISC	iron sulfur cluster
NCR	nitrogen catabolite repression
ROS	reactive oxygen species
TOR	target of rapamycin

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Replicative and Chronological Aging

The budding yeast *Saccharomyces cerevisiae* is a free-living unicellular eukaryote that has proven to be a valuable genetic model system to investigate mechanisms of aging that operate at the cellular level. Budding yeast undergo two types of aging: replicative and chronological. When nutrients are readily available in the environment, yeast reproduce by an asymmetric cell division process known as budding, during which a bud emerges, grows, and separates to yield a smaller daughter cell and a larger mother cell. Mother cells do not divide indefinitely and the number of daughter cells produced by a mother cell is known as the replicative life span (Mortimer and Johnson 1959). When nutrients in the environment become limiting, cell division ceases and yeast remain viable in a non-dividing state for a period of time known as the chronological life span (CLS) (Müller et al. 1980). Viability in this context is defined as the ability to resume the budding process when nutrients become available in the environment, which is a biologically meaningful measure of how well yeast cells survive in a non-dividing state. Exciting progress has been made in uncovering the molecular events that underlie aging in yeast, which has contributed to our understanding of mechanisms of aging and longevity that are evolutionarily conserved from lower to higher organisms (reviewed in Bishop and Guarente 2007; Fontana et al. 2010; Kaeberlein 2010; Kenyon 2005; Laun et al. 2008; Parrella and Longo 2010; Sinclair 2005; Smith et al. 2007; Srinivasan et al. 2010; Vijg and Campisi 2008).

Chronological Aging and Stationary Phase

Chronological aging in yeast is the process by which non-dividing cells lose viability due to a series of time- and metabolism-dependent changes and is distinguished from loss of viability due to the effects of deleterious mutations or adverse environmental factors (e.g., temperature extremes, toxic substances). Chronological aging in yeast takes place during a relatively inactive non-dividing state known as stationary phase. After preferred nutrients, such as glucose, are exhausted during logarithmic growth, alternative nutrients, such as ethanol or amino acids, support growth at a reduced rate during the diauxic shift growth phase. Following the diauxic shift is a period of slow growth termed the post-diauxic phase, during which yeast continue to adapt to nutrient scarcity and prepare for stationary phase. Depending on the nutrient limitation(s) faced during stationary phase, most yeast cells typically arrest as unbudded cells early in the cell cycle prior to the G_1/S transition (Hartwell 1974). During stationary phase, yeast cells derive energy from respiration, exhibit reductions in transcription and translation, accumulate storage carbohydrates, reinforce the cell wall, and acquire resistance to a variety of environmental stresses, yet remain responsive to environmental stimuli (reviewed in Gray et al. 2004; Herman 2002). In addition to exhibiting resistance to environmental stress, stationary phase yeast also up-regulate pathways that reduce or remove molecular damage due to metabolic stress. For example, stationary phase cells employ multiple mechanisms

to combat reactive oxygen species (ROS) damage and up-regulate pathways that degrade oxidatively damaged macromolecules (Chen et al. 2004, 2005; Longo et al. 1996).

Thus, stationary phase is not simply a winding down of cellular processes that drive growth and cell division in the presence of nutrients. Rather, yeast in stationary phase actively respond to reductions in nutrient availability by undertaking programmatic changes in gene expression and cell physiology that favor long-term survival in the non-dividing state. Because they retain the capacity to resume mitosis, viable stationary phase yeast are quiescent but not senescent, which is defined by the inability to resume or continue cell division. Stationary phase yeast are similar in many respects to non-dividing differentiated cells in the G_0 state that age chronologically in human tissues. This similarity combined with the evolutionary conservation of regulatory pathways that affect aging highlights the relevance of the budding yeast model system for the investigation of fundamental processes of cellular aging in post-mitotic human cells.

Interestingly, stationary phase yeast derived from glucose-containing rich medium undergo differentiation into two cell types, quiescent and non-quiescent (Allen et al. 2006; Aragon et al. 2008; Davidson et al. 2011). Quiescent cells resemble G_0 cells insofar as they are unbudded, resistant to stress, responsive to the environment, long-lived, and able to re-enter the cell cycle. Non-quiescent cells are more heterogeneous, contain budded and unbudded cells, lose the ability to re-enter the cell cycle relatively quickly, respire at a relatively low rate, give rise to an elevated number of respiration deficient (petite) colonies, and are more prone to apoptosis. The formation of these two cell types in stationary cultures may represent a survival strategy in which the population of replicatively older non-quiescent cells contribute nutrients (i.e., following cell death) and genetic diversity (i.e., due to mutation) to the population of replicatively young quiescent cells that persist the longest in the non-dividing state.

Adaptations that Circumvent Chronological Aging

It is important to note that yeast have evolved strategies to respond to nutrient scarcity other than stationary phase, such as filamentous growth and sporulation. Filamentous growth is a nutrient-seeking growth habit that depends on formation of elongate pseudohyphae. Filamentous growth is characterized by a number of cell biological changes, such as cell shape change, polar budding, suppression of asymmetric mother cell division, which lead to formation of chains of elongate cells arranged end-to-end (Gimeno et al. 1992). Diploid yeast strains respond to nitrogen limitation by forming pseudohyphae; haploid cells form filaments in response to glucose limitation (Zaman et al. 2008). Under conditions of nitrogen starvation in the presence of non-fermentable carbon sources, diploid yeast undergo meiosis and sporulation to generate ascospores that are resistant to desiccation and numerous environmental insults (Neiman 2005). Ascospores are capable of persisting in the environment for long periods of time. In addition to

filamentous growth and sporulation, yeast are capable of adhesion, flocculation, and biofilm formation, typically in response to nutrient limitation or environmental stress (Verstrepen and Klis 2006). Adhesion allows yeast to remain attached to surfaces where nutrients are available, and flocculation is an adaptive response that protects cells at the center of flocs from environmental stress. Laboratory yeast strains typically harbor mutations in *FLO* genes (e.g., *FLO8*) that render them deficient in filamentous growth and flocculation (Liu et al. 1996), which is valuable for technical reasons, such as the isolation of clonal cell lines (i.e., pure colonies).

Nutrient Limitation Leads to Chronological Aging

Stationary phase and chronological aging in yeast are brought about by limitation of one or more nutrient(s) required for growth. In the natural environment, prototrophic yeast strains require simple carbon and nitrogen sources, salts, and trace elements for growth, and limitations of any required substance can lead to cessation of growth and chronological aging. Laboratory yeast strains typically contain auxotrophic marker alleles that are useful for genetic manipulation precisely because of the specific nature of the nutrient-limited growth these alleles confer. It has become clear that limitation of "natural" nutrients, such as ammonium sulfate, leads to changes in gene expression and cell physiology that are different from changes brought about by limitations in "supplemental" nutrients that complement auxotrophic deficiencies (Saldanha et al. 2004). For example, nitrogen or sulfur limitation leads to "glucose sparing" growth whereas leucine or uracil limitation (in *leu2* or *ura3* auxotrophs) leads to "glucose wasting" growth (Brauer et al. 2008). Differences in limitation of "natural" versus "supplemental" nutrients also lead to differences in stationary phase survival of yeast (Boer et al. 2008), and increased availability of essential "supplemental" nutrients extends the CLS of auxotrophic yeast strains (Gomes et al. 2007). These findings conform to our expectation that chronological aging in a free-living organism such as yeast is triggered by cessation of growth due to nutrient limitation. However, it is clear that not all nutrient limitations are equivalent in terms of their impact on cellular physiology, and that limitations in different required nutrients can elicit dramatically different physiological responses and states in stationary phase cells that undergo chronological aging.

It is tempting to conclude that studies of chronological aging and longevity in yeast should focus on limitations of "natural" nutrients to avoid potentially artificial or artifactual effects of limitations of "supplemental" nutrients needed by auxotrophic strains. However, this conclusion may be premature at this point because it is not yet clear what conditions for nutrient limitation are relevant to human cells. Importantly, humans are not prototrophic organisms. In normal healthy humans, nine amino acids are essential and must be obtained from the diet, and another six amino acids are conditionally essential depending on growth rate (Pencharz and Ball 2003). Amino acid requirements for human cells are important in the context

of aging for at least four reasons. First, the level of essential amino acids in toto is an important factor in maintaining cell and tissue function during aging. For example, elevated levels of essential amino acids offset the debilitating effects of sarcopenia in older women (Dillon et al. 2009). Second, certain essential amino acids appear to be more important than others for optimal function in certain cell and tissue types (Millward et al. 2008). For example, branched side chain amino acids (BCAA; isoleucine, leucine, and valine), especially leucine, play important roles in maintaining function in skeletal muscle and nervous tissue (Drummond and Rasmussen 2008; Layman 2003; Murin and Hamprecht 2008). Third, the requirements for individual essential amino acids in the diet change with age (McLarney et al. 1996; Young and Borgonha 2000), which likely reflects age-dependent changes in requirements at the cell and tissue level. Fourth, amino acid requirements vary with pathophysiological state in diseased cells and tissues (Soeters et al. 2004). Such altered requirements in aging-related diseases could contribute to the aging process, as well as provide a potential opportunity to be exploited as part of a therapeutic regimen. The absolute amount of essential amino acids, the ratio of individual essential amino acids, and age-dependent changes in these two parameters contribute to the optimum dietary intake of essential amino acids that support human health and longevity. Model organisms such as yeast may be valuable in this context. Although it is difficult to study how amino acids affect longevity in humans, it should be relatively easy to genetically engineer experimental organisms (such as yeast) to recapitulate specific amino acid requirements in human cells (at the level of specific enzymatic deficiencies) to test relationships between levels of essential amino acids, chronological aging, and longevity.

Calorie Restriction Promotes Chronological Longevity

Whereas nutrient limitation induces chronological aging in yeast, calorie restriction (CR) induces chronological longevity. CR is arguably the most established and promising intervention for life span extension based on the wide variety of species studied so far. Because reduced levels of nutrients, rather than reduced levels of calories per se, extend life span in some species (see below), this intervention is also known as dietary restriction (DR), a term that allows more latitude in terms of underlying mechanism(s). In many animals, the maximum life span is obtained when intake during CR is restricted to approximately two-thirds of the amount of food consumed ad libitum. This does not mean that CR is without consequences to the organism; the fecundity of many species is reduced by CR. Depending on the species, CR can be achieved with different feeding schedules, including continuous (i.e., daily) CR, intermittent CR, modest CR, or mid- to late-life CR (Piper and Bartke 2008). Even modulation of the perception of food availability appears to mimic the life span extending effects of CR in *Drosophila* (Libert et al. 2007). In general, life span extension by CR can be interpreted as a universal response to nutrient scarcity that has evolved to maximize survival until nutrient availability is sufficient for optimal reproduction.

In terms of chronological longevity in yeast, CR is achieved by either growth in low (e.g., 0.4%) glucose medium or growth in normal (e.g., 2%) glucose medium followed by transfer to, and washing with, water (Fabrizio and Longo 2003; Piper 2006). In low glucose medium, "adaptation and regrowth" may be observed, depending on the yeast strain, and is due to resumption of growth of surviving cells that utilize metabolites released from dead and apoptotic cells (Fabrizio et al. 2004; Herker et al. 2004). This phenomenon has been termed "altruistic aging" (Longo et al. 2005) and makes sense as a survival strategy for a free living single-celled microorganism that has evolved to grow quickly in the presence of food to maximize population size rather than individual longevity. Similarly, prolonged life span in water is likely an adaptation to adverse situations that arise naturally in the wild (e.g., when rain washes yeast cells from fruit into soil). Life span extension by CR in yeast appears to depend on the specific conditions experienced by non-dividing cells. Extracellular levels of organic acids, such as acetic acid, and pH play critical roles in determining chronological longevity (Burtner et al. 2009). Thus, although there is considerable interest in understanding intracellular signaling pathways that regulate the effect of CR on life span (see below), the question of what external factors are integrated to yield the CR response has only been answered in part.

It is worth noting that the difference between chronological longevity brought about by CR and chronological aging brought about by nutrient limitation is more than a matter of "less" nutrients versus "no" nutrients, respectively. In other words, there is a qualitative component as well as a quantitative component to this difference. Two important considerations are the nature of the conditions that constitute "less" versus "no" nutrients and the nature of the transition from "less" to "no" nutrients. For example, in higher organisms, life span extension is only attained if the calorie restricted diet is high quality (i.e., it contains sufficient essential vitamins, trace elements, and so forth, to support growth, albeit at reduced rates or with reductions in adult body mass). In yeast, a series of metabolic decisions are made during the transition from "less" to "no" nutrients that affect energy storage and resource utilization that have a significant impact on CLS. The availability of amino acids, which serve both as building blocks for protein synthesis as well as a non-fermentable carbon source, is an important factor. Thus, one of the key decisions made by yeast in response to amino acid depletion during this transition is the up-regulation of autophagy.

Autophagy Supports Chronological Longevity

Autophagy is a lysosome- or vacuole-mediated degradation system that responds to reductions in available nutrients, especially amino acids. Autophagy is constitutively active at low levels in virtually all eukaryotic cells, and is induced by nutrient limitation or CR as well as different forms of environmental and cellular stress. Autophagy is generally considered to be a non-specific process, but specialized autophagic pathways exist to handle specific targets (Yang and Klionsky 2009). Autophagy accomplishes two main functions – recycling and removal. Recycling

maintains intracellular pools of building blocks, such as amino acids, that are needed for de novo synthesis. Removal targets cellular components ranging in size from small molecules to large organelles that are functionally impaired due to molecular damage, misfolding, and/or aggregation. Recycling and removal are particularly important during chronological aging because nutrient-limited non-dividing cells accumulate damage for two principal reasons: reduced rates of de novo synthesis and the absence of "dilution" of cellular damage during cell growth and division.

Studies in many organisms have revealed important roles for autophagy in forestalling chronological aging and promoting chronological longevity (Cuervo 2008a; Hubbard et al. 2011; Madeo et al. 2010; Vellai et al. 2009; Yen and Klionsky 2008). In *C. elegans*, autophagy is required for normal longevity (Melendez et al. 2003). In mammalian cells, autophagic protein turnover undergoes age-related decline (Cuervo and Dice 2000; Del Roso et al. 2003; Ward 2002). The decline in turnover of mitochondria by autophagy has been proposed to exacerbate ROS production with age (Kundu and Thompson 2005; Lemasters 2005). In skeletal muscle, this age-related decline in autophagy is attenuated by CR (Wohlgemuth et al. 2010).

Our studies have shown that autophagy is required for chronological longevity of yeast grown to stationary phase in synthetic media (Alvers et al. 2009). We found that autophagy-deficient mutant strains exhibited reduced chronological longevity compared to autophagy-proficient control strains in three different standard synthetic minimal and complete media. Genome-wide screens for mutations that reduce CLS in synthetic complete media likewise uncovered an important role for autophagy in maintaining chronological longevity (Fabrizio et al. 2010; Matecic et al. 2010). We also found that autophagy-deficient strains were as long lived as control strains in rich undefined medium, in agreement with the findings of Powers et al. (2006). This indicates that autophagy is required for longevity when amino acid availability is limited, but not when amino acid levels are high prior to or during stationary phase.

The *atg1*Δ and *atg7*Δ mutants used in our studies are deficient in multiple selective and non-selective autophagic pathways, including macroautophagy, microautophagy, the cytoplasm to vacuole targeting (CVT) pathway, piecemeal microautophagy of the nucleus (PMN), ribophagy, ER-phagy, turnover of fructose-1,6-bisphosphatase by vacuole import and degradation (VID), and selective degradation of mitochondria (mitophagy) and peroxisomes (pexophagy) (Nair and Klionsky 2005; Yang and Klionsky 2009). Chaperone mediated autophagy is not present in budding yeast. In our experiments, an *atg11*Δ strain exhibited a normal CLS, indicating that the CVT, pexophagy, and mitophagy pathways are not required for chronological longevity in synthetic media (Alvers et al. 2009; Kanki et al. 2009; Okamoto et al. 2009). Thus, selective mitophagy per se does not appear to play a significant role in maintaining chronological longevity. Taken together, these findings support a role for macroautophagic turnover and amino acid recycling in chronological longevity.

Amino Acid Homeostasis Promotes Chronological Longevity

Amino acid homeostasis consists of five primary processes: uptake, synthesis, utilization, recycling, and catabolism. In yeast, these processes are regulated by multiple, overlapping, global, and amino acid-specific pathways. Amino acids are among the most important nutrients recycled by autophagy, and maintenance of amino acid levels by autophagy is required for cell survival during nitrogen starvation (Onodera and Ohsumi 2005). Amino acid limitation is a potent inducer of autophagy in a wide range of eukaryotes from yeast to mammalian cells, and amino acid starvation in autophagy-deficient cells leads to rapid loss of viability. Thus, it is not surprising that amino acid availability has been found to play an important role in modulating yeast CLS. We and others have found that levels of essential amino acids affect chronological longevity (Alvers et al. 2009; Gomes et al. 2007). Traditionally, minimal and complete synthetic glucose media are prepared according to formulations that contain specific amounts of essential and nonessential amino acids (e.g., Amberg et al. 2005; Sherman 2002; Styles 2002). Inclusion of five-fold elevated concentrations of essential amino acids in minimal synthetic glucose medium extended CLS and increased resistance to oxidative, pH, and thermal stress during chronological aging (Gomes et al. 2007). Furthermore, Gomes et al. found that standard levels of essential amino acids resulted in aberrant cell cycle arrest and cellular DNA content consistent with programmed cell death. Thus, it is clear that limiting amounts of essential amino acids can have an adverse impact on chronological longevity that is distinct from the life span extending effects of CR. For this reason, yeast CLS measurements are routinely done using synthetic media that contain elevated levels of essential amino acids (Fabrizio and Longo 2003; Powers et al. 2006).

Interestingly, different essential amino acids are not equally effective in promoting chronological longevity. We analyzed the requirements for individual essential amino acids and learned that not all essential amino acids extend CLS when present at elevated levels (Alvers et al. 2009). This argues against the notion that elevated levels of essential amino acids extend CLS purely by a nitrogen supplementation mechanism. We tested three essential amino acids and found that elevated levels of leucine, but not histidine or lysine, resulted in a pronounced extension of CLS. Elevated levels of uracil, the only other essential nutrient, did not affect CLS. Furthermore, elevated leucine levels extended CLS in both autophagy-deficient and autophagy-proficient strains. In agreement with this, genetically complementing the *leu2*Δ auxotrophy in strain BY4742 with *LEU2* extended CLS whereas complementation of *his3*Δ or *ura3*Δ had only minimal effects on CLS (Alvers et al. 2009). Complementation of the *lys2*Δ marker with *LYS2* extended longevity in autophagy-proficient, but not autophagy-deficient strains. Thus, leucine promoted chronological longevity to the greatest extent among the essential nutrients we studied.

Moreover, nonessential amino acids promote chronological longevity in minimal media in both autophagy proficient and autophagy deficient strains. But, there is no strict correlation between number and amounts of nonessential amino acid

supplements in synthetic media and chronological longevity (Alvers et al. 2009). Thus, the effect of nonessential amino acids is not simply a matter of providing an additional source of nitrogen. Furthermore, we found that the non-essential BCAA isoleucine and valine, and their precursor threonine, had the most pronounced effect on chronological longevity. To our knowledge, our studies were the first to demonstrate roles for specific individual nonessential amino acids in promoting chronological longevity in *S. cerevisiae*. This indicates that during chronological aging in synthetic media the availability of specific nonessential amino acids is more important to amino acid homeostasis than availability of all nonessential amino acids. This may also apply to the role of autophagy during chronological aging: recycling of specific amino acids by autophagy may be more important than recycling of bulk amino acids or nitrogen.

Down-Regulation of General Amino Acid Control Extends Chronological Life Span

The observation that nonessential amino acids extend CLS suggested a regulatory role rather than nutritional effect. Cellular responses to amino acid availability are regulated in large part by the target of rapamycin (TOR) and general amino acid control (GAAC) pathways, both of which are highly conserved from yeast to human. GAAC is an elaborate regulatory network that coordinates synthesis of amino acids, purines, and other metabolites when they are in short supply and need to be synthesized by the cell (Hinnebusch 2005). A hallmark of GAAC is that reduced levels of one amino acid can exert "general" or global control on anabolic pathways for many amino acids as well as other nutrients. At the heart of GAAC is the transcription factor Gcn4p that participates in regulation of transcription of hundreds of target genes. *GCN4* expression is regulated at the translational level by Gcn2p. Gcn2p indirectly senses amino acid levels by responding to levels of uncharged tRNAs and regulating translation initiation accordingly, including translation of *GCN4* mRNA (Hinnebusch 2005).

Given the prominent role of GAAC in responding to nutrient limitation, we tested if the non-essential amino acids isoleucine, valine, and threonine extended CLS by down-regulating the GAAC pathway. We showed that Gcn4p levels were reduced in synthetic minimal medium containing isoleucine, valine, and threonine compared to medium without these amino acids (Alvers et al. 2009). Furthermore, we showed that constitutive expression of *GCN4* suppressed extension of CLS by isoleucine, valine, and threonine. Thus, at least some nonessential amino acids can bring about extension of CLS by down-regulating GAAC. However, deletion of *GCN4* does not extend CLS (Fig. 8.1). Similar results have been obtained in the context of replicative life span (RLS). Steffen et al. reported that *GCN4* is required for extension of RLS by 60S ribosomal protein mutants and CR, but deletion of *GCN4* does not similarly extend RLS (Steffen et al. 2008). To our knowledge, our studies were the first to demonstrate a link between down-regulation of GAAC and chronological longevity in *S. cerevisiae*.

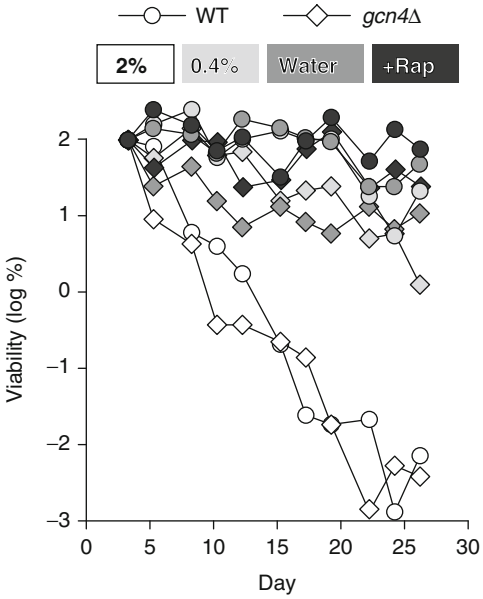

Fig. 8.1 General amino acid control is not required for extension of CLS by CR or rapamycin. CLS measurements were done with yeast strains in the BY4742 genetic background (*MAT*alpha *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0) grown in synthetic dextrose (SD) minimal medium as described (Alvers et al. 2009). Viability is expressed in terms of colony forming units (CFU) per mL and is plotted as the log of the percent viability on day three of the experiment. Wild type (WT, *ho*Δ) and *gcn4*Δ strains were grown in: normal (2%) glucose SD; low (0.4%) glucose SD; normal glucose SD followed by washing with water beginning on day three (Water); or normal glucose SD plus 10 nM rapamycin (+Rap)

To extend these studies, we wished to determine if GAAC played a role in the extension of CLS by rapamycin-dependent inhibition of the TOR pathway. Generally speaking, GAAC and TOR signaling act in a "yin-yang" fashion to manage cellular growth in relation to nutrient availability. GAAC up-regulates autophagy and synthesis of amino acids, purines, and other metabolites at a global level in response to amino acid limitation whereas the TOR pathway up-regulates protein synthesis and cell growth, and down-regulates autophagy and cellular stress response when nutrients are plentiful (De Virgilio and Loewith 2006a). *S. cerevisiae* has two partially functionally redundant TOR complexes. TOR complex 1 (TORC1) up-regulates anabolic processes, including amino acid utilization, and down-regulates catabolic processes, including autophagy, as well as expression of a number of stress response transcription factors. The functions of TORC2 are less well understood, but regulation of the actin cytoskeleton and cell polarity are important regulatory targets. In humans, aberrant regulation of TOR signaling is associated with aging and pathophysiological changes in multiple diseases (De Virgilio and Loewith 2006b).

In yeast, unlike in higher eukaryotes, the GAAC and TOR pathways communicate at the level of Gcn2p, which functions immediately upstream of Gcn4p. In the presence of ample amino acids, Gcn2p activity is down-regulated by phosphorylation under control of the TORC1 complex (De Virgilio and Loewith 2006a). Thus, in yeast, it is conceivable that Gcn4p may contribute to extension of CLS due to inhibition of TORC1 activity by rapamycin. However, we find that *GCN4* is not required for extension of CLS by rapamycin treatment (Fig. 8.1). Furthermore, we showed that *GCN4* is not required for extension of CLS by two CR interventions: growth in low glucose and washing with water (Fig. 8.1). Thus, although down-regulation of GAAC can extend CLS in yeast, as discussed above, extension of CLS by rapamycin and CR apparently involves branches of the TORC1 signaling pathway that do not include GAAC.

Deletion of the GAAC Target Gene LEU3 Extends Chronological Life Span

Because *GCN4* regulates diverse cellular functions, there are many potential mechanisms by which down-regulation of *GCN4* may extend CLS. Down-regulation of *GCN4* down-regulates autophagy, but it is unlikely that this extends CLS given that autophagy is up-regulated during chronological aging and is required for chronological longevity, as discussed above. *GCN4* is required for cellular responses to oxidative stress and DNA damage (Mascarenhas et al. 2008), and thus down-regulation of *GCN4* should shorten CLS. Given the large number of potential avenues by which *GCN4* might influence CLS, we focused on the link between chronological longevity and BCAA that emerged in our studies. The best known *GCN4* target in this context is *LEU3*. Leu3p is a key transcriptional regulator of BCAA synthesis and is known to regulate transcription of *LEU1*, *LEU2*, *LEU4*, *ILV2*, and *ILV5* as well as genes involved in BCAA uptake (Kohlhaw 2003). We found that deletion of *LEU3* resulted in a profound extension of CLS, and that elevated levels of leucine, which extend CLS in a *LEU3* strain, did not further extend CLS in the *leu3*Δ strain (Alvers et al. 2009). This points to down-regulation of *LEU3* expression as an important mechanism by which down-regulation of *GCN4* expression extends CLS.

These results raise an interesting question: how does deletion of *LEU3* extend CLS? We originally suggested that deletion of *LEU3* extends CLS by increasing cellular leucine availability and relieving an imbalance in synthesis of BCAA (Alvers et al. 2009). This suggestion was based on the observation that three conditions extended CLS in our studies: supplemental BCAA, conversion of *leu2*Δ to *LEU2*, or deletion of *LEU3* (Alvers et al. 2009). The notion that balanced synthesis of BCAA contributes to chronological longevity is consistent with the relative abundance of BCAA in the yeast proteome. Leucine, isoleucine, and valine codons account for 9.6, 6.5, and 5.6%, respectively, of codons present in annotated verified open reading frames (ORFs) in the *Saccharomyces* Genome Database (Alvers

et al. 2009). Because BCAA account for 21.7% of the amino acids in the yeast proteome, reduced amino acid availability may exert a direct effect on protein synthesis. Alternatively, or in addition, levels of the nonessential amino acids isoleucine and valine may exert an indirect effect on the levels of the essential amino acid leucine. Isoleucine and valine not only regulate enzymes required for their synthesis (i.e., threonine deaminase Ilv1p and acetolactate synthase Ilv2p) by feedback inhibition, but also regulate Leu1p by cross-pathway (non-end product) control (Jones and Fink 1982). Thus, for these reasons, we proposed that *leu3*Δ extended CLS by balancing BCAA levels (Alvers et al. 2009).

However, regulation of CLS in a *leu3*Δ strain may be more complicated and other factors may also contribute to extension of CLS. We have found that autophagy is up-regulated in a *leu3*Δ strain compared to a WT control (Fig. 8.2a, b). However, the regulation of autophagy does not appear to be altered in a *leu3*Δ strain insofar as autophagy is down-regulated by elevated levels of supplemental amino acids (Fig. 8.2c, d). This suggests that enhanced autophagy may contribute to extension of CLS in a *leu3*Δ strain, which agrees with our previous observations that autophagy promotes chronological longevity.

The fact that autophagy is upregulated in a *leu3*Δ strain is not unexpected given the number of cellular processes affected by Leu3p transcriptional regulation. At first glance, the Leu3p regulon appears relatively simple in terms of the number of

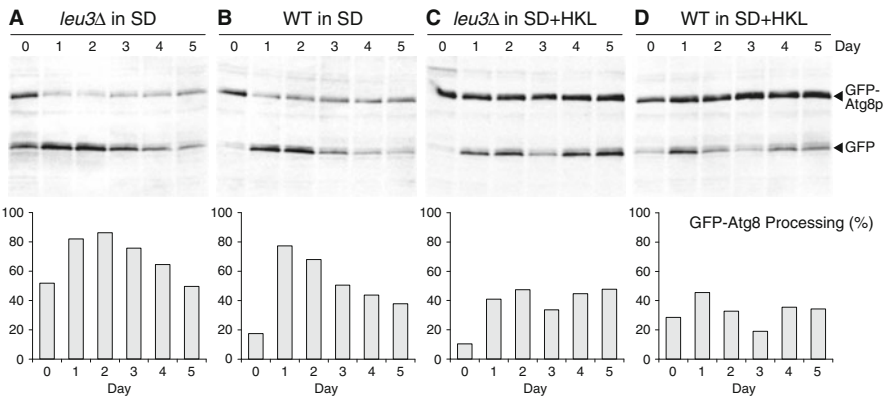

Fig. 8.2 Macroautophagy is up-regulated in a *leu3*Δ strain. Activation of autophagy in *leu3*Δ and wild type (WT) strains was measured over 5 days of a CLS experiment performed as described (Alvers et al. 2009). Strains in the BY4742 background were transformed with plasmid pCuGFPAUT7(416) (*URA3*), grown in synthetic dextrose medium (Sherman 2002) with standard (SD) or three-fold elevated levels (SD+HKL) of histidine, lysine, and leucine, and a western blotting assay was used to measure cellular levels of GFP and the GFP-Atg8p fusion protein (Klionsky et al. 2007). The percent conversion of GFP-Atg8p to GFP on each day reflects the extent of autophagic activation and is plotted in graphs below western blots. Equivalent amounts of cells, based on cell density measurements, were collected, processed, and analyzed on blots. Day zero samples were collected during mid-log growth. Quantification was done using ImageJ software [% processing = 100 × GFP ÷ (GFP + GFP–Atg8p)]

genes that bind Leu3p and the "condition invariant" nature of regulation by Leu3p (Harbison et al. 2004). However, Leu3p binds at detectable levels to only about 3% of genes whose expression is affected by *LEU3* deletion (Boer et al. 2005; Tang et al. 2006). Because of this, it has been estimated that Leu3p may contribute indirectly to the regulation of up to 10% of all yeast genes (Tang et al. 2006). Importantly, deletion of *LEU3* does not simply result in reduced transcriptional activation. Leu3p also functions as a transcriptional repressor when levels of its obligate coactivator 2-isopropyl malate are low (Kohlhaw 2003). Thus, deletion of *LEU3* results in increased expression of many genes involved in amino acid biosynthesis, including five genes involved in BCAA synthesis, when glucose is in excess and nitrogen is limiting (Boer et al. 2005). In addition, deletion of *LEU3* may influence CLS via its effect on nitrogen assimilation (see below).

We also tested the hypothesis that CLS is influenced by 3-isopropyl malate methyl ester, a secreted signaling molecule implicated in stimulation of filamentous growth during amino acid limitation (Dumlao et al. 2008). We reasoned that accumulation of 3-isopropyl malate in *leu2*Δ mutants may favor Tmt1p-dependent methyl esterification during chronological aging. However, we found that *tmt1*Δ and *TMT1* strains (in a *leu2* background) yield indistinguishable CLS (Fishwick and Aris, unpublished results), indicating that signaling by 3-isopropyl malate methyl ester does not influence CLS under the conditions we examined.

Nitrogen Assimilation and Chronological Longevity

Given the influence of autophagy and amino acid homeostasis on CLS, we wished to explore potential relationships between nitrogen utilization and chronological longevity. *S. cerevisiae* can utilize a wide range of nitrogen sources, but exhibits preferences based on the metabolic investment required to catabolize them (best = glutamine > serine, ammonia > glutamate > proline = poor). In the presence of preferred nitrogen sources, nitrogen catabolite repression (NCR) down-regulates a wide variety of genes needed for utilization of nonpreferred nitrogen sources (Hofman-Bang 1999). In the absence of preferred nitrogen sources and NCR, a number of genes required for nitrogen assimilation are activated, including *GAT1*, *GDH1*, *GLN1*, and *GLN3* (Fig. 8.3a). The transcription factors Gat1p and Gln3p are arguably the most important regulators of nitrogen assimilation, although nitrogen assimilation is known to be regulated at multiple levels (Zaman et al. 2008). Gln3p is the major regulator of NCR-sensitive gene expression, although many gene products are also controlled at the post-transcriptional level (Kolkman et al. 2006). Gln3p target genes are also up-regulated during release from NCR brought about by rapamycin mediated inhibition of TORC1, although rapamycin treatment does not faithfully mimic the pattern of Gln3p dephosphorylation observed during down-regulation of NCR (Tate et al. 2009). Gln3p stimulates expression of Gat1p, and both of these transcription factors stimulate expression of multiple downstream targets, including Gdh1p and Gln1p (Fig. 8.3a). *GDH1* expression is also regulated by Leu3p as well as the HAP transcriptional complex, which regulates many

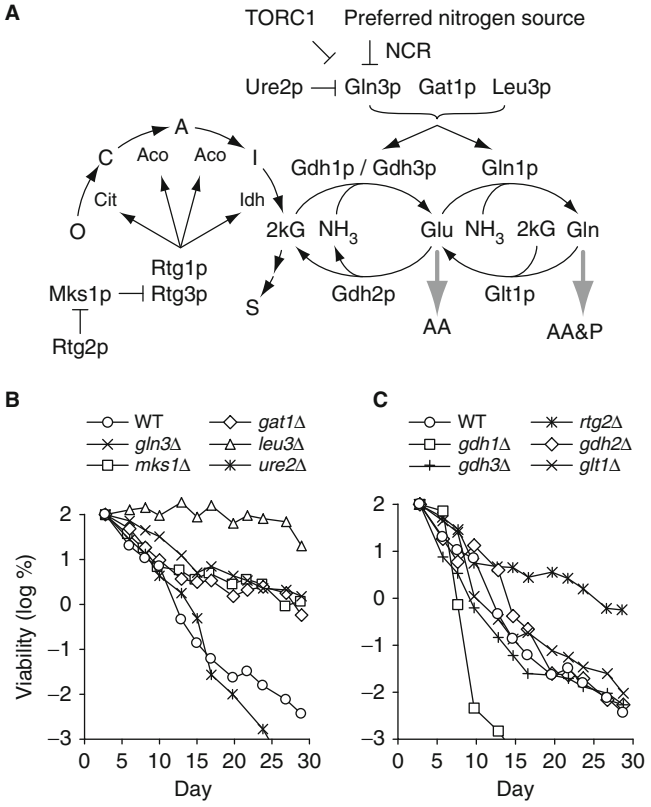

Fig. 8.3 Role for nitrogen assimilation in chronological longevity. **a** Nitrogen / ammonia assimilation pathway in *S. cerevisiae*. The TCA cycle intermediates are oxaloacetate (O), citrate (C), *cis*-aconitate (A), isocitrate (I), 2-ketoglutarate (2-kG), and succinate (S). Gene products are discussed in the text. AA, amino acids. P, purines and pyrimidines. Consumption of ATP, NAD^+, and $NADP^+$ are omitted for simplicity. Inhibition by TORC1 and Ure2p are diagrammed separately but participate in nitrogen catabolite repression (NCR). Interactions between NCR and the retrograde signaling pathway as well as negative regulation by Dal80p and Deh1p are not shown. **b** and **c** CLS measurements were done using deletion mutants in the BY4742 genetic background grown in SD minimal medium as described in Fig. 8.1. Viability is expressed in terms of colony forming units (CFU) per mL of culture and is plotted as the log of the percent viability on day three of the experiment. The results shown are representative of at least two independent experiments

aspects of mitochondrial function (Buschlen et al. 2003). *GDH1* encodes the major $NADP^+$-linked glutamate dehydrogenase isozyme that catalyzes the first step in nitrogen assimilation: formation of glutamate from ammonia and 2-ketoglutarate (2kG) (Fig. 8.3a). Nitrogen is also assimilated during conversion of glutamate to glutamine by glutamine synthetase encoded by *GLN1*. Because glutamate and glutamine serve as primary amine donors for synthesis of amino acids and other amine containing molecules, Gdh1p and Gln1p are central regulators of nitrogen assimilation (Hu et al. 1995). *GDH3* encodes an isozyme that consumes 2kG at a

lower rate and is glucose repressed. The third enzyme capable of synthesizing glutamate, NAD^+-dependent glutamate synthase, is encoded by *GLT1*. *GDH2* encodes an enzyme that catalyzes an anaplerotic reaction that regenerates 2kG.

To investigate the relationship between nitrogen assimilation and chronological longevity, we measured the CLS of a number of mutant strains impaired in different aspects of nitrogen utilization. Deletion of *GLN3* or *GAT1*, as well as *LEU3*, extends CLS, although deleting *GLN3* or *GAT1* does not prolong CLS to the same extent as deleting *LEU3* (Fig. 8.3b). Deletion of *GLN3* has been previously shown to extend CLS in synthetic complete media (Matecic et al. 2010; Powers et al. 2006). Deletion of *URE2*, which encodes an inhibitor of Gat1p and Gln3p, yields a CLS equivalent to the wild type control, suggesting that increased Gat1p and Gln3p activities do not reduce CLS. Extension of CLS in *gln3*Δ, *gat1*Δ, and *leu3*Δ strains suggests that reductions in assimilation of ammonia may contribute to chronological longevity. This raises the question: does elimination of the enzymatic machinery required for assimilation of ammonia extend CLS? The answer to this question appears to be "no" because a *gdh1*Δ strain is relatively short-lived, and *gdh2*Δ, *gdh3*Δ, or *glt1*Δ deletion strains are not more long-lived than the wild type control strain (Fig. 8.3c). The reduced CLS in the *gdh1*Δ mutant may be due to the pleiotropic effects of a greater than ten-fold decline in cytoplasmic glutamate concentration that is characteristic of *gdh1*Δ strains (Hofman-Bang 1999). We were not able to test deletion of *GLN1* because it is an essential gene. Deletion of *GAP1*, which encodes a general amino acid permease controlled by Gln3p and Gat1p, has no effect on CLS (data not shown).

Also important in this context is the retrograde signaling (mitochondrion to nucleus) pathway, which coordinates cellular and mitochondrial function (Liu and Butow 2006). Retrograde signaling has been implicated in coordinating metabolism, stress resistance, and genome stability during yeast replicative aging (Borghouts et al. 2004; Jazwinski 2005), but relatively little is known about the role of the retrograde response in chronological aging in yeast. One of the primary functions of retrograde signaling is to regulate carbon flow through the tricarboxylic acid (TCA) cycle and production of 2kG. The retrograde transcription factors Rtg1p and Rtg3p achieve this by regulating expression of four TCA pathway enzymes upstream of 2kG (Fig. 8.3a). Nuclear localization of Rtg1p and Rtg3p is inhibited by the cytoplasmic sequestration factor Mks1p that is regulated in turn by Rtg2p. Puzzlingly, deletion of either *MKS1* or *RTG2* results in extension of CLS and to an extent similar to deletion of *GLN3* or *GAT1* (Fig. 8.3b, c). The result with *rtg2*Δ notwithstanding, the extension of CLS in the *gln3*Δ, *gat1*Δ, and *mks1*Δ strains is consistent with increased aerobic respiration due to increased flow of carbon through the TCA cycle. That is, reduced consumption of 2kG by ammonia assimilation and increased utilization of 2kG by the TCA cycle is consistent with increased aerobic energy production. Increased CLS in *tor1*Δ strains has been attributed to increased aerobic respiration due to increased mitochondrial gene expression (Bonawitz et al. 2007). Such an increase in aerobic respiration would require increased carbon flow through the TCA cycle. This is consistent with the general idea that under the nutrient limiting conditions experienced during chronological aging, the balance

between production of energy and synthesis of amino acids plays an important role in post-mitotic cell survival.

In the paragraphs above, we have discussed how autophagy, amino acid availability, and nitrogen assimilation are associated with chronological longevity in yeast. In the paragraphs below, we address connections between amino acids and two important paradigms in the aging field: the mitochondrial theory of aging and life span extension by CR.

Amino Acid Homeostasis and the Mitochondrial Theory of Aging

A group of well-known and closely allied mitochondria-related theories of aging have provided a mechanistic basis for aging at the cellular level that underscores the importance of molecular damage due to mitochondrially produced ROS. The free radical theory of aging and the mitochondrial theory of aging constitute central paradigms in aging research (Attardi 2002; Beckman and Ames 1998; Harman 1956, 1972, 2003; Miquel et al. 1980; Wallace 2005). Mitochondrial dysfunction, ROS production, and oxidative stress have been linked to chronological aging in many species and life span extending interventions generally mitigate these problems (Merry 2004).

During chronological aging in yeast, a main source of cellular damage is ROS produced by mitochondria (Chen et al. 2005). To combat oxidative damage, yeast possess multiple oxidant defense systems, including catalases, glutathione, glutathione peroxidase and oxidoreductase, and superoxide dismutases (SOD). These mechanisms minimize oxidative stress during stationary phase and are key factors in influencing CLS (Fabrizio and Longo 2003; Garrido and Grant 2002; Longo 2004; Piper 2006). Yeast lacking Sod1p (cytoplasmic Cu/ZnSOD) or Sod2p (mitochondrial MnSOD) have reduced viability in stationary phase (Longo et al. 1996, 1999) and over-expression of *SOD1* or *SOD2* increases CLS (Bonawitz et al. 2006; Harris et al. 2003, 2005). Yeast in stationary phase up-regulate transcription of *SOD1*, *SOD2*, and *GLR1* (glutathione reductase) following exposure to menadione (Cyrne et al. 2003). Oxidative damage to mitochondrial DNA (mtDNA) is linked to instability of the mitochondrial genome and loss of respiration (Doudican et al. 2005).

Although the mitochondrial / ROS theory of aging is well established, there are exceptions to the existence of a strict correlation between aerobic respiration, ROS production, and chronological aging. In fact, there is a large and growing body of evidence that runs counter to mitochondrial theories of aging, which has lead to revision and reconsideration of some key elements of these theories (e.g., Lapointe and Hekimi 2010; Ristow and Zarse 2010). In yeast, deletion of *TOR1*, CR, and mild uncoupling increase respiration, but reduce ROS production and oxidative damage, which are linked to extended CLS (Barros et al. 2004; Bonawitz et al. 2007; Gomes et al. 2007). Similar salubrious effects of mild uncoupling on life span have been observed in higher organisms (e.g., Andrews 2010; Dietrich and Horvath 2010). Conversely, reductions in gene products required for oxidative phosphorylation do

not necessarily lower ROS production and extend CLS. On the contrary, a mutation in mitochondrial RNA polymerase (Rpo41p) that causes a defect in mitochondrial translation of gene products required for oxidative phosphorylation results in a significant increase in ROS production and a severe reduction in CLS that can be rescued by over-expression of *SOD1* or *SOD2* (Bonawitz et al. 2006). These results indicate that increased longevity is associated with increased respiration, which may be due to increased electron transport chain efficiency and/or reductions in ROS production and concomitant molecular damage.

Amino acids are important in this context because they are able to serve as an aerobic carbon source that supports aerobic energy production during chronological aging. Rich media contain abundant nonessential amino acids that can serve this purpose. Certain amino acids, such as BCAA, phenylalanine, and methonine, cannot serve as carbon sources because they are metabolized via the Ehrlich pathway (Hazelwood et al. 2008). Nevertheless, other amino acids function as aerobic carbon sources following exhaustion of glucose during the diauxic shift, post-diauxic growth, and stationary phase. Aerobic growth prior to stationary phase has been shown to promote chronological longevity (Brauer et al. 2005; Piper et al. 2006). Furthermore, amino acids are also important because that are used as building blocks for protein synthesis that accompanies reprogramming of the cellular proteome during adaptation to the non-dividing state.

It is also important to consider the relevance of amino acid biosynthetic pathways to chronological longevity. De novo synthesis of many amino acids involves at least one step carried out in mitochondria. BCAA are synthesized by a superpathway in which catalytically similar steps are carried out and regulated by shared gene products, namely Bat1p, Bat2p, Ilv2p, Ilv3p, Ilv5p, and Ilv6p. All of these enzymes, except Bat2p, function in the mitochondrial matrix and are potential targets of ROS damage. Perhaps the best understood case of oxidative damage to a mitochondrial matrix enzyme is aconitase. Aconitase is a bifunctional iron sulfur cluster (ISC) containing enzyme that catalyzes two steps in the TCA cycle and stabilizes the mitochondrial genome. Over-expression of mitochondrial Sod2p extends CLS by protecting aconitase from oxidative damage during chronological aging (Fabrizio et al. 2003; Harris et al. 2003). ISC synthesis is known to take place in the mitochondrial matrix due to the oxidizing environment present there. However, ISC enzymes are susceptible to oxidative damage, irrespective of their localization within the cell. Leu1p is a cytoplasmic ISC containing enzyme required for synthesis of leucine that is susceptible to inactivation by ROS, but is protected under aerobic conditions by SOD (Wallace et al. 2004). The same is true for Lys4p, a cytoplasmic ISC enzyme required for lysine synthesis. Thus, the extent to which amino acid biosynthetic enzymes are targets of oxidative inactivation may also play an important role in chronological longevity.

In addition to amino acid availability and de novo synthesis, amino acid recycling may play an important role in chronological longevity. The main cellular pathways for amino acid recycling – autophagy and the ubiquitin/proteasome system – function in both recycling and damage removal. Because of this, it is difficult to parse the relative significance of recycling and removal. Clearly, maintaining

intracellular amino acid pools by recycling is important, especially when levels of environmentally available amino acids are low, as discussed above. Damage removal is important because it eliminates damage *after* exposure to ROS whereas cellular oxidant defense systems inactivate ROS *before* damage occurs. This is particularly valuable for non-dividing cells wherein damage cannot be "diluted out" by cellular growth and division, as discussed above. Autophagy is the only known mechanism for degrading large intracellular structures and organelles such as mitochondria, the importance of which has been codified in the lysosomal-mitochondrial axis theory of aging (Terman et al. 2006). Recent studies of ours indicate that when amino acids are limiting, autophagy is needed to turnover damaged mitochondria in order to promote chronological longevity, but under certain conditions of amino acid availability mitochondrial damage is reduced and autophagy is not required for chronological longevity (Aris et al. unpublished results). This suggests that interrelationship between amino acid availability, mitochondrial damage, and autophagy is important for chronological longevity.

Amino Acid Homeostasis and Extension of Life Span by Calorie Restriction

CR is the most widely recognized intervention for extension of life span and there has been considerable debate about the mechanism(s) by which CR extends life span in yeast and other species, including the way in which CR affects mitochondrial ROS production. Multiple signaling pathways have been implicated in life span extension by CR, including the insulin/insulin-like growth factor (I/IGF), Ras/protein kinase A, and TOR pathways (Stanfel et al. 2009). These pathways play crucial roles in integrating information from growth signals and levels of available nutrients, including levels of amino acids. In budding yeast, inhibition or abrogation of TOR signaling appears to mimic CR and promotes chronological longevity via a cell autogenous mechanism that involves modulation of mitochondrial translation and aerobic energy production (Bonawitz et al. 2007; Cheng et al. 2007; Powers et al. 2006). Similarly, inactivation of the TOR pathway extends CLS in *Drosophila* (Kapahi et al. 2004), and inhibition of TOR or translation extends life span in *C. elegans* (Hansen et al. 2007; Henderson et al. 2006; Pan et al. 2007). Multiple downstream targets have been the focus of studies seeking to elucidate underlying mechanisms, including up-regulation of multiple stress response pathways that impact production, repair, accumulation, and clearance of different types of molecular damage (Cuervo 2008b; Guarente 2008).

An exciting development in the field is the emerging importance of diet composition in life span extension by CR. It is becoming increasingly clear that CR-mediated life span extension is not simply a matter of reduced caloric intake and that amino acid sensing and utilization play key roles (Mair et al. 2005; Piper et al. 2005). Restriction of specific essential amino acids has been found to extend life span in animals and appears to do so by mimicking CR. Reducing the amount of the essential amino acid methionine can mimic many of the effects of CR / DR in

rats, including increased life span (Orentreich et al. 1993). A diet deficient in methionine also extends life span in mice and yields many of the physiological benefits associated with CR (Miller et al. 2005). A careful analysis of the effects of specific essential amino acids in *Drosophila* reveals that methionine restriction confers extended life span without the decrease in fecundity observed under traditional CR conditions (Grandison et al. 2009). Moreover, the *Drosophila* I/IGF signaling pathway links amino acid sensing to reduced life span when amino acids are abundant (Grandison et al. 2009). The benefit of restricting nutrients, rather than calories, likely involves, at least in part, a reduction in oxidative stress and damage. Methionine restriction reduces oxidative damage and increases mitochondrial biogenesis as well as expression of an uncoupling protein in rat brain (Naudi et al. 2007). Reduced consumption of protein, but not fat or carbohydrates, leads to lower levels of ROS and oxidative stress in rat liver, which may be attributable to methionine restriction (Ayala et al. 2007). These results highlight the importance of amino acids as modulators of life span and point to amino acid sensing pathways and effects on translation and energy production / utilization as potential mediators.

Finally, the availability of sugars / carbon sources is integrated with the availability of amino acids / nitrogen sources to influence chronological longevity. Glucose is unique in terms of the metabolic preference exhibited for this sugar by many organisms including yeast. In yeast, sugar / carbon source availability is detected by the Ras/PKA pathway whereas amino acid / nitrogen source availability is detected by the TOR pathway. When glucose is readily available, *S. cerevisiae* grows with minimal respiration, even under aerobic conditions, a phenomenon known as the Crabtree effect. To achieve this, numerous genes involved in aerobic respiration and utilization of alternative carbon sources are subject to glucose repression (Carlson 1999; Verstrepen et al. 2004). In contrast, *Kluyveromyces lactis* is a budding yeast closely related to *S. cerevisiae* that is Crabtree negative, meaning that glucose fails to down-regulate respiration and mitochondrial function when oxygen is available for aerobic respiration. An interesting result in this regard is that *K. lactis* does not appear to enjoy extension of CLS due to CR in low glucose medium (Oliveira et al. 2008).

One possibility is that *S. cerevisiae* favors fermentation and rapid growth when glucose is plentiful but must shift to aerobic respiration to achieve chronological longevity when glucose is exhausted. This physiological strategy to ferment glucose and produce ethanol may have conferred a selective advantage to yeast during evolution because of the protection of environmental resources afforded by ethanol (Thomson et al. 2005), and its oxidation product acetic acid. But, when glucose is exhausted, *S. cerevisiae* must carry out extensive metabolic reprogramming to aerobic metabolism to support long-term survival in the non-dividing state. This metabolic and proteomic reprogramming takes place during the transition from rapid fermentative growth in log phase to slow aerobic growth during the diauxic and post-diauxic phases to low level aerobic respiration in stationary phase. If there is sufficient availability of amino acids and non-fermentable carbon sources

(including certain amino acids), then yeast are able to complete this transition to aerobic metabolism, which supports chronological longevity.

Concluding Perspectives

As our knowledge of how diet influences longevity has increased, relatively simple ideas have given way to increasingly complex ones. The idea that longevity is simply a matter of reducing caloric intake and respiration-dependent energy production has given way to a more complex set of ideas that relate nutrient availability to cellular metabolic decision-making, damage control, and longevity. Dietary nutrient balance is emerging as an important longevity factor that affects a multitude of metabolic processes, including amino acid and protein homeostasis. But, many significant questions remain. For example, increased essential amino acid availability supports chronological longevity in yeast, yet limitation of a specific essential amino acid in mice and flies brings about extension of life span, apparently in a manner that mimics CR. Perhaps the most exciting aspect of the relationship between nutrient balance and longevity is the potential for human dietary intervention. Altering the composition of the diet offers fundamental advantages over adding a pharmacological compound to the diet. Perhaps the most important advantage is the potential for widespread acceptance and implementation in society. Put simply, a "nutrient balance" strategy for extending life span and/or health span has the potential to reach a much larger portion of our population than a "calorie restriction" strategy.

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Chapter 9
DNA Damage and DNA Replication Stress in Yeast Models of Aging

William C. Burhans and Martin Weinberger

Abstract DNA damage is an important factor in aging in all eukaryotes. Although connections between DNA damage and aging have been extensively investigated in complex organisms, only a relatively few studies have investigated DNA damage as an aging factor in the model organism *S. cerevisiae*. Several of these studies point to DNA replication stress as a cause of age-dependent DNA damage in the replicative model of aging, which measures how many times budding yeast cells divide before they senesce and die. Even fewer studies have investigated how DNA damage contributes to aging in the chronological aging model, which measures how long cells in stationary phase cultures retain reproductive capacity. DNA replication stress also has been implicated as a factor in chronological aging. Since cells in stationary phase are generally considered to be "post-mitotic" and to reside in a quiescent G0/G1 state, the notion that defects in DNA replication might contribute to chronological aging appears to be somewhat paradoxical. However, the results of recent studies suggest that a significant fraction of cells in stationary phase cultures are not quiescent, especially in experiments that employ defined medium, which is frequently employed to assess chronological lifespan. Most cells that fail to achieve quiescence remain in a viable, but non-dividing state until they eventually die, similar to the senescent state in mammalian cells. In this chapter we discuss the role of DNA damage and DNA replication stress in both replicative and chronological aging in *S. cerevisiae.* We also discuss the relevance of these findings to the emerging view that DNA damage and DNA replication stress are important components of the senescent state that occurs at early stages of cancer.

Keywords DNA damage · DNA replication stress · Replicative aging · Chronological aging · Genome instability

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Introduction

Genome instability is broadly considered to be an important factor in aging in all eukaryotic organisms. Support for this proposition includes accumulating evidence that genome instability and the rate at which this instability occurs increase with age, as well as the accelerated aging phenotypes associated with defects in many genome maintenance programs. Furthermore, there is now compelling evidence that in complex organisms, the activity of some (although not all) DNA repair pathways declines with age. As was discussed in an excellent recent review of the connections between DNA damage and aging in higher eukaryotes (Freitas and de Magalhaes 2011), proof of the validity of this proposition requires the demonstration that enhanced DNA repair extends lifespan, which has not yet been observed. It also remains a matter of debate whether the accelerated aging phenotypes observed in progeroid syndromes are relevant to intrinsic aging that occurs in the absence of mutations in genome maintenance proteins. Collectively, however, the evidence from many lines of investigation suggests that DNA damage is a significant causal factor in normal aging (Freitas and de Magalhaes 2011 and references therein).

As predicted by the longstanding free radical theory of aging, reactive oxygen species (ROS) that damage DNA and other macromolecules also have been strongly implicated as pro-aging factors in all eukaryotes. Growth signaling through conserved RAS, TOR, AKT and other pathways is a third factor that plays an important role in aging. Some of the pro-aging effects of growth signaling can be attributed to increased levels of intracellular ROS detected when growth signaling pathways are activated, which leads to oxidative damage to DNA. Recent studies have established that growth signaling can also cause DNA damage independently of ROS by producing DNA replication stress – that is, inefficient DNA replication – in cells undergoing genome duplication (Bartkova et al. 2005). The unique structure of replicating DNA, which requires unwinding of double-stranded template DNA to allow for template-directed DNA synthesis, increases the susceptibility of DNA at stalled replication forks to DNA damage. For example, single strand scissions that occur with little consequence elsewhere in the genome can cause more deleterious doublestrand DNA (dsDNA breaks) when they occur within regions of unwound DNA at replication forks. The unwound DNA at replication forks is also recombinogenic. Factors that slow or stall replication forks and thus contribute to DNA replication stress include reduced pools of deoxynucleotide triphosphates (dNTPs) required for DNA synthesis, DNA secondary structures that are difficult for polymerases to replicate and proteins bound to DNA that block replication fork progression (reviewed in Burhans and Weinberger 2007).

In the budding yeast model organism *S. cerevisiae*, two separate, but overlapping paradigms exist for assessing factors that contribute to aging. Replicative aging studies measure the number of times cells divide before they senesce and eventually die. Although this model is relevant to aging of somatic cells of complex eukaryotes, the asymmetry of cell division in budding yeast suggests that replicative aging studies may be especially informative as a model for aging of asymmetrically dividing stem cells in these organisms. Chronological aging studies measure the survival and/or

reproductive capacity of cells that have entered stationary phase due to the depletion of glucose and other nutrients from medium. The chronological aging model is frequently employed to investigate conserved factors that impact aging in cells of complex eukaryotes that have entered into a postmitotic state due to downregulation of growth signaling pathways during differentiation. However, the interpretation of many earlier chronological aging studies was based on the erroneous assumption that cells in stationary phase cultures uniformly reside in a non-dividing "quiescent" state. In fact, recent studies described in detail by Werner-Washburne et al. (Chapter 6, this volume) indicate that a significant fraction of cells in stationary phase cultures are not quiescent, and that some cells in these cultures continue to divide. These findings have important implications for understanding chronological aging and its relationship to aging in complex eukaryotes.

In this chapter we review evidence that DNA damage is a significant causal factor in aging of the model organism *S. cerevisiae* assessed using both the replicative and chronological aging models. One goal of our discussion is to integrate information about how DNA damage contributes to aging in this organism with the results of studies that were focused on the impact on aging of growth signaling pathways and oxidative stress. Many of these studies point to DNA replication stress as a source of DNA damage that arises as a consequence of growth signaling and oxidative stress. This integrative view suggests a new paradigm for understanding aging and age-related diseases that may serve as a guide for future studies.

DNA Damage and Replicative Aging in Budding Yeast

The replicative aging model is based on the seminal discovery more than 60 years ago that budding yeast cells are not immortal (Mortimer and Johnston 1959). Instead, they divide a finite number of times, which defines their replicative lifespan as measured by micromanipulating daughter cells from mother cells each time a mother cell divides. The replicative lifespan of individual cells that have lost reproductive capacity can also be assessed by counting "bud scars" deposited on the surface of cells each time they divide. Old mother cells are also usually larger than young cells and often have also lost the capacity to mate with cells of the opposite mating type (i.e., they are sterile). Importantly, there is an asymmetric component to replicative aging that leads to "resetting" of the aging clock of daughter cells when they are born to middle-aged mothers. Resetting of this clock allows daughter cells produced by the division of older mother cells to be born "young" – that is, they retain the capacity to divide as many times as their mothers eventually will divide. However, this asymmetry breaks down in very old mothers, which produce daughter cells that are sometimes as large as their mothers and exhibit a shorter lifespan. The asymmetric component of replicative aging in budding yeast has clear parallels in the asymmetric division of stem cells in metazoans.

Replicative aging and ERCs. Early efforts to understand the nature of the finite replicative lifespan of budding yeast were focused on a search for "senescence"

factors that accumulate in, and are retained by, mother cells each time they divide. This led to the discovery that replicative aging is related to the accumulation in mother cells of extrachromosomal ribosomal DNA (rDNA) circles (ERCs) generated by instability in the highly repeated rDNA locus (Sinclair and Guarente 1997). At least some of this instability occurs in association with the replication of rDNA, because deletion of the *FOB1* gene encoding a protein that forms a polar barrier to DNA replication forks in each rDNA repeat suppresses rDNA recombination and reduces the number of ERCs that accumulate compared to age-matched wild type cells. The reduced number of ERCs detected in *fob1Δ* cells is accompanied by an approximately 2-fold extension of replicative lifespan compared to isogenic wild type cells. ERC formation also is suppressed by the formation of heterochromatin in the rDNA locus, a process that is regulated by the NAD+-dependent histone deacetylase Sir2, as well as other proteins (Guarente 2000). Rtg2p, which is a key factor in retrograde responses to mitochondrial dysfunction that extend replicative lifespan in rho 0 cells (Jazwinski 2005), extends replicative lifespan in rho+ cells as well, independently of its role in retrograde responses. Both replicative aging and ERC formation are accelerated in *rtg2Δ* cells, but in the absence of changes in heterochromatin-mediated silencing of the rDNA locus (Borghouts et al. 2004).

Each rDNA repeat harbors an origin of replication capable of initiating DNA replication. Consequently, once formed, ERCs containing rDNA sequences replicate to high copy numbers (Sinclair and Guarente 1997). Replication of ERCs is accelerated in *sir2Δ* cells by increased frequency of initiation of DNA replication in the rDNA locus (Pasero et al. 2002). However, except in very old cells, ERCs are largely retained in mother cells when they divide. This identifies ERCs as a prime candidate for the "senescence" factor that accumulates in mother cells postulated earlier to explain the asymmetric nature of replicative aging. How the accumulation of ERCs in mother cells might cause the loss of reproductive capacity that underlies replicative aging remains unclear. One possibility is that factors required for DNA replication or transcription that interact with rDNA sequences are titrated off of chromosomes by binding to accumulated ERCs, thus blocking events required for cell division (Sinclair and Guarente 1997).

Replicative aging and age-dependent genetic instability. If accumulating DNA damage is a component of replicative aging, the expectation is, of course, that replicatively aged cells would have a hypermutation phenotype. In fact, pedigree analysis of a standard laboratory strain revealed that the daughters of replicatively aged mother cells exhibit a greater than 100-fold increase in mutations leading to loss of heterozygocity (LOH) (McMurray and Gottschling 2003). Subsequently this mutator phenotype was attributed to the loss of mitochondrial DNA in daughter cells born of old mothers and consequent defects in the biogenesis of iron-sulfur clusters, which are present in a number of proteins required for the maintenance of nuclear genomes (Veatch et al. 2009). Somewhat surprisingly, however, this mutator phenotype occurred independently of the Fob1p-dependent polar replication fork barrier in the rDNA locus.

The analysis of genetic instability in association with replicative aging has been limited by the small number of old mother cells in aging populations cultured in liquid medium. Recently, new approaches have been devised that depend on transient selection against daughter cells, which leads to the accumulation of a large number of synchronously aging mother cells until the production of daughter cells is restored (Jarolim et al. 2004; Lindstrom and Gottschling 2009). Fortuitously, similar to most natural isolates of *S. cerevisiae,* daughter cells produced by old mothers in strains employed in the Mother Enrichment Program (MEP) approach to investigating aging retain mitochondrial genomes (indicated by respiratory competence) (Lindstrom and Gottschling 2009). This provides the opportunity to analyze genetic instability in old mother cells using colony-based assays in the absence of effects associated with the loss of mitochondrial DNA in the daughter cells that form colonies.

The results of a recent study by Lindstrom et al. that employed the MEP approach to investigate age-dependent genome instability revealed the existence of replicative age-related increased genome instability in the rDNA locus that depends on Fob1p (Lindstrom et al. 2011). Interestingly, this instability is unrelated to an age-dependent decline in Sir2p reported earlier (Dang et al. 2009), which was confirmed in this more recent study. Unlike the uniformly non-reciprocal recombination events detected earlier as a function of age in cells that have lost respiratory competence, instability in the rDNA locus of aging respiratory competent cells is manifested as age-dependent increases in LOH associated with both reciprocal and non-reciprocal recombination events. Reciprocal recombination reflects resolution of Holliday junctions via crossing over, while non-reciprocal recombination events likely reflect break-induced replication.

Although instability in the rDNA locus in MEP cells depends on Fob1p, the aging process that underlies rDNA instability in this experimental system does not depend on Fob1p, nor is Fob1p strictly required for the accumulation of ERCs. Lindstrom et al. postulate that aging in these cells is instead caused by DNA replication stress that acts cooperatively with the Fob1p replication fork barrier to generate age-dependent instability in the rDNA locus. Since most cells in complex eukaryotes retain respiratory competence, the age-dependent genetic instability revealed by this study is more likely to be conserved compared to the age-dependent instability driven by loss of mitochondrial DNA reported earlier.

The results of this study are generally consistent with earlier findings by Ganley et al. that point to Fob1p-dependent instability in the rDNA locus of old mother cells as a factor that can limit replicative lifespan (Ganley et al. 2009). Although rDNA instability was reportedly enhanced by ERCs in this earlier study, it also occurred independently of ERC accumulation. Deletion of *HPR1* encoding a component of the RNA polymerase II complex also shortens replicative lifespan in concert with destabilization of the rDNA locus, but in the absence of ERC accumulation (Merker and Klein 2002). Conversely, overexpression of Rtgp suppresses ERC accumulation but does not impact replicative aging (Borghouts et al. 2004). Deletion of *AFO1* encoding a mitochondrial ribosomal protein also extends replicative lifespan independently of the influence of ERC accumulation (Heeren et al. 2009). Furthermore

deletion of the genes encoding the growth signaling proteins Sch9p or Tor1p also extends replicative lifespan in a fashion that mimics the effects of caloric restriction, and the additive effects on longevity of deleting *FOB1* from *sch9Δ* and *tor1Δ* cells is consistent with a role these proteins in replicative aging that does not depend on ERCs (Kaeberlein et al. 2005). All these findings suggest that ERC accumulation is not a prerequisite for replicative aging.

Replicative aging that does not depend on the accumulation of ERCs raises questions, of course, regarding the nature of factors that promote rDNA instability and trigger senescence independently of ERCs in old mother cells, but not their daughters. In addition to their preferential retention of ERCs, mother cells also retain oxidatively damaged proteins in a Sir2-dependent fashion (Aguilaniu et al. 2003). It is possible that in addition to the failure of ERCs to segregate into daughter cells, exclusion from daughter cells of damaged proteins that interfere with DNA replication and other aspects of genome maintenance promotes recovery of rDNA stability. This latter model does not exclude the possibility that ERC accumulation promotes aging. However, some of the findings described above clearly point to rDNA instability as an important determinant of replicative lifespan that does not always depend on elevated levels of ERCs.

Replicative aging and DNA replication stress. The role for DNA replication stress in destabilization of the rDNA locus that underlies replicative aging proposed by Lindstrom et al. is supported by the results of a number of additional studies. This includes, for example, the observation that reducing the intracellular level of DNA polymerase alpha destabilizes rDNA (Casper et al. 2008). Therefore, in principle, replication stress can induce instability in the rDNA locus. A role for replication stress in replicative aging is also supported by the observation that at the end of their replicative lifespans, aged mother cells enter into a senescent state that based on DNA content measurements often appears to occur in S phase (Pichova et al. 1997). We believe that DNA replication stress may contribute to the pro-aging phenotype of *rtg2Δ* cells as well (Borghouts et al. 2004), a possibility that has not been described by others. Defects in Rtg2p promote more rapid expansion of trinucleotide repeats (Bhattacharyya et al. 2002), which occurs via mechanisms that include the formation of unusual DNA structures in repeat sequences during DNA replication (Lopez Castel et al. 2010). Rtg2 is required to maintain dNTP pools required for efficient DNA replication and repair, and *rtg2Δ* cells exhibit lower levels of dNTPs (Hartman 2007).

Although rDNA instability induced by low polymerase activity is Fob1p-independent, other studies clearly implicate Fob1p-dependent replication stress in rDNA instability. For example, doublestrand DNA (dsDNA) breaks occur in close proximity to the Fob1p-dependent replication fork barrier in the rDNA locus (Weitao et al. 2003b). These breaks could be caused by incomplete DNA replication or by spontaneous breaks in unwound single strand DNA at paused replication forks, which (as discussed earlier) are uniquely susceptible to single strand scissions that can lead to dsDNA breaks (reviewed in Burhans and Weinberger 2007). In addition, both replication fork pausing and dsDNA breaks are elevated in strains expressing

a hypomorphic allele of *DNA2*, which encodes an essential DNA helicase required for DNA replication and repair, and deletion of *FOB1* suppresses these phenotypes (Weitao et al. 2003b). Similar phenotypes are detected upon inactivation of Sgs1p, the budding yeast RecQ helicase (Weitao et al. 2003a). In all eukaryotes, RecQ helicases play an important role in repair of stalled replication forks (Singh et al. 2009).

Perhaps not surprisingly, deletion of *SGS1* shortens replicative lifespan (Sinclair and Guarente 1997). Although the shorter lifespan of *sgs1Δ* cells was initially reported to coincide with the accelerated accumulation of ERCs, subsequent studies indicated that the number of ERCs in aged *sgs1Δ* cells is similar to the number of ERCs detected in age-matched wild type cells (Heo et al. 1999; McVey et al. 2001). Therefore, the mechanism by which inactivation of Sgs1p accelerates aging does not depend on the accelerated accumulation of ERCs. The accelerated aging phenotype of *sgs1Δ* cells is of course reminiscent of the accelerated aging phenotypes associated with defects in RecQ helicases in humans (Singh et al. 2009).

Strains expressing hypomorphic alleles of *DNA2* employed in the study described above also exhibit a shorter replicative lifespan, as do several strains harboring hypomorphic mutations in other proteins required for DNA replication (Hoopes et al. 2002). These proteins include the Rad27p flap endonuclease required for the maturation of Okazaki fragments during DNA replication and the DNA polymerase alpha homologue Pol1p. Similar to deletion of *SGS1*, a mutation in *DNA2* accelerates replicative aging in the absence of the accelerated accumulation of ERCs in mother cells, providing another example of ERC-independent replicative aging related to DNA damage (Hoopes et al. 2002).

Although it is not entirely clear how the accelerated replicative aging phenotype of these strains is related to normal (i.e. intrinsic) aging, the increased rDNA instability that accompanies accelerated aging in the *dna2* and *sgs1Δ* strains suggests some degree of relevance to replicative aging in wild type cells. Furthermore, inhibition of glucose signaling by deleting *HXK2*, *SCH9* or *TOR1* (Riesen and Morgan 2009) or by caloric restriction imposed by reducing the glucose concentration in medium extends replicative lifespan in concert with reduced recombination in the rDNA locus (Riesen and Morgan 2009; Smith et al. 2009). These findings also point to reduced stability in the rDNA locus as an intrinsic aging factor in the replicative aging model.

An intrinsic role for DNA replication stress and DNA damage in replicative aging is also supported by microarray experiments that profiled changes in gene expression in old mother cells. In one study, compared to young cells, old cells or prematurely aged cells harboring a mutation in *DNA2* were found to upregulate the expression of *SGS1* as well as many other DNA repair genes and genes involved in different DNA damage responses (Lesur and Campbell 2004). *SGS1* and other DNA damage response and repair genes were similarly upregulated in old cells in a second study (Laun et al. 2005). In this latter study, *SGS1* and other genes encoding proteins that respond to DNA damage and replication stress also were represented in the overlap between datasets of genes upregulated in old cells

with those shown earlier to be upregulated by a mutation in Orc2p (Ramachandran et al. 2006). Orc2p is an essential protein required for initiation of DNA replication, and defects in Orc2p function cause DNA replication stress, genome instability and apoptosis.

DNA Damage and Chronological Aging in Budding Yeast

Senescence in the replicative and chronological aging models. Accumulating evidence suggests that in complex eukaryotes, stem cell senescence promotes aging by blocking the homeostatic maintenance and repair of tissues (Rodier and Campisi 2011). The asymmetric cell division shared by stem cells and budding yeast cells is often cited in support of the potential utility of budding yeast replicative aging studies to provide insights into factors that lead to stem cell senescence. Senescence is also an important component of early stages of preneoplastic disease (Rodier and Campisi 2011), which budding yeast replicative aging experiments are also thought to model.

In contrast, the chronological aging model – which examines the survival or reproductive capacity of cells in stationary phase – is more often employed to model aging of differentiated cells in complex eukaryotes that have entered into a non-dividing "postmitotic" quiescent state. In the world of mammalian cell biology, however, confusion exists regarding the definition of quiescence, which is often mistaken for the physiologically distinct senescent state (Blagosklonny 2011). Although neither senescent nor quiescent cells divide, in contrast to quiescent cells that retain the ability to respond to growth signals triggered by mitogens and nutrients, senescent cells cannot respond to these signals and have lost their capacity to reproduce. Similar confusion regarding this and related issues reigns in the somewhat different realm of stationary phase biology and chronological aging in budding yeast. For example, most chronological aging studies have measured chronological lifespan by assessing the ability of cells in stationary phase cultures to form colonies. Many of these studies (including a few published by our laboratory) inaccurately refer to the loss of reproductive capacity detected by this approach as loss of viability. In fact, it was recently discovered that loss of reproductive capacity of cells in stationary phase precedes loss of viability (Aragon et al. 2008), and therefore reflects the establishment of a senescent state, similar to the senescent state detected in replicatively aged budding yeast cells as well as in mammalian cells.

Furthermore, although chronological aging of stationary phase cells is usually considered as a model for aging in postmitotic cells, at least some of the events that drive stationary phase cells into a senescent state must occur during transitions into stationary phase before cells stop dividing. This logically follows from the observation that senescence of stationary phase cells is inhibited by caloric restriction imposed by a reduction in the initial concentrations of glucose in medium. Since glucose is consumed as cells undergo the transition to stationary phase and is absent from stationary phase cultures, the decrease in the initial concentration of

glucose must impact the senescence of stationary phase cells before this transition is completed and cells cease to divide.

Another point of confusion in the stationary phase and chronological aging literature has been that until recently, it was generally assumed that most cells in stationary phase reside in a uniform quiescent, non-dividing state until they eventually die. As is described in more detail by Wener-Washburne et al. (Chapter 6, this volume), in fact a large fraction of cells in stationary phase cultures are not quiescent (Allen et al. 2006). The fraction of non-quiescent cells in stationary phase cultures is even larger in experiments performed using defined medium rather than rich medium (Madia et al. 2009). The larger fraction of non-quiescent stationary phase cells in defined medium – which is the medium employed in most prior studies of chronological aging – is likely due to the enhanced accumulation of acetic acid that occurs in this medium as cells enter stationary phase (Burtner et al. 2009). Acetic acid accumulation leads to a substantially lower pH in defined compared to rich medium stationary phase cultures (Weinberger et al. 2010). Low pH activates the same growth signaling pathways triggered by glucose in yeast and by glucose or mitogens in mammals (reviewed in Weinberger et al. 2010). Therefore, sustained growth signaling by low pH as acetic acid accumulates in defined medium stationary phase cultures likely inhibits quiescence and promotes senescence.

Yet another misconception regarding chronological aging experiments is that because the number of cells in stationary phase cultures does not change substantially with time, these cells are not dividing. However, in the defined medium employed in most chronological aging experiments, the fraction of cells in S phase first declines to 0% at day 1 of chronological aging experiments and then steadily increases with time in stationary phase (Madia et al. 2009). Thus, some cells in defined medium stationary phase cultures appear to be re-entering the cell cycle, most likely due at least in part to increased growth signaling by low pH as acetic acid accumulates in this medium. Re-entry into the cell cycle is accompanied by a parallel increase in the non-quiescent fraction of stationary phase cells observed at the same time points (Madia et al. 2009). Senescent cells in stationary phase eventually die by an apoptotic mechanism (Fabrizio et al. 2004; Herker et al. 2004) that leads to their destruction, similar to mammalian cells undergoing apoptosis, and this occurs more frequently in dividing cells with buds (Weinberger et al. 2010). Consequently, although the number of dividing cells in stationary phase at specific time points in chronological aging experiments can be quite low, this low number reflects the steady-state turnover of cells that during longer periods of time (such as those typically employed in chronological aging experiments) is represented by a larger fraction of cells that continue to divide.

Chronological aging, DNA damage and DNA replication stress. The more nuanced view of stationary phase biology described above has important implications for understanding chronological aging of budding yeast and its relationship to aging and age-related diseases in complex eukaryotes. In most mammalian cells, senescence is a consequence of DNA damage induced by oxidative stress and/or DNA replication stress. In many types of tumors, preneoplastic stages of

tumorigenesis are accompanied by loss of reproductive potential and the induction of senescence markers triggered by sustained oncogenic growth signaling in cells that have not yet become fully transformed. Additional mutations that abrogate checkpoint responses to DNA damage and replication stress restore reproductive potential, and thus convert these cells to a malignant, neoplastic state. Although earlier investigations of how senescence occurs in preneoplastic cells were focused on oxidative stress (Chen et al. 2007), the emerging view of the senescent state detected in preneoplastic cells is that it is largely induced by DNA replication stress (Negrini et al. 2010). Therefore, one can argue that in addition to modeling events that occur in postmitotic differentiated cells of mammals, chronological aging experiments model events related to DNA damage and DNA replication stress that lead to senescence of mammalian cells during transitions to quiescence. This includes senescence of stem cells that frequently transition into a quiescent state as well as senescence of cells transitioning to quiescence during differentiation that occurs at early stages of neoplastic disease.

However, a role for DNA damage or DNA replication in chronological aging has not been extensively explored. This is related in part to the prevailing (albeit inaccurate) view that cell division is not a significant factor in chronological aging experiments. For example, this view underlies a recently published argument that replication stress is unlikely to be a major factor in chronological aging, because based on measurements of budding index, only a small fraction of cells in stationary phase cultures are potentially in S phase (Fabrizio and Longo 2008). This argument of course fails to consider that the number of budded cells in stationary phase cultures underestimates the number of cells undergoing the steady state process of cell division and cell death described above that over time can occur in a large fraction of cells.

Despite the paucity of studies that address these issues, however, there is some evidence that DNA damage and DNA replication stress are significant determinants of chronological lifespan. For example, an analysis of genetic instability using colony sectoring approaches similar to those that detected LOH in replicatively aged cells clearly points to a similar age-dependent increase in LOH during chronological aging (Qin et al. 2008). A role for DNA damage and DNA replication stress in chronological aging is also consistent with the significantly shorter chronological lifespan that results from the inactivation of Rad27p (Laschober et al. 2010), the flap endonuclease required for Okazaki fragment maturation that (as discussed earlier) also promotes longevity in the replicative aging model. Surprisingly, in contrast to the replicative lifespan-shortening effects of deleting *SGS1* described earlier, it has been reported that deletion of *SGS1* does *not* impact chronological lifespan (Madia et al. 2008; Ringvoll et al. 2007). However, compared to wild type cells *sgs1Δ* cells frequently undergo adaptive regrowth promoted by the apoptotic release of nutrients into the medium (Madia et al. 2008). Adaptive regrowth leads to an apparent increase in reproductive capacity as measured by colony-forming units at later time points of chronological aging experiments, and this increase can mask an inherently shorter chronological lifespan. In fact, in both studies that reported the absence of effects of deleting *SGS1* on chronological lifespan (Madia et al. 2008; Ringvoll

et al. 2007), the reproductive capacity of *sgs1Δ* cells was significantly lower than wild type cells at early time points before adaptive regrowth occurred.

A role for Sgs1p in promoting chronological longevity via maintenance of genetic stability is also supported by the higher mutation frequency detected in chronologically aging *sgs1Δ* compared to wild type cells (Madia et al. 2008). This latter study also reported that compared to wild type cells, *sgs1Δ* cells less frequently enter into a quiescent state in stationary phase. In the context of the well-characterized role of Sgs1p in stabilizing stalled DNA replication forks, these findings are consistent with a role for a defective response to replication stress that impacts chronological aging of *sgs1Δ* cells.

Chronological lifespan is also shortened by inactivation of Sic1p (Weinberger et al. 2010; Zinzalla et al. 2007), a cyclin-dependent kinase inhibitor that blocks S phase entry of cells in stationary phase. *sic1Δ* cells suffer replication stress caused by premature entry into S phase that leads to a reduction in the number of active origins of replication, which leads in turn to a deficit in the number of replication forks (Lengronne and Schwob 2002). Mutations in the mRNA decapping enzymes Lsm1p and Lsm4p that lead to stabilization of mRNA also shorten chronological lifespan (Palermo et al. 2010), and this also occurs via a mechanism that likely involves enhanced replication stress. Strains harboring these mutations progress through S phase more slowly than wild type cells, which means they suffer from replication stress (Palermo et al. 2010). Our prediction that *lsm* mutations cause replication stress by promoting the accumulation of excess histones (Burhans and Weinberger 2010) was recently confirmed by others (Herrero and Moreno 2011).

Ribonucleotide reductase (RNR) is essential for the synthesis of dNTPs required for DNA replication and repair. Additional evidence that replication stress can impact chronological aging is provided by the observation that deletion of the *RNR1* gene encoding a subunit of RNR dramatically shortens chronological lifespan (Powers et al. 2006). Replication stress induced by the ribonucleotide reductase inhibitor hydroxyurea also shortens chronological lifespan (Palermo et al. 2010). The shorter chronological lifespan of hydroxyurea-treated cells is observed at low concentrations of hydroxyurea that do not impact the growth of cells in exponential cultures. The increased sensitivity to hydroxyurea in chronological aging experiments likely reflects the downregulation of RNR expression that occurs during the transition to stationary phase (Gasch et al. 2000). Downregulation of RNR is expected to cause a decrease in dNTP pools. Presumably, inhibition of residual RNR activity by low levels of hydroxyurea reduces dNTP pools in these cells further, below a threshold required for efficient DNA replication, thus causing replication stress and a shorter chronological lifespan. Chronological lifespan is also shortened in a strain harboring a hypomorphic allele of *MEC1* (Weinberger et al. 2007), the essential function of which is to upregulate RNR (Desany et al. 1998). Furthermore, the elevated levels of apoptosis detected in this strain (*mec1-21*) during chronological aging are suppressed by ectopic expression of the RNR subunit Rnr1p (Weinberger et al. 2007). Not unexpectedly, dNTP pools in *mec1-21* cells were recently shown to be reduced compared to wild type cells (Fasullo et al. 2010). *rtg2Δ* cells also exhibit a shortened chronological lifespan (Barros et al. 2004) – as

we argued above for the shortened replicative lifespan detected in these cells, this may also be related to the reduced dNTP pools reported in these cells (Hartman 2007).

Replication stress as a cause of intrinsic aging in the chronological aging model. The findings summarized above provide strong evidence that in principle, replication stress can impact chronological aging. It is likely that replication stress also plays a role in intrinsic aging in this model in the absence of mutations or drugs that induce replication stress. For example, profiling of gene expression in non-quiescent stationary phase cells that more frequently senesce compared to quiescent cells revealed that non-quiescent cells harbor elevated levels of transcripts encoding DNA repair and other proteins that respond to DNA damage and/or DNA replication stress (Allen et al. 2006). Furthermore, a strong correlation exists between chronological lifespan extension and a reduction in the frequency with which cells fail to arrest in G0/G1 in stationary phase under a variety of experimental conditions (Weinberger et al. 2010). Shorter-lived cells that in the absence of experimental manipulations capable of extending lifespan remain in S phase in stationary phase cultures are likely undergoing replication stress due to reduced dNTP pools. A role for replication stress in intrinsic chronological aging is also suggested by the more frequent death of budded compared to unbudded cells in stationary phase cultures, and the frequency of budded cell death is accelerated further in cultures established in medium containing elevated concentrations of glucose to enhance growth signaling (Weinberger et al. 2010). The rapid loss of reproductive capacity observed in high glucose medium is accelerated further in the *mec1-21* strain.

Additional evidence that replication stress is an intrinsic aging factor is provided by the observation that RNR activity is limiting in wild type cells during transitions to stationary phase, even in the absence of exposure to hydroxyurea. This is indicated by suppression of apoptosis in stationary phase cells ectopically expressing the RNR subunit Rnr1p (Weinberger et al. 2007). Furthermore, we recently determined that ectopic Rnr1p expression also substantially increases chronological lifespan (data not shown). Although the effects of ectopic Rnr1p expression might be related to enhanced repair of DNA damage, Rnr1p expression also reduces the number of stationary phase cells that remain budded, most likely because they are no longer trapped in S phase due to limiting dNTPs (Weinberger et al. 2007 and data not shown).

A role for DNA replication stress in chronological aging is not consistent, however, with a recent report that during chronological aging in defined medium, mutations measured using a colony-forming assay occur more frequently in the quiescent fraction of cells in stationary phase (Madia et al. 2009). However, the cells employed in these experiments are not quiescent – close inspection of flow cytometry measurements of DNA content in these cells indicates they are mostly in S phase (Fig. S1, Madia et al. 2009), perhaps due to the sustained growth signaling by low pH that occurs in defined medium. Furthermore, although the frequency with which mutations were detected in quiescent cells was higher than in non-quiescent cells, non-quiescent cells senesce and die more frequently than quiescent

cells (Allen et al. 2006). It remains possible, therefore, that non-quiescent cells suffering DNA damage and/or replication stress often cannot form colonies required to detect mutations.

The impact of growth signaling and oxidative stress on chronological age-related DNA damage and replication stress. In all eukaryotes, activation of growth signaling pathways is an important component of aging. In both the replicative and chronological models of aging in budding yeast, activation of conserved RAS, TOR, and SCH9/AKT signaling pathways promotes aging, and inactivation of these pathways promotes longevity (reviewed in Weinberger et al. 2010; see also above). Most efforts to understand the underlying mechanisms in the chronological aging model have focused on the negative regulation of oxidative stress responses by activated growth signaling pathways, which leads to elevated intracellular levels of reactive oxygen species (ROS) that damage DNA and other cellular components. However, as has been reported in other model systems, increased oxidative stress and oxidative damage do not always correlate with a shorter lifespan – for example, inactivation of catalases extends the chronological lifespan of budding yeast in concert with elevated levels of both hydrogen peroxide and oxidative damage to cellular components (Mesquita et al. 2010). This suggests the existence of factors that contribute to chronological aging other than (or in addition to) oxidative damage to DNA and other molecules.

The strong inverse correlation between chronological lifespan and the failure of stationary phase cells to efficiently arrest in G0/G1 when growth signaling is active (Weinberger et al. 2010) suggests that sustained growth signaling can also promote aging by inducing replication stress in cells that are driven into S phase while in stationary phase, where dNTPs and other factors required for efficient DNA replication are reduced or absent (Weinberger et al. 2007). A key element of this model is that the multiple events required for the coordinated entry into and progression through S phase are regulated by different growth signaling pathways, not all of which may be active in chronological aging experiments. This is perhaps best exemplified by the discovery that constitutive activation of Ras2p (which shortens chronological lifespan) induces the transcription of the *CLN3* gene, but not the transcription of *RNR1* and other genes encoding proteins required for DNA replication (Wang et al. 2004). *CLN3* encodes the cyclin-dependent kinase activator Cln3 that promotes entry into S phase. Ectopic expression of Cln3p shortens chronological lifespan in concert with a higher frequency of mutations and an increased number of stationary phase cells that remain in S phase (Weinberger et al. 2007). A similar failure to activate the full panoply of growth signaling pathways required for normal cell division, including efficient progression through S phase, may be responsible for the rapid death of stationary phase cells induced by adding glucose, but not other nutrients, to stationary phase cultures (Granot et al. 2003).

At least one form of ROS produced downstream of growth signaling – superoxide anions – also increases the number of cells that remain in the cell cycle in stationary phase (Weinberger et al. 2010). This is similar to the more frequent growth arrest of MnSOD-defective mouse cells in S phase when they are driven into quiescence by

contact inhibition (Sarsour et al. 2008), and both observations and may be related to inhibitory effects of superoxide anions on DNA replication reported in mammals (Chaudhuri et al. 2010; Zanetti et al. 2001). Therefore, an additional possibility is that growth signaling also induces replication stress by inducing superoxide anions that trap stationary phase cells in S phase.

Interestingly, stationary phase *rad27Δ* and *sgs1Δ* cells exhibit elevated levels of ROS detected by a somewhat non-specific ROS probe (Ringvoll et al. 2007), and we recently determined that these strains harbor elevated levels of superoxide anions (data not shown). Elevated levels of superoxide anions have also been detected in *sic1Δ* cells (Weinberger et al. 2010) that (as discussed above) suffer replication stress due to a reduced number of active origins of DNA replication, and in DNA repair mutants, where it may serve as a DNA damage signal (Rowe et al. 2008). In the context of the inhibitory effects of superoxide anions on G0/G1 arrest in stationary phase, superoxide produced as part of a DNA damage response could participate in a self-amplifying regulatory loop that enhances both oxidative and replicative stress in stationary phase cells.

Relevance of DNA Damage and DNA Replication Stress in Yeast Aging Models to Aging and Cancer in Metazoans

There is little doubt that DNA damage is an important component of aging in all eukaryotes – the debate is now mostly focused on how this damage occurs. Certainly oxidative damage to DNA is an important factor, particularly to mitochondrial DNA (Salmon et al. 2004), although the relationship between oxidative DNA damage and aging has rarely been directly addressed in budding yeast aging experiments. DNA replication stress that results in DNA damage and genome instability is an additional feature of aging shared by both the budding yeast replicative and chronological aging models. The extensive overlap between effects of experimental manipulations that impact replication stress and aging in both the replicative and chronological aging models reflects recent findings that suggest that senescence and/or apoptosis of dividing cells is a previously unrecognized feature of both aging models. Although the production of ERCs induced by replication stress does not appear to be a feature of aging in complex eukaryotes, as described above there is plenty of evidence that replication stress can promote aging in the absence of ERC formation in both the budding yeast replicative and chronological aging models.

In fact, recent studies of mice expressing a hypomorphic allele of the replication protein Mcm2 point to replication stress as a causal factor in aging of mammals (Pruitt et al. 2007). The longstanding connections between the mammalian WRN and BLM RecQ helicases that respond to replication stress and the defects in these proteins that cause accelerated aging also point to a role for DNA replication stress in aging in mammals. This is not entirely surprising given the unique susceptibility of DNA replication forks to DNA damage.

The source of replication stress that triggers responses that require proteins such as WRN and BLM in mammals and Sgs1p in budding yeast remains more of a mystery. Based on the results of yeast experiments, we proposed the model described above that replication stress can arise as a consequence of "imbalanced" growth signaling that in budding yeast leads to trapping of stationary phase cells in S phase (Weinberger et al. 2007). The replicative lifespan-extending effects of inactivating Sch9p and Tor1p might be explained by a reduction in "imbalanced growth signaling" that prematurely drives dividing cells into S phase as well.

We proposed this model as a potential explanation for replication stress that had been recently implicated in the senescent state of preneoplastic cells, which are also trapped in S phase (Di Micco et al. 2006). This model was recently validated by the discovery that in preneoplastic cells at early stages of cancer, uncoordinated activation of pathways that enforce cell proliferation (such as pathways that depend on E2F), but not pathways that upregulate dNTP metabolism, triggers senescence by driving cells into S phase in the absence of sufficient dNTP pools to support efficient DNA replication and maintain genome stability (Bester et al. 2011). As in budding yeast, dNTP metabolism is downregulated in quiescent mammalian cells (Hakansson et al. 2006). Subsequent findings in yeast described in this review predict that an additional component of this recently described mechanism for triggering senescence in mammals is sustained growth signaling coupled to the downregulation of dNTP metabolism in stem cells or differentiating cells that are transitioning into quiescence.

Whether DNA replication stress plays a role in intrinsic aging in complex eukaryotes is a larger mystery that remains almost completely unexplored. Relevant to this question, a recent review of factors that cause genome instability in cancer cells suggests that replication stress is not an initiating event in neoplastic disease. Instead, the general consensus appears to be that cancer is initiated by mutations that activate oncogenic growth signaling, which is generally viewed as the proximal cause of DNA replication stress (Luo et al. 2009). However, the rapid death of dividing stationary phase budding yeast cells when growth signaling is enhanced by high levels of glucose or by adding glucose, but not other nutrients to stationary phase cultures coupled to numerous reports that high glucose inhibits DNA replication and induces AKT-dependent growth signaling and DNA damage in mammalian cells (reviewed in Weinberger et al. 2010) suggests an alternative possibility – that in complex eukaryotes, replication stress is induced both by oncogene activation and by hyperglycemia that leads to the mutational activation of oncogenes. Hyperglycemia is a risk factor in neoplastic and other age-related diseases and this model provides a potential link between diet and these diseases. Since the underlying mechanism (glucose-induced replication stress that leads to DNA damage and senescence) may contribute to aging as well, this model also provides a potential link between diet and intrinsic aging.

Integration of the information regarding DNA damage and DNA replication stress in budding yeast aging models presented in this review with information from studies of aging in complex eukaryotes suggests a model for how DNA damage and replication stress ultimately impact aging in budding yeast as well as aging

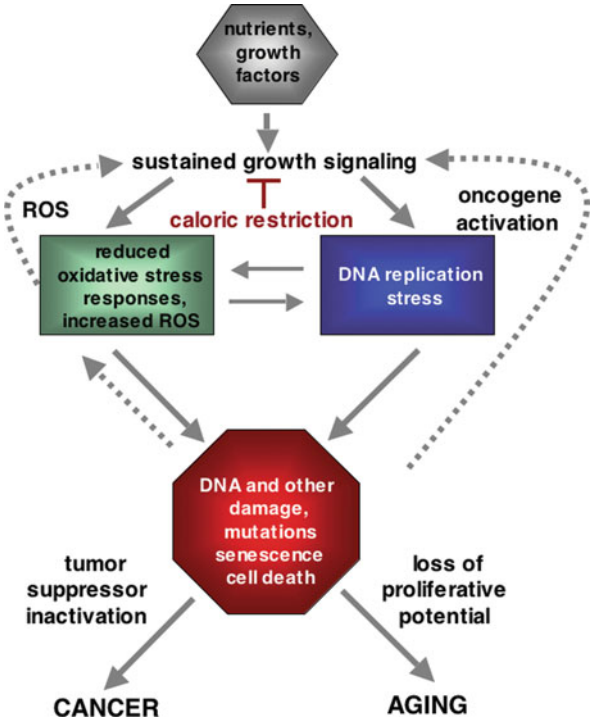

Fig. 9.1 Model for how DNA damage and DNA replication stress impact aging and age-related diseases based on studies of *S. cerevisiae* and other organisms. According to this model, sustained activation of growth signaling pathways by mitogens and/or glucose and other nutrients elevates levels of ROS by inhibiting oxidative stress responses. It also induces DNA replication stress by driving cells into S phase in the absence of sufficient dNTPs and other factors required for efficient DNA replication. Elevated ROS also contribute to replication stress by trapping cells in S phase or by signaling growth (*rightward pointing arrow*). At least in budding yeast, replication stress can also elevate ROS (*leftward pointing arrow*). Both elevated ROS and DNA replication stress cause DNA damage and mutations that lead to senescence and cell death. In mammals, DNA damage that causes mutations in oncogenes can amplify growth signaling that promotes additional ROS and DNA replication stress. Rarely, senescent preneoplastic cells regain the capacity to reproduce when additional mutations occur in tumor suppressor genes required to maintain the senescent state, and this leads to tumor formation. Maintenance of the senescent state promotes aging. This model also predicts that caloric restriction attenuates growth signaling and therefore reduces ROS, DNA damage and DNA replication stress and their downstream consequences for aging and cancer

and age-related diseases in metazoans (Fig. 9.1). Most studies of aging have been focused on effects of oxidative stress. The powerful tools available to investigators who study aging in the model organism budding yeast are ideally suited to explore the broader impacts of DNA damage and DNA replication stress, in addition to oxidative stress, on aging summarized in this review and how they might be mitigated by caloric restriction.

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Chapter 10
Yeast Aging and Apoptosis

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Abstract A concerted balance between proliferation and apoptosis is essential to the survival of multicellular organisms. Thus, apoptosis per se, although it is a destructive process leading to the death of single cells, also serves as a pro-survival mechanism that ensures healthy organismal development and acts as a life-prolonging or anti-aging program. The discovery that yeast also possess a functional and, in many cases, highly conserved apoptotic machinery has made it possible to study the relationships between aging and apoptosis in depth using a well-established genetic system and the powerful tools available to yeast researchers for investigating complex physiological and cytological interactions. The aging process of yeast, be it replicative or chronological aging, is closely related to apoptosis, although it remains unclear whether apoptosis is a causal feature of the aging process or vice versa. Nevertheless, experimental results obtained during the past several years clearly demonstrate that yeast serve as a powerful and versatile experimental system for understanding the interconnections between these two fundamentally important cellular and physiological pathways.

Keywords Apoptosis · Necrosis · Replicative aging · Chronological aging · Cell death

The Historical Background of Apoptotic Cell Death

The phenomenon of cell death has kept scientists occupied for more than 150 years since it was first described by the German scientist Carl Christoph Vogt (1842). Initially, research in this area mainly focused on the description of specific morphological cell death markers. A contemporary of Carl Vogt, the German physician Rudolf Virchow, treated the topics related to cell death in his internationally recognized lecture on "Cellular Pathology as Based upon Physiological

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and Pathological Histology" using terms such as necrosis, necrobiosis, mortification, softening or degeneration. Around 1880 Karl Weigert (together with Julius Cohnheim) identified the phenomenon of "coagulation necrosis" (Weigert 1880). We now understand that some of these experimental findings were misinterpreted by the authors. However, the observation that necrotic cells lose their nuclei was important for further research and eventually led to the discovery of autolysis (self-digestion) (Jacoby 1900), pyknosis (irreversible condensation of chromatin) (Schmaus and Albrecht 1894), karyolysis (complete dissolution of the chromatin) and karyorhexis (destructive fragmentation of the nucleus) (Klebs 1889) or chromatin margination (Arnheim 1890). As soon as cell staining techniques became available, spontaneous cell death was investigated as a physiological event. Breakthrough publications by Walther Flemming describing chromatolysis (Flemming 1885) led to numerous subsequent publications on cell death in lactating mammary glands (Nissen 1886), during ovarian follicle development (Ruge 1889), in breast cancer cells (Ströbe 1892) and many other cell types and tissues (for review see Glücksmann 1951). All of these studies described a specific mode of cell death we now call apoptosis. However, in these earlier times pathologists mostly considered cell death to be the result of external insults. Thus, death was thought to occur passively, being merely inflicted on cells.

Modern apoptosis research begins with the seminal paper by Kerr et al. (1972) which drew the attention of the scientific world to the previously ignored fact that the ordered and programmed removal of cells of multicellular organisms under a variety of physiological conditions is as important to organismal viability as is the production of these cells. For the first time, a clear distinction was made between apoptosis, a tightly regulated, non-inflammatory and mostly unnoticed process, and necrosis, a process usually accompanied by inflammation. Within the next few years additional morphological and biochemical characteristics of apoptotic cells as well as molecular determinants regulating the apoptotic program have been identified. The activation of endonucleases has been shown to be involved in the process of apoptosis (Williams et al. 1974), serving as the first non-morphological marker of this process. Subsequently, cleavage of DNA between nucleosomes, resulting in DNA "laddering", was found to accompany apoptotic cell death (Wyllie 1980). In addition, the externalisation of phosphatidylserine to the outer leaflet of the plasma membrane was identified as an early apoptotic event some years later (Fadok et al. 1992).

At about the time Kerr defined the term apoptosis, Sydney Brenner successfully introduced *Caenorhabditis elegans* as a genetic model system. The observation that cell death occurs during development of *C. elegans* (Sulston 1976) and the evidence that cell death in worms is caused by a specific, exclusive process (Horvitz et al. 1982) led to the discovery of the first *ced* mutants (Hedgecock et al. 1983). Horvitz and his colleagues could show that 131 of the 1090 somatic cells formed during the life of worms are destined to die during development (Ellis and Horvitz 1986).

In 1986 the human BCL-2 gene was cloned (Cleary et al. 1986; Tsujimoto and Croce 1986). It was first assumed that BCL-2 was an oncogene encoding a protein that promotes cell proliferation, because in non-Hodgkin follicular lymphoma, a

common human blood cell cancer, the gene is activated by the immuno-globulin heavy chain enhancer leading to vigorous proliferation of B-cells. The interaction between BCL-2 and the enhancer is enabled by the well-known chromosome translocation (14;18) near the tips of these two chromosomes. Two years later, Vaux and colleagues (1988) demonstrated that high levels of BCL-2 protected cells from death upon growth factor removal.

This finding immediately drew the attention of the scientific community to apoptosis and marked the beginning of intensive research on the molecular machinery initiating and executing apoptosis. The fact that disruption of the apoptotic program eventually caused tumor proliferation electrified many scientists all over the world. After this point in time various hallmarks of apoptosis were discovered in quick succession, including the involvement of the tumor suppressor p53 in apoptosis regulation (Chiou et al. 1994; Yonish-Rouach et al. 1991), the role of different **c**ysteine-dependent **asp**artate-directed prote**ases** (caspases) (Miura et al. 1993; Yuan et al. 1993) and their activation pathways (Alderson et al. 1995; Chinnaiyan et al. 1997), the role of apoptotic protease activating factor 1 (Apaf-1) and mitochondrial cytochrome *c* release (Liu et al. 1996). Additional pro- and anti-apoptotic proteins such as BAX (Oltvai et al. 1993), BCL-X_L (Muchmore et al. 1996), BIM (Bouillet et al. 1999), or inhibitor of apoptosis genes (IAP) like p35 (Birnbaum et al. 1994; Clem et al. 1991) were also identified.

In very general terms, at least three rather different biological purposes or scenarios appear to activate the apoptotic machinery in cells:

(I) Cells commit suicide during the process of morphogenesis early in life.
(II) Cells that have accumulated damage beyond a certain threshold are removed by apoptosis, thereby ensuring proper functioning of organs and tissues in adult life.
(III) Senescent cells undergo apoptosis, a process that up to now has been studied mainly in cultured mammalian cells at the Hayflick limit (replicative senescence) (Hampel et al. 2004). In contrast, some cell lines, (such as fibroblasts) are resistant to radiation-induced or p53-induced apoptotic stimuli at the Hayflick limit. However, this resistance to apoptosis is not a general feature of senescent cells.

A prominent example of morphogenic apoptosis in the embryo which has found its way into numerous textbooks is the formation of digits of the mouse paw during embryogenesis (Wood et al. 2000). In this work, the authors compared wild type mice and homozygous PU.1 null mice, which do not produce mature macrophages, osteoclasts, eosinophils or B-cells. PU.1 null mice also exhibit retarded and aberrant neutrophil development, undergo delayed T-lymphocyte differentiation and are completely devoid of professional phagocytes as a consequence of an ETS family transcription factor mutation. Apoptotic cells were identified in this study by acridine orange staining and by electron microscopic analysis of apoptotic bodies, which were either free (PU.1 null) or engulfed (WT; wildtype) by professional

phagocytes. The defect of PU.1 null mice in the process of engulfment was partly compensated for by mesenchymal cells.

Another role of apoptosis is the quality control of germ cells in the nematode and in mammals. To eliminate any haploids harboring mutations caused by premeiotic DNA synthesis, faulty segregation at the meiotic divisions and, perhaps also heteroplasmic mitochondrial mutations (Cree et al. 2008), a very high proportion of the female germ cells undergo apoptosis and only a minute fraction survives (De Felici et al. 2007). A comparable quality control mechanism has been observed in mammalian male meiosis (Odorisio et al. 1998).

Later, apoptotic markers like DNA fragmentation as indicated by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling method) assay were detected in the mouse system (Fernandez-Teran et al. 2006). Furthermore the signaling function of externalized phosphatidylserine and other possible surface markers was studied in more detail (Hamon et al. 2002; Henson and Hume 2006), and these studies revealed that externalized phosphatidylserine signals to macrophages (which subsequently engulf the apoptotic cells) and also signals downregulation of the inflammatory response (Cvetanovic et al. 2006).

During morphogenic development single mutations in genes encoding components of the apoptotic machinery mostly result in mild phenotypes. Occasionally an embryonic or perinatal lethal phenotype can be detected, like in the DNAse IIα homozygous knock-out mutant (Evans and Aguilera 2003; Kawane et al. 2001, 2003). However, this might not be due to a loss of apoptotic functions. Instead, it may be related to a defect in an essential "day-job" function of the DNAse IIα, for instance in haematopoiesis.

Remarkably, not only are proteins pivotally important to the execution of the programmed cell death process conserved to a certain degree, but also the signals and mechanisms that underlie the removal of apoptotic cells appear to be conserved between widely divergent animal species, including *D. melanogaster*, nematodes and vertebrates. However, there is also evidence that the well-characterized mitochondrial pathway of cell death in the invertebrates *C. elegans* and *D. melanogaster* is not universally conserved among animals (Oberst et al. 2008). Nevertheless, the multiple stimuli (pathways) that converge on apoptotic processes and the presumed multiplicity of apoptotic mechanisms requires detailed elucidation and poses a challenge to current research. For instance, little is known about the signals (intracellular and extracellular) leading to the embryonic morphogenic apoptosis described above or the degree of conservation between the pathways culminating in embryonic and other forms of apoptosis.

Yeast as a Model Organism to Study Cell Death

Yeast was introduced as an experimental organism almost 100 years ago and has emerged as one of the most important and useful model systems developed since the mid-twentieth century. This eukaryotic organism offers the advantage of a short generation time and, in contrast to many higher eukaryotes, genetic tractability.

The complete genome of *Saccharomyces cerevisiae* has been sequenced, and this information together with the relatively facile genetic manipulations that are possible in this organism has generated an enormous collection of available mutants and research tools. Since many basic features of eukaryotic physiology are conserved across phyla, this model system has been employed to delineate many of the molecular players, pathways and subroutines underlying human disease, including the roles and mechanisms of apoptosis.

In earlier studies the yeast system was employed to study the actions of mammalian pro- and anti-apoptotic proteins in live cells that offered the advantages of conserved biological processes and reduced complexity but were also believed to lack the apoptotic machinery. The heterologous expression of mammalian p53, for example, was observed to cause a severe defect in cell proliferation, while BAX triggered yeast cell death. However, in these studies yeast cells killed by expression of mammalian BAX did not exhibit apoptotic changes like nuclear fragmentation, internucleosomal DNA fragmentation, chromatin margination or any other classical morphological feature of apoptosis (Bischoff et al. 1992; Jurgensmeier et al. 1997). The lack of a typical apoptotic phenotype and of obvious apoptotic players led to the assumption that yeast, though an ideal model organism to study components of the mammalian apoptotic machinery, does not undergo apoptosis itself.

This assumption was challenged in 1997 when Frank Madeo and co-workers published a pioneering study on a temperature-sensitive cdc48 mutant strain. At non-permissive temperatures this strain exhibits many of the same markers of apoptosis detected in multicellular organisms, but in the absence of heterologous "apoptotic" genes. Similar to mammalian cells, dying cdc48 cells exhibit DNA fragmentation detectable using the TUNEL assay, exposure of phosphatidylserine at the outer layer of the cytoplasmic membrane (as indicated by Annexin V staining), ROS accumulation (quantifiable using dihydrorhodamine or dihydroethidium) and chromatin condensation and fragmentation (Madeo et al. 1997). Madeo et al. also demonstrated that the occurrence of these events was highly coordinated, implying the presence of a molecular machinery in yeast that executes apoptosis.

Since then, a large number of studies have demonstrated the existence of conserved apoptotic regulators in yeast. Various exogenous triggers (e.g. acetic acid, aspirin, UV radiation, paclitaxel, edelfosine, arsenic, bleomycin, amiodarone, heat, salt, sugar, several heavy metals, hypochlorous acid and many others) (reviewed in Carmona-Gutierrez et al. 2010) as well as dysfunction of conserved mitochondrial, proteasomal, or peroxisomal pathways and defective N-glycosylation and actin dynamics have been shown to culminate in apoptotic death of yeast cells. These studies highlight the potential the yeast system offers for further understanding programmed cell death, including in higher eukaryotes.

The Molecular Machinery Executing Yeast Apoptosis

Since the discovery of yeast apoptosis in a strain harboring a mutation in the gene *CDC48*, multiple pro- and anti-apoptotic regulators of yeast cell death have been identified and further characterized (Fig. 10.1).

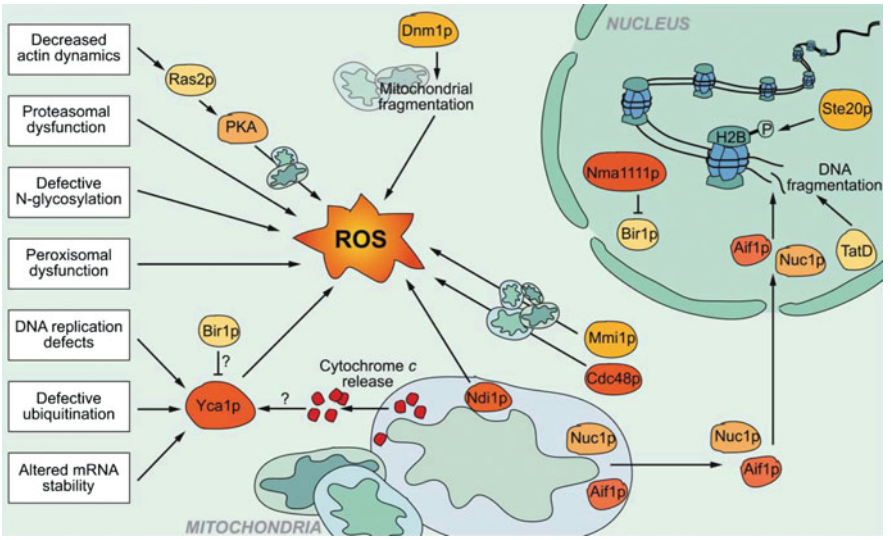

Fig. 10.1 The basic molecular machinery of yeast apoptosis. Various proteins of the molecular machinery executing mammalian cell death are conserved in yeast, including the yeast caspase Yca1p, the apoptosis-inducing factor Aif1p, yeast endonuclease G (Nuc1p), Cdc48p, the serine protease HtrA2/Omi (Nma111p), yeast AMID (Ndi1p), the survivin Bir1p and many others. Furthermore, yeast apoptosis involves complex processes like histone modification, mitochondrial fragmentation and cytochrome *c* release and is linked to impaired proteasomal and peroxisomal function, cytoskeletal perturbations and defects in ubiquitination, N-glycosylation, DNA replication, and RNA stability

In mammalian cells, numerous caspases function as the core of the apoptotic machinery; upon activation caspases initiate and execute the regulated disintegration of the cell. About ten years ago, it was discovered that plants, fungi and protozoa encode caspase-related proteases termed metacaspases (Uren et al. 2000). Shortly after these discoveries, the role of the yeast metacaspase Yca1p in the execution of yeast apoptosis was described (Madeo et al. 2002). While overexpressing Yca1p triggers cell death with typical apoptotic markers that require its protease activity, the absence of Yca1p protects cells against apoptosis induced by hydrogen peroxide (Madeo et al. 2002). In the following years, disruption of *YCA1* has been shown to result in reduced cell death and/or ROS accumulation in a variety of cell death scenarios, including death induced by exogenous triggers such as the histone deacetylase inhibitor valproic acid (Mitsui et al. 2005), cadmium (Nargund et al. 2008) or acetic acid (Guaragnella et al. 2010) as well as death caused by deregulation of essential cellular processes, including altered mRNA stability (Mazzoni et al. 2005), breakdown of ubiquitination control (Bettiga et al. 2004), insufficient clearance of insoluble protein aggregates (Lee et al. 2010) or DNA replication failure (Weinberger et al. 2005). Homologs of metazoan proteins that regulate the mitochondrial pathway of apoptosis also have been identified in yeast, including the flavoprotein Aif1p and the nuclease Nuc1p, the yeast homologs of mammalian

apoptosis-inducing factor (AIF) and endonuclease G (EndoG) respectively. Like their mammalian counterparts, Aif1p and Nuc1p reside within the mitochondria of healthy cells and are released into the cytosol upon apoptotic stimuli (Buttner et al. 2007; Wissing et al. 2004). Both proteins have been shown to translocate to the nucleus, where they degrade chromosomal DNA and thus provoke cellular demise. While death mediated by Aif1p depends on the presence of the cyclophilin A Cpr1p (Wissing et al. 2004), yeast EndoG requires components of the permeability transition pore (PTP) and phosphorylation of histone H2B for efficient cell killing (Buttner et al. 2007). H2B phosphorylation at a specific serine residue (S10) has been shown to be a universal prerequisite of oxidative stress-induced death of mammalian and yeast cells (Ahn et al. 2005).

The NADH dehydrogenase Ndi1p, the yeast homolog of AMID (AIF homologous mitochondrion-associated inducer of cell death), is another protein that plays a role in the mitochondrial pathway of apoptosis in yeast (Li et al. 2006). Besides their lethal roles in the execution of apoptosis, Aif1p, Nuc1p and Ndi1 fulfill day-time jobs in healthy cells, where they are involved in the maintenance of mitochondrial respiratory function and mitochondrial DNA (Li et al. 2006; Wissing et al. 2004; Zassenhaus and Denniger 1994). The crucial role of mitochondria in the apoptotic program is also emphasized by the fact that mitochondrial dynamics have an important impact on cellular apoptosis. It was clearly demonstrated that the induction of apoptosis is accompanied by mitochondrial fission, although mitochondrial fission is neither sufficient nor a prerequisite for apoptosis (Heath-Engel and Shore 2006). The key component of mitochondrial fission is the Dynamin-related GTPase Dnm1p, which forms helical structures that are capable of constricting the mitochondrial membranes. To be fully functional Dnm1p needs the help of two additional proteins, namely Fis1p and Mdv1p. Fis1p is permanently localized to the outer mitochondrial membrane and in concert with the adaptor protein Mdv1p is responsible for the recruitment of Dnm1p to mitochondria (Lackner and Nunnari 2009; Naylor et al. 2006). Both Mdv1p and Dnm1p are essential for mitochondrial fragmentation and cell death after the application of different apoptotic insults (Bink et al. 2010; Fannjiang et al. 2004; Palermo et al. 2007). Surprisingly, Fis1p plays an opposite, anti-apoptotic role by preventing mitochondrial fission and cell death, in contrast to its "normal role" in healthy cells (Fannjiang et al. 2004). The pro-apoptotic function of Dnm1p seems to be well conserved. It has also been demonstrated in mammals that mitochondrial fragmentation precedes mitochondrial cytochrome c release and that a dominant-negative Drp1 variant (the mammalian homologue of DNM1) delays, but does not completely abolish apoptosis (Frank et al. 2001; Heath-Engel and Shore 2006; Scheckhuber et al. 2007). Interestingly, it has been reported that during apoptosis, BAX always co-localizes with Drp1 at mitochondria and that these mitochondrial regions become the future mitochondrial scission sites (Karbowski et al. 2002). Also, a deletion of Mdm30p, a F-box protein involved in mitochondrial fusion, results in an abnormal mitochondrial morphology and a dramatically increased resistance to apoptotic stimuli such as H_2O_2 (Palermo et al. 2007).

Keeping in mind the crucial role of mitochondria in the apoptotic program, the mitochondrial proteome of apoptotic- and non-apoptotic cells has been compared (Braun et al. 2006; Rinnerthaler et al. 2006). It was found that Mmi1p translocates from cytosol to the outer mitochondrial membrane in cells subjected to different stressors that induce cell death. The relocalization of this highly conserved protein under these conditions establishes a functional link between microtubules and mitochondria (Rinnerthaler et al. 2006). It was subsequently determined that the human homologue of Mmi1p, TCTP (translationally controlled tumour protein), inserts into the membrane of isolated rat mitochondria (Susini et al. 2008) and accumulating evidence indicates that TCTP exerts an anti-apoptotic function either by direct (Susini et al. 2008) or indirect (Liu et al. 2005; Yang et al. 2005) inhibition of BAX-induced cell death.

Additional mitochondrial proteins that are involved in the apoptotic program are Uth1p and Yme1p. Uth1p, a protein of the outer mitochondrial membrane and an eponymous member of the SUN (*SIM1*, *UTH1*, *NCA3*) family (Camougrand et al. 2000) has proven to be a jack-of-all-trades. This marvelous protein is involved in such diverse functions as cell wall biogenesis (Ritch et al. 2010), oxidative stress responses (Bandara et al. 1998) and mitophagy (Camougrand et al. 2000). In a genetic screen UTH1 was also demonstrated to be essential for BAX-induced cell killing in yeast. However, in a strain from which UTH1 has been deleted, BAX still localizes to mitochondria, which results in cytochrome c release (Camougrand et al. 2003, 2004). Yme1p localizes to the inner mitochondrial membrane and functions as an AAA-protease (Leonhard et al. 1999; Palermo et al. 2007). The BAX-induced cytochrome c release from yeast mitochondria is accompanied by degradation of cytochrome c oxidase and this degradation is strictly dependent on Yme1p. Therefore it is not surprising that deletion of *YME1* results in delayed BAX-induced cell death (Manon et al. 2001).

The yeast homolog of mammalian HtrA2/Omi, the pro-apoptotic serine protease Nma111p (nuclear mediator of apoptosis) plays a role in the execution of apoptosis, because its deletion diminishes and its overexpression enhances the development of apoptotic markers induced by heat shock and oxidative stress (Fahrenkrog et al. 2004). Interestingly, one of the rare IAP (inhibitor-of-apoptosis) proteins identified in yeast, Bir1p, has been shown to be a substrate of Nma111p (Walter et al. 2006). Another protein with a proven anti-apoptotic function in yeast is the E3 ubiquitin protein ligase Bre1p. The anti-apoptotic feature of this ligase is linked to the ubiquitination of one of its substrates, namely the histone H2B that for example plays an essential role in checkpoint activation in case of DNA damage (Walter et al. 2010). Multiple other proteins have been shown to be involved in the regulation of yeast apoptosis, including the nuclease Tat-D (Qiu et al. 2005), Ost2p, which is the yeast homologue of the mammalian defender of apoptosis-1 (DAD1) protein (Hauptmann et al. 2006), and Ste20p, a kinase that mediates pheromone-induced apoptosis in yeast (Severin and Hyman 2002). Other proteins include Stm1p, a DNA-binding protein that contributes to apoptosis (Ligr et al. 2001), the mitochondrial protein Ysp1p, which is essential for apoptosis-induced mitochondrial fragmentation (Pozniakovsky et al. 2005a), the anti-silencing protein Asf1p, inactivation of which strongly affects the nucleosome turnover resulting

in the induction of apoptosis (Yamaki et al. 2001) and many others (reviewed in Carmona-Gutierrez et al. 2010). Furthermore, apoptotic processes such as the release of cytochrome *c* from mitochondria (Ludovico et al. 2002; Manon et al. 1997), the opening of a high conductance channel within the mitochondrial membrane (Pavlov et al. 2001), dissipation of mitochondrial membrane potential, and mitochondrial fragmentation (Pozniakovsky et al. 2005b) have been observed in yeast, in addition to higher eukaryotes. As mentioned above, apoptotic death can be induced exogenously e.g. by acetic acid (Ludovico et al. 2001), oxidative stress (Madeo et al. 1999; Narasimhan et al. 2001), SICD (sugar induced cell death; Granot et al. 2003) or salt stress (Huh et al. 2002; Wadskog et al. 2004). There are also two important scenarios where the induction of apoptosis occurs endogenously – specifically, replicative and chronological aging. Considering all of the above observations, the relevance of apoptosis in yeast physiology can no longer be ignored.

Reactive Oxygen Species in Apoptosis and Aging of Yeast and Higher Organisms

Reactive oxygen species (ROS) are key regulators of aging as well as apoptotic execution. Thus, it is not surprising that the exposure of *C. elegans* to oxidative stress during development leads to an increased number of cells that undergo apoptosis. However, apoptosis and aging do not seem to be correlated in this animal (see below).

In multicellular animals apoptosis is well documented as an anti-aging mechanism. Damaged cells are apoptotically removed from tissues followed by the replacement of these cells by homeostatic mechanisms that, when they fail, contribute to aging (Schmitt et al. 2007). Although several different forms of molecular damage can trigger the apoptotic process, the most generally accepted mechanism of damage relevant here is the oxidative damage exerted primarily by ROS. Cellular oxidative damage (genotoxic and cytotoxic) induced directly or indirectly via ROS in the living cell (Halliwell and Gutteridge 2007; Aung-Htut et al. Chapter 2, this volume) is present in most aged and apoptotic cells in all model systems. The exposure of cells to several externally added oxidants, like hydrogen peroxide, tert-butyl hydroperoxide, diamide and others can mimic internal oxidative stress. Furthermore, the role of extramitochondrial sources of ROS, such as NADPH oxidases (Krause 2007), in the regulation of apoptosis should be taken into consideration (our own unpublished results).

The phenomenon of replication stress (Burhans and Weinberger 2007; Weinberger et al. 2007, 2010) is an example where the primary defect is not oxidative stress but creates oxidative stress secondarily. Upon cell cycle arrest at a checkpoint, the cell has in principle two possible ways to react, depending on the duration of the cell cycle arrest and the state of the cell. First, the cell is able to repair the damaged DNA and continue normal cell cycle progression or secondly, the cell activates the apoptotic program when DNA repair mechanisms fail (Jacotot

et al. 2000; Pietenpol and Stewart 2002). Furthermore, loss of checkpoint control has been shown to trigger apoptosis (Luo et al. 2001; Wang et al. 2003).

This is intriguing, because senescent yeast mother cells often die in the middle of an ongoing cell cycle (Pichova et al. 1997), displaying markers of apoptosis (Laun

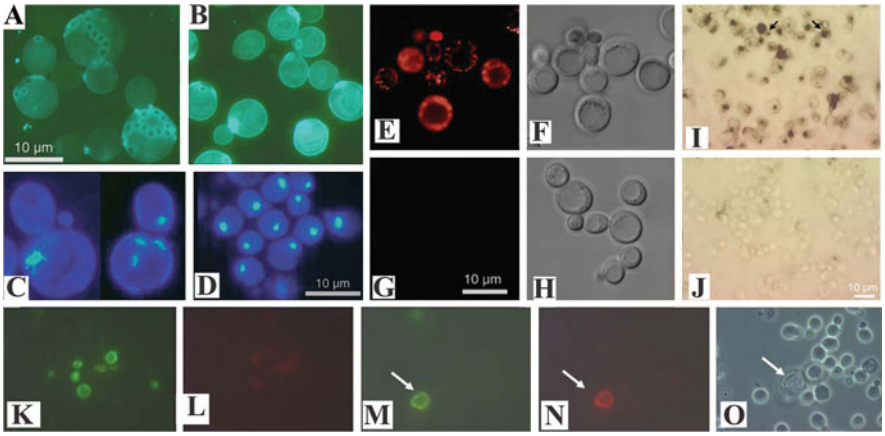

Fig. 10.2 Yeast exhibits markers of apoptosis during replicative aging. Using a method developed in our laboratory for the enrichment of senescent (terminal) yeast mother cells we investigated cytological markers of oxidative stress and of apoptosis in senescent cells (Fraction V) and, for comparison, in young daughter cells (Fraction II) that had undergone the same elutriation procedure (Laun et al. 2001). **a** Fraction V cells after staining with Calcofluor White M2R and viewing under a fluorescence microscope. **b** Fraction II cells treated exactly as in **a**. The majority of these cells are virgins with just one (*dark*) birth scar. The cells are smaller than in **a**. **c** Fraction V cells after staining of nuclei with DAPI. Note diffuse chromatin and, occasionally, multiple nuclei. **d** Fraction II cells treated exactly as above. Note compact well-defined nuclei. **e** Fraction V cells were stained with DHR (indicating ROS) and viewed and photographed under a confocal laser-scanning fluorescence microscope. The stained cells show typical mitochondrial morphology. **f** The same sample in phase contrast. **g** Fraction II cells treated as above show only very weak staining indicating a low mitochondrial ROS load. **h** The same sample as in **g** viewed in phase contrast. **i** Fraction V cells were fixed, cell walls were digested and strand breaks in DNA were detected by the TUNEL assay. Nuclei containing large amounts of DNA strand breaks were stained black by the diaminobenzidine $\pm$ H_2O_2 reaction after incorporation of fluorescein isothiocyanate (FITC)-labelled dUTP and treatment with anti-FITC antibody Fab fragment from sheep coupled with horseradish peroxidase. Positive staining was observed in about 20% of the 500 cells that were examined in each sample. In some cases (*arrows*), mother and daughter cells from a pair were both TUNEL positive, indicating that the last daughters of old apoptotic mother cells are sometimes also apoptotic. **j** Fraction II cells were treated and stained as in **i**. Practically no TUNEL-positive staining was observed. **k** Fraction V cells were stained for exposed phosphatidylserine with FITC-conjugated annexin V after digestion of cell walls with glusulase/lyticase and viewed under a fluorescence microscope. **l** The same sample was washed and stained with propidium iodide and viewed using the fluorescein filter set. This control shows that the annexin-positive cells in **k** are not lysed or damaged. **m** Fraction II cells stained for phosphatidylserine with FITC conjugated annexin V and viewed under a fluorescence microscope. A sample with a very infrequently observed annexin-positive cell (*arrow*) is shown. **n** The same sample stained with propidium iodide showing that the marked cell is lysed (*arrow*). **o** The same sample as in **m** and **n** shown in phase contrast. It is obvious here as well that the marked cell is lysed (*arrow*)

et al. 2001; Fig. 10.2) . The old mother cells either arrest with a daughter cell still attached (varying in size) or initiate a new cell cycle although the previous one is still not finished correctly (Nestelbacher et al. 1999). A lack of control mechanisms would therefore lead to aneuploid cells among the daughters of these mother cells. This is a striking parallel to many human cancer cells, which also have lost cell cycle control and are often aneuploid.

The correlation between apoptosis and oxidative stress seems to be general. Even in cases where the primary cause of apoptosis is unrelated to oxidative stress (for instance during faulty response to DNA damage, see above), the apoptotic destruction program that is turned on includes production of ROS (Marchetti et al. 2006; Weinberger et al. 2005).

The Interconnection Between Apoptosis and Aging

Only a few studies that explicitly address relationships between aging and apoptosis have been published so far. Although a correlation between apoptosis, senescence and aging has been described in some systems, an overlap between these two processes is essentially absent in *C. elegans* (Hengartner 1997). Surprisingly, mutations that compromise the machinery responsible for developmental apoptosis neither constrict the fitness and fecundity of this organism (Ellis and Horvitz 1986; Lettre and Hengartner 2006), nor do they influence its lifespan (Conradt and Xue 2005). The *C. elegans* model probably has the largest number of genetically defined aging mutants assigned to a specific signaling pathway, the IGFR (insulin like growth factor receptor) pathway, which also governs the formation of *dauer* larvae. The formation of dauer is mainly a response to stress conditions and no aging phenotype. However, these mutations have no influence on developmental apoptosis. Although apoptosis occurs in more than 10% of all cells during development and can also be induced in response to external oxidative stress, apoptotic markers are reportedly absent from senescent worms.

Caloric restriction, which is one of the rare methods of lifespan elongation that works in all known model systems of aging, attenuates age-related apoptosis and ROS production in addition to exending lifespan (compare Zhang and Herman 2002 and the literature references therein). Furthermore, genes involved in the IGFR pathway that also have a strong impact on *C. elegans* lifespan are conserved in mammals, where they are known to regulate apoptosis (Zhang and Herman 2002). More recently, it was reported that mutations in the IGFR pathway of mice also cause lifespan extension (Selman et al. 2007). Papa and Skulachev (1997) discuss the mitochondrial origin of oxygen radicals and their influence on both processes, aging and apoptosis. However, as mentioned above, in many cases manipulation of an "apoptotic gene" did not result in the expected lifespan elongation and vice versa, manipulation of a longevity gene did not effect apoptosis (for example, Conradt and Xue 2005).

Büttner et al. (2006) have provided an overview of the physiological situations in which yeast cells undergo apoptosis. Interestingly, both the chronological and

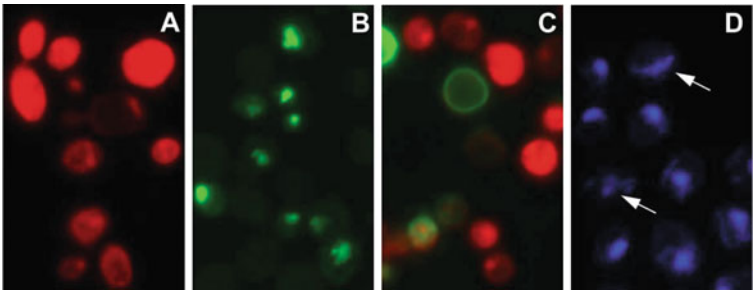

Fig. 10.3 Yeast exhibits markers of apoptosis during chronological aging. Chronologically aged yeast cultures of BY4741 die exhibiting typical markers of apoptosis and accumulate oxygen radicals (Herker et al. 2004). **a** Generation of reactive oxygen species (ROS) as indicated by the superoxide-driven oxidation of non-fluorescent dihydroethidium (DHE) to fluorescent ethidium. **b** Apoptotic DNA fragmentation visualized using the TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) assay. **c** Co-staining of Annexin V and propidium iodide to detect apoptotic phosphatidylserine externalization and necrotic loss of membrane integrity. **d** DAPI staining to detect nuclear fragmentation and chromatin condensation (*arrows*)

mother cell-specific aging processes of yeast cells, which represent distinct physiological scenarios, lead to apoptosis of terminally senescent cells. Fluorescence microscopic analysis of chronological dying yeast cells stained for specific apoptotic markers are depicted in Fig. 10.3.

Replicative aging depends on the number of cell generations a mother cell is able to undergo and is independent of calendar time. Analyzing a cohort of mother cells, the distribution of lifespans of the single cells follows the Gompertz law. During their lifespan, mother cells become much larger than their daughter cells and display a prolonged cell cycle time. They exhibit reduced translational activity of ribosomes as well as a massive accumulation of ROS indicated by strong DHR staining of the mitochondria. Their actin cytoskeleton collapses at the terminal stage and forms large F-actin clumps. Nuclear structure changes from compact to fuzzy. The whole aging process occurs in the presence of nutrients and has nothing to do with starvation (Breitenbach et al. 2003; Laun et al. 2001).

Contrary to this scenario for replicative aging, chronologically aged (stationary) yeast cells, which are reproductively still young, undergo a process of senescence due to lack of nutrients. During chronological aging, yeast cells in which the Yca1p metacaspase has been inactivated exhibit improved survival and reduced ROS production (Herker 2004). However, the avoidance of apoptotic death under these circumstances appears to provide a rather short-term advantage. In a direct competition assay, cells deleted of *YCA1* are outlived by wild type cells (Herker 2004), perhaps indicating that apoptotic death of old or damaged cells may be necessary for the maintenance of long-term viability of the total clonal yeast population.

In stationary phase cultures two quite different cell populations can be obtained by isolation on density gradients (Allen et al. 2006; Werner-Washburne et al. Chapter 6, this volume). The first population (cells from the lower and thus denser fraction) consists of cells that remain perfectly viable, resistant to stress and exhibit

a long chronological lifespan. This lifespan elongation is due to a process of differentiation involving changes of the cell wall and synthesis of reserve carbohydrates like trehalose and glycogen. In addition, they initiate a cell cycle arrest with a G1 content of DNA that resembles a true G_0 state. These cells represent the daughter cells resulting from the last cell division cycle that occurred in culture. The second population (cells from the upper fraction) in a stationary culture is rather heterogeneous, harbouring older mother cells and adherent daughter cells in unfinished cell cycles. These cells exhibit decreased viability and increased levels of oxygen radicals compared to cells found in the quiescent fraction. Eventually, both populations at very different times undergo apoptosis. The role of apoptosis in postmitotic aging has been described as an adaptive trait that was positively selected for during evolution (Buttner et al. 2006; Skulachev 2002). Selection for this trait may have been driven by the survival of adjacent yeast cells in times of nutrient scarcity.

This model was further elaborated by Longo and Skulachev who hypothesized that most aging processes (including those that occur in higher organisms) have an adaptive value and are "altruistic" (Longo et al. 2005). However this and similar hypotheses concerning multicellular organisms should be viewed skeptically because strong arguments to the contrary have been put forward (Kirkwood and Proctor 2003; Lithgow and Kirkwood 1996; Vijg 2000). For example: The vast majority of individuals in a wild population never reaches senescence, and therefore it is unclear how selection for the process of aging would occur. The argument that the force of biological selection is at work only until progeny has been procreated and can survive on their own, clearly contradicts a direct positive selection of aging and individual death. Nevertheless, models like the somatic mutation theory of aging (Medawar 1951; Orgel 1963; Szilard 1959), or the theory of antagonstic pleiotropy (Medawar 1951; Williams 2001) may be helpful to understand the evolutionary context of ageing. The latter theory favors the accumulation (during evolution) of late-acting deleterious mutations which are beneficial in youth but lead to an increase in mortality rates late in life.

Concerning yeast mother cell-specific aging, the death of senescent mother cells is absolutely irrelevant for the growing yeast strain or the growing yeast colony. A normal yeast wild-type strain displays a median lifespan of about 20–30 generations and a maximum lifespan of about 50–60 generations (Breitenbach et al. 2003). Taking into consideration that the fraction of cells which is senescent in a population of yeast cells is exceedingly small (Breitenbach et al. 2003) and the nutrients supplied by the death and lysis of the old mother cells are therefore negligible, the positive value of the demise of the senescent mother cells is unclear. Therefore, there must be more convincing reasons why those old mother cells undergo apoptosis.

One key observation is that cell division cycles of budding yeast are asymmetric, resulting in rejuvenated daughter cells that have reset their clock to zero (Jazwinski et al. 1989), and a mother cell which in every cell cycle acquires a gradual change in the parameters mentioned above. This asymmetric division is also found in metazoa, e.g. in the division of human stem cells. Here, the division also results in one new stem cell and one cell that undergoes cellular differentiation.

In living cells, metabolism unavoidably produces waste. Thus, there must be mechanisms to eliminate that waste either by removing it or at least by ensuring

that the load of waste is not passed on to progeny. The latter is achieved by asymmetric segregation of waste between mother and daughter cells to ensure that the species can survive over evolutionary time spans (Klinger et al. 2010).

Oxidation products of cellular components represent predominantly, but not exclusively, the main part of waste in yeast cells as well as in higher eukaryotic cells. One method for quantification of oxidative damage is the detection of carbonylated proteins. The carbonyl groups which exist on proteins due to oxidative reactions ("protein carbonyls") are conjugated with p-nitro phenylhydrazine to form hydrazones, which subsequently are decorated with standardized antibodies directed to p-nitro phenylhydrazone (the "oxy-blot" technique; Nystrom 2005; Requena et al. 2003). It has been clearly demonstrated that in yeast cells cytoplasmic as well as mitochondrial proteins are oxidized during the aging process as well as under stress conditions that are known to induce apoptosis (Aguilaniu et al. 2003). Carbonylation is observed on a small number of proteins and with a strong bias in the mother cell, not in the daughter cell (Aguilaniu et al. 2003; Erjavec et al. 2007; Erjavec and Nystrom 2007). The mechanism of this asymmetric segregation or asymmetric scavenging, which also occurs in *E. coli* and in higher eukaryotes (Erjavec and Nystrom 2007) is presently under investigation (Liu et al. 2010; Winkler et al. 2010). The group of Thomas Nyström was able to identify the protein complex responsible for the retention of damaged and aggregated proteins as well as the machinery that segregates protein aggregates during the mitotic cell cycle – the polarisome. In the polarisome, the interaction of the yeast formin Bni1p, with Myo2p and Sir2p is responsible for the retention of damaged material in the mother cell. Surprisingly, the daughter cells can clear themselves of protein aggregates by polarisome-dependent, actin cable-dependent retrograde transport (Liu et al. 2010).

Disruption of asymmetric segregation by introducing mutations into the yeast *SIR2* gene leads to the expected shortening of the lifespan (Erjavec et al. 2007). Almost all of the tested polarity mutants of the polarisome (Liu et al. 2010) show accelerated aging. It seems plausible that survival and evolutionary success of a (single-celled) species primarily depends on the ability to asymmetrically segregate damage during cell division cycles. The gradual accumulation of damage in mother cells leads to the activation of programmed cell death once a certain threshold is reached and finally to senescence and cell death. This kind of cell death clearly is evolutionarily neutral and is therefore not selected against; however it is not "altruistic".

What appears to be a genetic program of aging is more or less the genetic program(s) of stress responses, because the accumulation of damage causes aging cells to accumulate stress, most importantly, oxidative stress. Part of this stress response program is identical with the apoptotic program, which in a rudimentary form existed in evolution before the first multicellular organisms appeared and still exists today in many single celled organisms. However, the apoptotic response to stress has evolved to become an extremely complex system in higher metazoans. All at the moment known yeast "apoptotic" genes and the effect of their deletion on the aging processes (chronological and replicative) are summarized in Table 10.1. It is obvious that deletion of a pro-apoptotic gene does not always result in the expected lifespan

Table 10.1 The connection between apoptosis, replicative and chronological aging. List of all yeast genes known to be part of the apoptotic machinery. In nearly every case the deletion of one of these "apoptotic" genes impacts either chronological or replicative lifespan. The impact on replicative lifespan of deleting just four genes (*DNM1*, *UTH1*, *MMI1* and *FIS1*) has been examined so far. Although these four genes exhibit pro- as well as anti-apoptotic functions, the replicative lifespan in each case is increased. Therefore, the relationship between apoptosis and replicative lifespan remains unclear

Name	ORF	Chronological lifespan	Replicative lifespan
Pro-apoptotic			
YCA1	YOR197W	Decreased (Piper et al. 2006) /	n.a.
NUC1	YJL208C	Decreased on glucose, increased on glycerol (Buttner et al. 2007)	n.a.
AIF1	YNR074C	Increased (Wissing et al. 2004)	n.a.
CDC48	YDL126C	not viable	not viable
CPR3	YML078W	Increased (Powers et al. 2006)	n.a.
NDI1	YML120C	Increased (Li et al. 2006)	n.a.
DNM1	YLL001W	Increased (Palermo et al. 2007)	Increased (Scheckhuber et al. 2007)
MDV1	YJL112W	Unaltered (Powers et al. 2006)	n.a.
MDM30	**YLR368W**	Increased (Burtner et al. 2011; Palermo et al. 2007)	n.a.
UTH1	YKR042W	Decreased (Powers et al. 2006)	Increased (Kennedy et al. 1995; Weng et al. 2010)
YME1	YPR024W	Decreased (Francis et al. 2007; Palermo et al. 2007)	n.a.
NMA111	YNL123W	Increased (Walter et al. 2006)	n.a.
TAT-D	YBL055C	Increased (Powers et al. 2006)	n.a.
OST2	YOR103C	not viable	not viable
STE20	YHL007C	Decreased (Powers et al. 2006)	n.a.
STM1	YLR150W	Decreased (Powers et al. 2006)	n.a.
YSP1	YHR155W	Decreased (Powers et al. 2006)	n.a.
Anti-apoptotic			
FIS1		Decreased(Powers et al. 2006)	Increased (Scheckhuber et al. 2007)
MMI1	YKL056c	Increased (Powers et al. 2006)	Increased (Managbanag et al. 2008; Rinnerthaler et al. 2006)
BIR1	YJR089W	Overexpression increased (Walter et al. 2006)	n.a.
BRE1	YDL074C	Decreased (Burtner et al. 2011)	n.a.

elongation, but the trend is very clear: deletion of a pro-apoptotic gene results in a prolonged chronological lifespan, whereas deletion of an anti-apoptotic gene has the opposite effect. There are exceptions to this general trend, however. Deletion of Mmi1 is one of the rare mutations that prolongs replicative as well as the chronological lifespan (Managbanag et al. 2008; Powers et al. 2006; Rinnerthaler et al. 2006). Since MMI1 is an anti-apoptotic gene, one might assume the opposite effect would occur. In addition to its role as part of the apoptotic machinery, however, Mmi1 has a "day-job" as a ribosome associated protein. Recently it was demonstrated that heterozygous deletion of several ribosomal proteins can lead to a robust increase in yeast replicative lifespan, by as much as 45% (Chiocchetti et al. 2007). However, as can be seen in Table 10.1, at the time of writing too few replicative lifespan experiments have been performed to draw a general conclusion concerning the correlation between deletion of apoptotic genes and replicative lifespan.

The Comparison Between Yeast and Higher Cell Apoptosis and Aging

Not every aspect of apoptosis present in higher eukaryotic cells also exists in yeast. For example the important mechanisms of "engulfment" or apoptotic recognition, respectively, of the apoptotic cells (Krieser and White 2002) or the Fas-receptor are not part of the yeast apoptotic repertoire. On the other hand, distinct subroutines such as the extrinsic pathways (alpha factor; Severin and Hyman 2002) or the intrinsic mitochondrial pathway leading to apoptosis are surprisingly well conserved, including mitochondrial components that translocate to the nucleus upon cell death induction (AIF, endo-G). A growing number of genes and gene functions that were previously thought to be specific for apoptotic processes in metazoan eukaryotes are now known to exist in yeast as well. In fact, yeast provides a powerful genetic approach to the discovery of new "apoptotic" genes within reasonably short periods of time that has also made it possible to assign specific lethal or protective functions to various apoptosis-related proteins, which are often transferrable to the mammalian system. For example, one consequence of the discovery of yeast Cdc48p as an apoptotic regulator was the subsequent discovery that its human homolog p97/VCP acts as anti-apoptotic factor as well (Madeo et al. 1997).

Another notable stress mechanism is the UPR (unfolded protein response), the signaling pathway of the ER stress response. The ER is the major signal transducing organelle that senses and responds to changes of redox homeostasis and reacts to stress created by defects in the ribosome and protein synthesis machinery. Indirect evidence exists for a switch in ribosome specificity associated with the switch of a cell to the apoptotic mode, which includes a change in gene expression at the transcriptional as well as the translational level. Furthermore, ribosome function is modulated during the apoptotic response (Holcik and Sonenberg 2005; Thedieck et al. 2007) and this modulation is dependent on the synthesis of apoptosis-specific proteins via a switch in the protein synthesis machinery (Holcik and Sonenberg

2005). A similar modulation of ribosome function has been reported for the aging process (Singh 2004; Syntichaki et al. 2007).

In mammalian cells, this translational modulation – which is initiated by the unfolded protein response – leads to differential translation of ATF4 and Bip/GRP78 mRNAs, both encoding anti-apototic proteins, via PERK kinase and eIF2 phosphorylation. The kinases that mediate translational response to stress (starvation stress and ER stress) are antagonized by kinases that mediate nutrient signaling and growth signals.

The TOR (Target of Rapamycin) kinase is a highly conserved, central controller of cell growth and is active in two distinct multisubunit complexes in yeast, the rapamycin sensitive TORC1 and the rapamycin insensitive TORC2 complex. TORC1 controls protein synthesis and ribsosome biogenesis by regulating translation. Under regulation of phosphorylated S6 kinase and eIF-4B binding protein (4E-BP), two key regulators of translation initiation, TORC1 orchestrates the increased translation of mRNAs encoding growth control genes, at the same time that it represses genes encoding components of the apoptotic machinery. In this way, active TORC1 favors translation of mRNAs that drive cellular growth, which is supported by the timely expression of antiapoptotic proteins, to compensate for cellular stress derived from increased protein synthesis, i.e. activation of the UPR pathway. On the other hand, aberrant activation of the TOR pathway induces apoptosis, which is a crucial event in the early response to malignant development (Thedieck et al. 2007).

Ribosomal proteins (RP) have been shown to regulate activity of the prototypic apoptotic protein p53. Ribosomal proteins L23, L11, and L5 regulate P53 activity by abrogating *Mdm2*-induced degradation and RPL26 modulates p53 translation (He and Sun 2007). A variant ribosomal protein, S27L, is pro-apoptotic and synthesized under the direct control by p53 (He and Sun 2007). It will be interesting to learn whether these RPs are involved in a translational switch between growth and stress-induced apoptosis.

Conclusion

Regarding the relation between aging and apoptosis in yeast, two very obvious questions need to be answered. Firstly, we have to ask whether apoptosis is the cause or a consequence of senescence and secondly, whether the apoptotic killing of yeast cells is an adaptive trait.

The first question can be answered by comparing the mother cell specific lifespan or the chronological lifespan of deletion mutants corresponding to the “apoptotic” yeast genes identified so far with the lifespan of the wild type strain. In general, there is very little concordance between the mother cell-specific and chronological lifespan data obtained with the 4800 deletion mutants (Laun et al. 2006). This published data has been re-examined and compared with the data obtained in our laboratory for mutants in apoptotic genes (Laun et al. 2008). Interestingly, deletion mutants in genes coding for proapoptotic proteins mostly exhibit an

increased chronological lifespan, but no change in replicative lifespan. However, there is one important caveat: practically all apoptotic genes of yeast have a second function (we like to call this the "day-job") in growing cells and in many cases this second function is not essential for life. However, even if it is not essential for life, we cannot separate the aging or apoptosis phenotype from the phenotype of the second function through specific mutations (certainly not when dealing with deletion mutations). The final phenotype, especially if it is a shortened lifespan, could be due to the loss of the apoptotic function or, alternatively, due to the loss of the "day-job" function.

Another way to answer the first question is to look at the apoptotic phenotypes of long-lived mutants. For example, a mutation of AFO1 leading to a 60% (and statistically significant) elongation of the mother cell-specific lifespan (Heeren et al. 2009) does not prevent yeast apoptosis; rather, the apoptotic phenotypes appear at a later cell generation, in these cells that exhibit a longer lifespan. Taken together, these data support the notion that apoptosis is a consequence (not a cause) of mother cell specific aging. However, more systematic data are needed before this question can be definitely answered.

The answer to the second question – that is, whether apoptotic killing of yeast cells is an adaptive trait – seems to be "Yes", at least for the chronological aging model. The main supporting argument is that a substantial part of a stationary population dies and lyses either in spent medium or in water and the nutrients liberated can be used by surviving cells. However, for mother cell –specific (replicative) aging the answer is "No", because senescence occurs in spite of adequate food supply and the fraction of dying cells is very small.

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Chapter 11
Cellular Homeostasis in Fungi: Impact on the Aging Process

Christian Q. Scheckhuber, Andrea Hamann, Diana Brust, and Heinz D. Osiewacz

Abstract Cellular quality control pathways are needed for maintaining the biological function of organisms. If these pathways become compromised, the results are usually highly detrimental. Functional impairments of cell components can lead to diseases and in extreme cases to organismal death. Dysfunction of cells can be induced by a number of toxic by-products that are formed during metabolic activity, like reactive oxygen and nitrogen species, for example. A key source of reactive oxygen species (ROS) are the organelles of oxidative phosphorylation, mitochondria. Therefore mitochondrial function is also directly affected by ROS, especially if there is a compromised ROS-scavenging capacity. Biological systems therefore depend on several lines of defence to counteract the toxic effects of ROS and other damaging agents. The first level is active at the molecular level and consists of various proteases that bind and degrade abnormally modified and / or aggregated mitochondrial proteins. The second level is concerned with maintaining the quality of whole mitochondria. Among the pathways of this level are mitochondrial dynamics and autophagy (mitophagy). Mitochondrial dynamics describes the time-dependent fusion and fission of mitochondria. It is argued that this kind of organellar dynamics has the power to restore the function of impaired organelles by content mixing with intact organelles. If the first and second lines of defence against damage fail and mitochondria become damaged too severely, there is the option to remove affected cells before they can elicit more damage to their surrounding environment by apoptosis. This form of programmed cell death is strictly regulated by a complex network of interacting components and can be divided into mitochondria-dependent and mitochondria-independent modes of action. In this review we give an overview on various biological quality control systems in fungi (yeasts and filamentous fungi) with an emphasis on autophagy (mitophagy) and apoptosis and how these pathways allow fungal organisms to maintain a balanced cellular homeostasis.

Keywords *Podospora anserina* · *Saccharomyces cerevisiae* · Autophagy · Aging · Apoptosis

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Abbreviations

AIF	apoptosis inducing factor
Amid	AIF-homologous mitochondrion-associated inducer of death
COX	cytochrome c oxidase
CvT	cytoplasm-to-vacuole targeting
ER	endoplasmic reticulum
GFP	green fluorescent protein
MIPA	micropexophagic membrane apparatus
PAS	pre-autophagosomal structure
RNAi	RNA interference
ROS	reactive oxygen species
TOR	target of rapamycin

Introduction

The maintenance of the integrity of living systems depends on the existence of different quality control pathways (reviewed in Tatsuta and Langer 2008). Failure of these pathways may lead to severe impairments, diseases and even cell death. Especially mitochondria need careful surveillance, because as organelles of oxidative phosphorylation they are both prominent sources and targets of reactive oxygen species (ROS). ROS are capable to cause massive damage to biologically relevant molecules. Several lines of defence exist to prevent / minimise the toxic effects of ROS and other damaging agents (Fig. 11.1).

At the molecular level, various proteases have been identified that degrade aggregated mitochondrial proteins. At the organellar level mitochondrial quality surveillance is controlled by fusion and fission of mitochondria. This kind of dynamics is capable to restore the function of impaired organelles by content mixing. If mitochondria are damaged too severely, they can be removed by a specific degradation process termed mitophagy. If all quality control systems fail, cells can eventually be eliminated by apoptosis which constitutes a cellular level of quality control.

Autophagy

The controlled growth and function of any living being is not possible without continuous synthesis of proteins (e.g., structure proteins and enzymes) and organelles (e.g., nuclei, microbodies, mitochondria). However, equally important is the degradation and "recycling" of damaged and non-functional proteins and organelles which might cause severe problems to the cell if not sufficiently removed from the cytoplasm. Therefore, the cell has to achieve a carefully regulated homeostatic balance between the opposing processes of synthesis and degradation. In eukaryotic

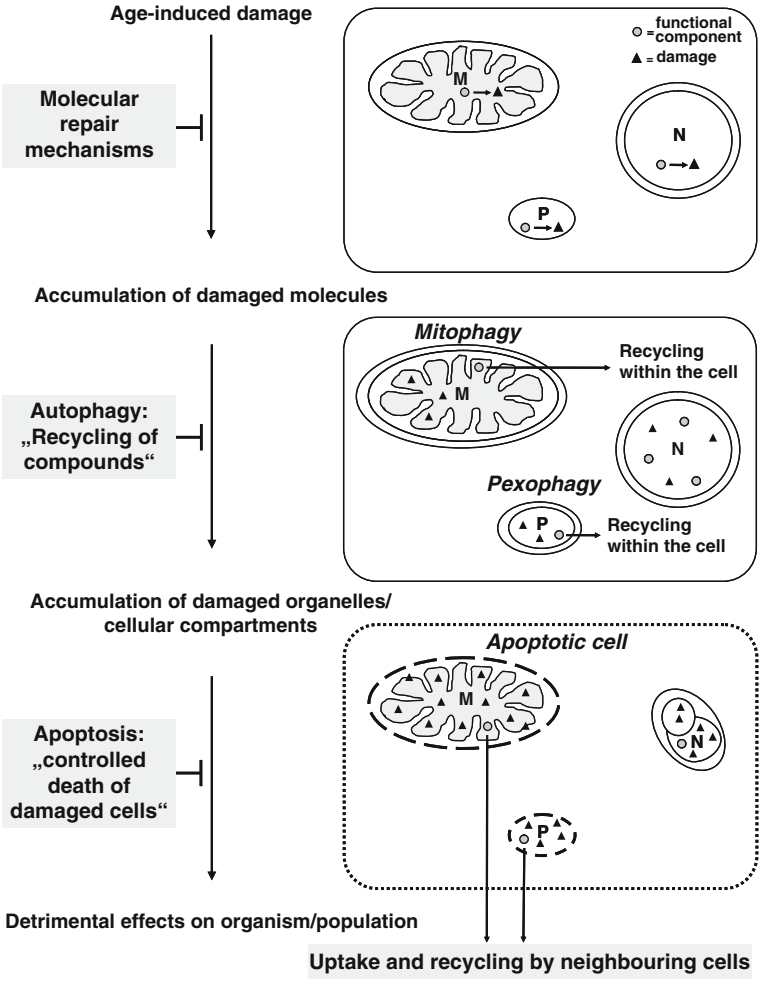

Fig. 11.1 Mitochondrial quality control systems and recycling of vital components. *Top panel*: Molecular repair mechanisms are capable to remove damage suffered during aging. Components of the DNA repair machinery and proteases belong to this first "line of defence". *Middle panel*: If molecular repair mechanisms are not able to cope with the damage load, whole organelles can be specifically degraded. Mitophagy and pexophagy allow the cell to get rid of dysfunctional mitochondria and peroxisomes, before they can cause further damage to the cell. Functional components can be efficiently "recycled" so that the cell still benefits from the degraded organelles. *Bottom panel*: The cell is able to eliminate cells via apoptosis when the progressive accumulation of damaged organelles leaves no other choice. Membranes are being dissolved (*dashed lines*) and cellular contents leak out of the apoptotic cells. These compounds can be internalised and utilised by neighbouring cells. If this last "line of defence" fails, detrimental effects on the individual organism or the population are the result. M: mitochondrion, N: nucleus, P: peroxisome

organisms, several systems were described which are capable to remove exhausted, damaged or unwanted cellular components. One intensively studied field dealing with this issue is autophagy, which can be sub-divided into several different pathways (reviewed in Todde et al. 2009). Macroautophagy is a non-specific uptake (bulk) or a specific (selective) uptake of components of the cytoplasm by so-called autophagosomes which are double membrane vesicles. After enclosing their prey, they fuse with vacuolar (fungi, plants) or lysosomal (animals) membranes, respectively. These degradation compartments contain an array of enzymes for efficient breakdown of macromolecules into their building blocks. Macropexophagy is a selective process for degrading targeted peroxisomes which has been mainly described in methylotrophic yeast species, *Candida boidinii* and *Hansenula polymorpha*, respectively (Veenhuis et al. 1978, 1983; Tuttle and Dunn 1995). In contrast to macroautophagy, microautophagy is not dependent on the engulfment of cytoplasmic material by autophagosomes. Here, the vacuolar membrane is able to form either tubular invaginations or even protrusions into the cytoplasm which ensures uptake of cellular components to be degraded in the lumen of the vacuole. Microautophagy can be sub-divided into several classes, including micropexophagy, piecemeal autophagy of the nucleus and mitophagy (selective degradation of dysfunctional mitochondria) (reviewed in Todde et al. 2009; Tolkovsky 2009).

Mitophagy

Mitophagy enables the cell to specifically degrade and "recycle" deteriorated mitochondria (reviewed in Tolkovsky 2009). One important factor contributing to mitochondrial damage is the formation of ROS within these organelles as toxic by-products of oxidative phosphorylation (reviewed in Starkov 2008). Oxidatively modified components of the respiratory chain can further increase ROS-mediated stress so that eventually a vicious cycle causing further oxidative insult to the cell is initiated. Therefore, energetically compromised mitochondria have to be removed from the mitochondrial population without affecting fully functional ones. In the past years, a number of yeast genes have been identified which influence mitophagy in the yeast *Saccharomyces cerevisiae* (Kennedy et al. 1995; Campbell and Thorsness 1998; Camougrand and Rigoulet 2001; Camougrand et al. 2003; Kissova et al. 2007; Nowikovsky et al. 2007; Tal et al. 2007) (Fig. 11.2).

UTH1 ("*youth 1*") encodes an outer mitochondrial membrane protein and was originally identified in a screen for genes regulating *S. cerevisiae* replicative lifespan (Kennedy et al. 1995). Independently, UTH1 was described as an apoptosis regulator in yeast, where it is required for activation of cell death by heterologous expression of mammalian pro-apoptosis factor BAX (Camougrand and Rigoulet 2001; Camougrand et al. 2003). The characterisation of a Δ*UTH1* mutant revealed that mitophagy is affected, suggesting a specific role for UTH1 in this process (Kissova et al. 2007). Moreover, Δ*UTH1* cells are more resistant to rapamycin mediated cell death (Camougrand et al. 2003). Rapamycin is capable of eliciting autophagy by inhibiting the TOR ("target of rapamycin") kinase which is involved in nutrient sensing and various other cellular functions (reviewed in De Virgilio and Loewith

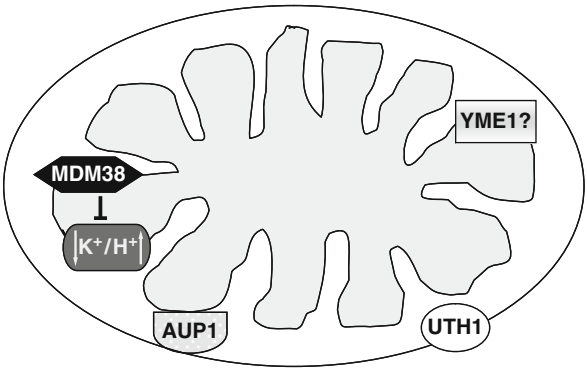

Fig. 11.2 Proteins involved in mitophagy in *S. cerevisiae*. UTH1 is located in the outer mitochondrial membrane. The exact localisation of AUP1 in the intermembrane space has, so far, been not determined. It is speculated, however, that it is either associated with the inner surface area of the outer membrane or the outer surface area of the inner membrane. MDM38 and YME1 are both localised in the inner membrane. MDM38 is a component of the K^+/H^+ exchanger which protects the mitochondrial matrix from K^+ overload. YME1 is assembled as homo-oligomers which form the i-AAA protease. Key functions of this protease involve degradation of damaged or misfolded proteins and chaperone-like activities to prevent the aggregation of unfolded proteins. Modified after Tolkovsky (2009)

2006). So far, the precise function of UTH1 has not been elucidated. Remarkably, *UTH1* loss of function mutants display (albeit moderate) longevity (Kennedy et al. 1995). Mitochondrial biogenesis is negatively affected in Δ*UTH1* strains. This was demonstrated by measuring reduced levels of respiratory chain cytochromes (aa_3, *b* and *c*) and lowered activities of a mitochondrial key enzyme, citrate synthase (Camougrand et al. 2000). It is speculated that the prolonged lifespan of the Δ*UTH1* mutants is due to a decreased production of ROS by fewer but more efficient mitochondria (Camougrand and Rigoulet 2001). Concrete information on the importance of autophagy (mitophagy) as a quality control system influencing the aging process is still scarce. In *Arabidopsis thaliana* it was shown that the disruption of autophagy genes leads to stress hypersensitivity and even premature senescence of leaves (Doelling et al. 2002; Hanaoka et al. 2002). The absence of autophagy leads to failure of nutrient redistribution and cell death in the investigated *A. thaliana* mutants. This process is probably under control of proteolytic systems. Meléndez et al. down regulated the expression of genes encoding autophagy components in the nematode *Caenorhabditis elegans* via RNAi (Meléndez et al. 2003). When the synthesis of the pre-autophagosome forming protein BEC-1 is inhibited in long-lived *daf2* (gene encoding an insulin receptor analog) mutants, the increase in median lifespan is reduced. These findings suggest that autophagy acts downstream of the insulin-like pathway in *C. elegans*. Autophagy also seems to be important for lifespan extension via caloric restriction in mammals. Here it was shown that autophagy declines progressively with the aging of rodents (Donati et al. 2001). This decline was less apparent in long-lived calorie-restricted rats. Moreover, autophagy was initiated less efficiently with age in the ad libitum-fed rats. This indicates that a

persistent metabolic state close to starvation allows a more efficient reaction towards nutrient limitation.

In contrast to Δ*UTH1* cells, deletion strains of *AUP1* display increased sensitivity against rapamycin (Tal et al. 2007). AUP1, which is localised in the mitochondrial inter-membrane space, is proposed to flag the organelles for selective degradation in stationary phase cells: in AUP1-positive but not in Δ*AUP1* cells, mitochondria become degraded by mitophagy after an incubation time of 3 days. Mitochondrial degradation in this experiment was monitored by observing a translocation of mitochondrial-targeted GFP to the vacuoles and aconitase degradation (Tal et al. 2007). Interestingly, AUP1-GFP itself seems to escape vacuolar degradation. It was shown that AUP1 has a potential phosphatase activity, but it is not known whether this is necessary for fulfilling its function during mitophagy. Similar to UTH1, the exact function of AUP1 during mitophagy remains to be clarified although the studies performed on this protein suggest that mitophagy is a specific process.

YME1 ("yeast mitochondrial escape") belongs to the family of AAA metalloproteases (reviewed in Shafer et al. 1999; van Dyck and Langer 1999). The protein is located in the inner mitochondrial membrane while the catalytic site faces the intermembrane space. Yeast mutants in which *YME1* is deleted contain fragmented mitochondria when grown on non-fermentable carbon sources (i.e. ethanol/glycerol) (Campbell and Thorsness 1998). Mitochondria were not only localised near vacuole invaginations but were also found to be taken up and degraded within the vacuolar lumen. Being important for processing of various proteins like COX2 (subunit of respiratory chain cytochrome-c oxidase, Nakai et al. 1995) and NDE1 (external mitochondrial NADH dehydrogenase, Luttik et al. 1998) it remains to be shown if it is the absence of YME1 itself or one of its processed target proteins which signals mitophagy.

MDM38 is situated in the inner mitochondrial membrane where it has an essential function in the regulation of K^+/H^+ distribution which is important for volumetric control of mitochondria (Nowikovsky et al. 2004; Froschauer et al. 2005). MDM38 depletion leads first to mitochondrial swelling due to K^+ influx into the matrix, then dissipation of the membrane potential ($\Delta\Psi_M$), fragmentation, and eventually degradation by mitophagy (Nowikovsky et al. 2007). If the K^+/H^+ exchange activity is inhibited by the ionophore nigericin, the phenotype of the Δ*MDM38* is reverted. Interestingly, a double mutant which is deficient in mitochondrial fission (Δ*MDM38* / Δ*DNM1*) contained swollen mitochondria displaying reduced $\Delta\Psi_M$, but no mitophagy was observed. These data show that the controlled division of mitochondria is necessary in Δ*MDM38* cells for mitophagy to occur.

In yeast and mammalian cells, mitochondrial fission can sometimes lead to the generation of depolarised mitochondria which are prone to degradation by autophagy (mitophagy) (Twig et al. 2008a). Therefore, fragmentation of mitochondria is hypothesised to be important for their turn-over (Twig et al. 2008a). Consequently, if components of the fission machinery in mammalian cells are down-regulated (e.g., by expression of a dominant negative form of the division protein DRP-1), a pronounced and specific reduction of mitochondrial autophagy is resulting (Arnoult et al. 2005; Barsoum et al. 2006; Twig et al. 2008a, b).

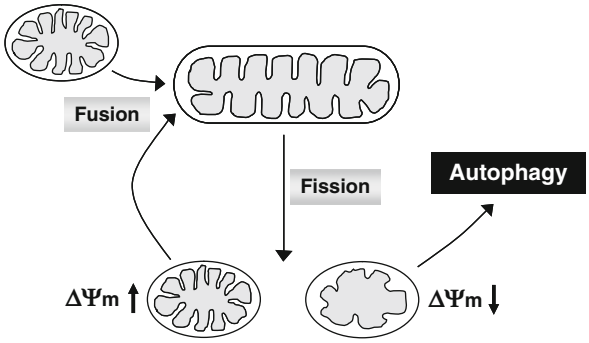

Fig. 11.3 Mitochondrial dynamics and autophagy collaborate to sort out dysfunctional mitochondria. Solitary mitochondria are able to fuse if they are functionally intact (normal $\Delta\Psi_M$). Fission of fused mitochondria may lead to the generation of dysfunctional mitochondria with decreased $\Delta\Psi_M$ (shown as a mitochondrion with aberrant cristae structure). Before these mitochondria can cause problems to the cell (e.g., by accidentally eliciting apoptosis) they are targeted for degradation by autophagy. The mitochondrial fusion machinery is down-regulated in these detrimental organelles to prevent the damaged mitochondria from re-fusing with the healthy subpopulation. Modified after Twig et al. (2008b)

Fission of mitochondria in conjunction with fusion of these organelles is regarded as part of a quality control system which might play an important role in sorting out dysfunctional mitochondria (reviewed in Twig et al. 2008b) (Fig. 11.3). An intriguing model has been put forward by Twig et al. which illustrates the combined tasks of mitochondrial dynamics and autophagy on recognising and eliminating mitochondria of poor quality (i.e., depolarised mitochondria) (Twig et al. 2008a). According to this model, mitochondria cycle between a solitary and a network state. Mitochondrial networks are formed by fusion of solitary mitochondria. After subsequent fission, the daughter mitochondria can follow one of two paths depending on their $\Delta\Psi_M$. If $\Delta\Psi_M$ is still intact, the mitochondrion is able to re-fuse again with the healthy population. However, if the daughter mitochondrion is depolarised and unable to recover its membrane potential, the amount of fusion protein OPA1 ("optic atrophy 1") is strongly reduced (Twig et al. 2008a). Being unable to fuse with healthy mitochondria it eventually ends up in the autophagosome. This model implicates that a high mitochondrial fission frequency results in a higher probability for segregation and elimination of dysfunctional mitochondria (Twig et al. 2008a). So far, it is not clear if non-mammalian systems contain a similar mitochondrial quality control system.

Studies performed in the filamentous ascomycete *Podospora anserina* and the yeast *S. cerevisiae* suggest that the absence of mitochondrial fission is not necessarily detrimental to the organism (Scheckhuber et al. 2007, 2008). *P. anserina* and yeast strains deleted for the fission gene *PaDnm1* / *DNM1* display healthy aging (i.e. increased lifespan without negative physiological effects after ascospore germination). So far, it is not known whether autophagy (mitophagy) is affected in

these mutants, which are characterised by pronounced mitochondrial elongation / network formation (Scheckhuber et al. 2007, 2008).

Pexophagy

Mitochondria are not the only organelles which are selectively degraded. In fact, peroxisomes have been the first organelles which were reported to be eliminated by selective autophagy (pexophagy) (Bormann and Sahm 1978; Veenhuis et al. 1978, 1983). Peroxisomes are involved in different metabolic tasks, e.g. catabolic degradation of fatty acids, detoxification of ROS and synthesis of secondary metabolites (reviewed in van der Klei and Veenhuis 2002; Schrader and Yoon 2007). Methylotrophic yeast species (i.e., *Candida boidinii*, *Hansenula polymorpha*, *Pichia pastoris*), when grown on methanol as a carbon source, contain peroxisomes equipped with enzymes needed for CH_3OH metabolism. Macropexophagy in *H. polymorpha* can be observed when cells grown on CH_3OH are shifted to media not requiring enzymes of CH_3OH degradation (Veenhuis et al. 1978, 1983). The phosphoprotein ATG11 and the peroxisomal membrane protein ATG30 are both needed for initiating binding to the so-called pre-autophagosomal structure (PAS) (He et al. 2006; Farré et al. 2008). Other factors needed in early steps for macropexophagy to occur are PEX3 (assembly of peroxisome membranes) and PEX14 (uptake of proteins into the peroxisomal matrix) (Bellu et al. 2001, 2002). So-called pexophagosomes sequester the peroxisome to be eliminated and subsequently fuse with the vacuolar membrane. In the lumen of the vacuole, the peroxisome eventually is degraded.

Micropexophagy has been examined in detail in *P. pastoris* (Tuttle and Dunn 1995; Yuan et al. 1997, 1999; reviewed in Sakai et al. 2006; Farré et al. 2007). After engulfment of the peroxisome by the vacuolar membrane but before membrane fusion, a micropexophagic membrane apparatus (MIPA) is formed on the peroxisomal surface (reviewed in Sakai et al. 2006). ATG8, which also plays a pivotal role in macroautophagy and the CvT (cytoplasm-to-vacuole targeting) pathway, is considered vital for MIPA formation (Huang et al. 2000; Xie et al. 2008).

Studies examining potential links between pexophagy and aging are still scarce. However, recently Aksam et al. examined the role of peroxisomal Lon protease and pexophagy on the vitality of *H. polymorpha* cells (Aksam et al. 2007). Loss of either *HpPln* (encoding peroxisomal Lon) or *ATG1* (encoding a cytosolic protein kinase required for vesicle formation during autophagy and the Cvt pathway) leads to elevated cellular peroxisome content. Notably, intracellular ROS content is also significantly increased. The authors suggest that an uneven distribution of catalase activities in the peroxisomes as witnessed by cytochemical analysis might be the reason for this effect. Not surprisingly, cell viability is decreased in Δ*pln1*, Δ*atg1* single and especially in Δ*pln1* / Δ*atg1* double mutants (Aksam et al. 2007). These findings highlight the importance of peroxisomal protein quality control via proteolysis and autophagic removal of an excessive population of peroxisomes on cell survival (Aksam et al. 2007).

Incompatibility

So far, links between the incompatibility reaction and the aging process have not been identified in *P. anserina*. Nonetheless, incompatibility is a developmentally important and well-studied cell death process in this fungus which led to the unravelling of interesting mechanistic insights that are briefly summarised here.

Autophagy was found to be strongly induced during the cell death reaction by incompatibility in *P. anserina* which constitutes the non-self recognition system in this organism (reviewed in Pinan-Lucarré et al. 2007). This phenomenon can be observed following fusion between individuals if they contain certain differences at specific *het* loci (reviewed in Saupe et al. 2000) (Fig. 11.4). *idi-6* and *idi-7* (also known as *PaAtg8*) were found to be induced during cell death by incompatibility (Pinan-Lucarré et al. 2003). *idi-6* encodes a homolog of *S. cerevisiae* PRB1 (vacuolar serine protease of the subtilisin family [Moehle et al. 1987]) while the yeast ortholog of PaATG8, ATG8, is known to be involved in the expansion of the phagophore during autophagosome formation (Nakatogawa et al. 2007). Pinan-Lucarré et al. showed that the autophagy marker GFP-PaATG8 translocates to autophagosomes soon after inducing the incompability reaction (Pinan-Lucarré et al. 2003). Autophagosomes were identified by their characteristic double membrane and contents. Moreover, degradation intermediates (autophagic bodies) were found in the vacuolar lumen if a protease inhibitor which delays the breakdown of proteins was applied (Pinan-Lucarré et al. 2003). When cell death by incompatibility is initiated it is generally limited to the fusing cell, but in some cases also the cells which are immediately adjacent are also affected.

Interestingly, in *P. anserina* it was found that autophagy delays cell death by incompatibility and promotes cellular survival, because mutants in which genes encoding components of the autophagy machinery were deleted (Δ*PaAtg*) display

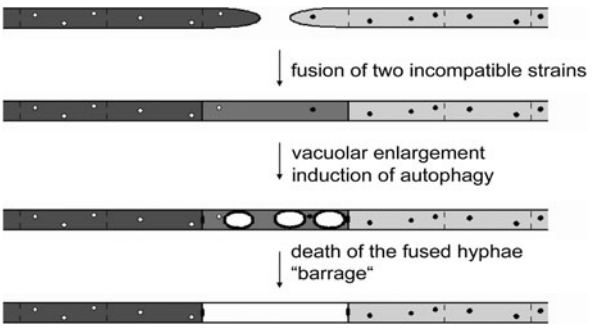

Fig. 11.4 The incompatibility reaction in *P. anserina*. After fusion of two incompatible strains, vacuoles enlarge and autophagy is induced. In addition to autophagy in the original fused hyphal segment, autophagy in adjacent hyphal segments may prevent the diffusion of pro-death signals. Moreover, closure of septa to neighboring hyphae inhibits spreading of these signals. Finally, the fused hyphae die forming a macroscopically visible so-called barrage between the two strains (modified after Saupe et al. 2000)

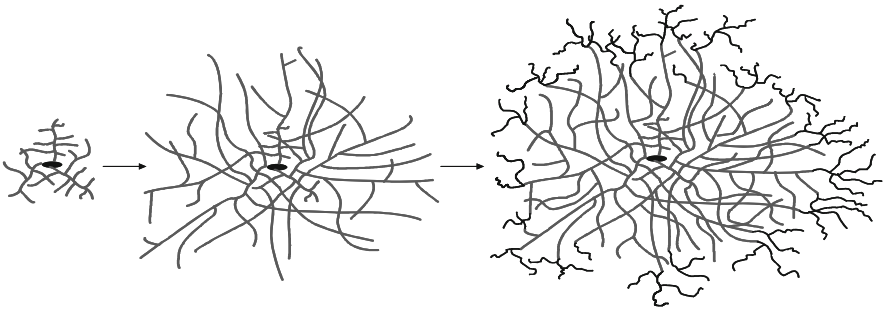

Fig. 11.5 Senescence in *P. anserina*. The filamentous ascomycete *P. anserina* is characterised by a strain-specific onset of senescence. After germination of the ascospore, a branched mycelium is formed. Approximately 20 days after germination – depending on the strain and the culture conditions – the hyphae start to change morphology and become hyperbranched as manifestation of senescence

increased cell death by incompatibility (Pinan-Lucarré et al. 2005). It is speculated that the induction of autophagy in the neighbouring cells of the degraded fusion cell eliminates detrimental material (e.g., damaged organelles, toxic compounds) leaking out of the degraded fusion cell. Together with sealing of pores in the hyphal septa by Woronin bodies (Jedd and Chua 2000), autophagy would help to prevent the spreading of "pro-death" signals and subsequent death of the colony as a whole. In addition to cell death by incompatibility *P. anserina* is valuable system to study cell death during senescence (reviewed in Esser 1974; Osiewacz 2002; Lorin et al. 2006; Scheckhuber and Osiewacz 2008) (Fig. 11.5). It is tempting to hypothesise that autophagy also plays an important role (cyto-protective or cell-killing) during replicative aging in this fungal model system. Future studies are expected to deal with this interesting field of experimental research.

Apoptosis

As soon as molecular and organellar scavenging systems are no longer able to cope with the age-accumulated damage within a cell, apoptosis may become activated. During the last about ten years a huge body of evidence arose, demonstrating apoptosis-like mechanisms in lower eukaryotes and especially in fungi. From these studies it became clear that fungi contain apoptosis factors homologous to those of the mammalian apoptosis pathways. In general, fungi exhibit characteristics of (meta)caspase-dependent apoptosis as well as of mitochondrial apoptosis pathways involving homologs of the human apoptosis-inducing factor AIF (reviewed in Büttner et al. 2006; Eisenberg et al. 2007; Hamann et al. 2008).

The first evidence for the involvement of apoptosis in aging processes of fungi came from studies of the baker's yeast *S. cerevisiae* where markers of apoptosis in

aged mother cells were demonstrated (Laun et al. 2001). Based on these findings, it is attractive to assume that failure of intracellular scavenging mechanisms in aged fungal cells results in the activation of programmed cell death.

Role of Metacaspases in Fungal Apoptosis

Metacaspases are sequence and functional homologs of the mammalian caspases, which are cystein-dependent aspartate specific proteases. Such protease activity can also be measured in fungal extracts and could be assigned to the presence of metacaspases. During the last years it became clear that metacaspases have biochemical properties distinct from mammalian caspases. Bacterial and yeast extracts containing metacaspase Yca1 as well as plant metacaspases preferentially exhibit arginine/lysine-specific endopeptidase activities but cannot cleave caspase-specific substrates residue instead of an aspartate residue in case of the caspases (Watanabe and Lam 2005). Moreover, studies with total protein extracts of wild-type and metacaspase deletion strains of *P. anserina* demonstrate a metacaspase-dependent arginine-peptidase activity, while the cleavage of caspase-specific substrates is not metacaspase-dependent (Hamann et al. 2007). In addition, the metacaspase LmjMCA of the protozoan parasite *Leishmania major*, which does not exhibit any activity against caspase substrates, but efficiently cleaves arginine-containing substrates, can functionally replace the yeast metacaspase Yca1 (Gonzalez et al. 2007). Taken together, these data strongly implicate that fungal metacaspases (and those in plants and protozoans) are up-stream regulators of a proteolytic cascade involving proteases with caspase-like cleavage activity.

Although featuring some biochemical properties different from the mammalian caspases, several studies demonstrate a functional homology of this group of enzymes. Lack of yeast metacaspase YCA1 results in resistance against different apoptotic stimuli like hydrogen peroxide (Madeo et al. 1999, 2002), valproic acid (Mitsui et al. 2005), hyperosmotic shock (Wadskog et al. 2004; Silva et al. 2005), arsenic (Du et al. 2007), Mn^{2+} (Liang and Zhou 2007) and others. Moreover, overexpression of *YCA1* results in stimulation of apoptotic cell death (Madeo et al. 2002). In *P. anserina*, and in *Candida albicans* deletion of metacaspase genes results in increased resistance against hydrogen peroxide (Hamann et al. 2007; Cao et al. 2009) and in *S. pombe* deletion of the gene encoding metacaspase PCA1 delays a specific form of lipid-induced cell death (Low et al. 2008). These data stress the central role of fungal metacaspases in a variety of cell death scenarios. However, until now only in a few cases a link between activity of these enzymes and aging processes has been established. The chronological aging of stationary yeast cultures has repeatedly been described to be delayed by deletion of the gene encoding the yeast metacaspase YCA1 (Madeo et al. 2002; Herker et al. 2004; Lee et al. 2007; Almeida et al. 2008). An influence of metacaspase presence on the replicative lifespan has been described in *P. anserina*: lack of PaMCA1, one of the two metacaspases in this fungus, results in 2.5 fold lifespan extension. Moreover, senescent *P. anserina*

possess a metacaspase-dependent peptidase activity which is lacking in juvenile cultures (Hamann et al. 2007). The direct involvement of this metacaspase in apoptotic processes in this fungus is further supported by the increased resistance of the *PaMca1* deletion strain against the apoptosis inducer etoposide (Scheckhuber et al. 2007). The key regulators of metacaspase activity are reactive oxygen species. It is therefore suggestive to assume that an increased ROS level occuring during aging results in the activation of a metacaspase-dependent cell death reaction. This idea is supported by the observation, that metacaspase deletion *S. cerevisiae*, *P. anserina* and *C. albicans* strains are more resistant against ROS (Madeo et al. 2002; Hamann et al. 2007; Cao et al. 2009). Thus, metacaspases seem to be sensors of cellular dysfunction via increased ROS levels. These increased ROS levels might be the result of mitochondrial dysfunction, a common hallmark of aged cells.

Mitochondria-Dependent Apoptosis

In mammals, not only caspase-dependent apoptotic pathways are described to detect cellular dysfunction. There are other pathways known which focus directly on the functionality of the mitochondria. One prominent factor of this pathway is the apoptosis-inducing factor AIF. Homologs of this factor can also be found in fungi and it could be demonstrated that the yeast AIF homolog controls apoptosis in this fungus (Wissing et al. 2004). Comparable to the mammalian situation, yeast AIF1 is located in the mitochondria and becomes translocated to the nucleus upon apoptotic stimulus. Deletion of this factor results in increased survival after hydrogen peroxide treatment. Overexpression of *Aif1* per se does not result in increased apoptosis, but depends on an apoptosis-stimulus like hydrogen peroxide. Unlike in mammals, where AIF-dependent apoptosis is found to be caspase-independent, the AIF1-mediated cell death in yeast is at least partially dependent on the metacaspase YCA1 (Wissing et al. 2004). The involvement of this pathway in aging processes in yeast is confirmed by the observation that deletion of *Aif1* results in delayed chronological aging (Wissing et al. 2004).

Another metacaspase – (and AIF1-) – independent mitochondrial apoptosis signaling pathway consists of the endonuclease G. This endonuclease is normally located in the mitochondria. During apoptosis, the mammalian homolog translocates to the nucleus. Exclusion of the yeast EndoG from mitochondria leads to an apoptotic phenotype. Moreover, yeast EndoG has an interesting impact on the chronological lifespan. On glucose medium, deletion results in increased cell death; however, on medium containing glycerol as sole carbon source, deletion of *EndoG* increases survival during chronological aging and promotes survival upon treatment with apoptosis inducers like e.g. hydrogen peroxide and acetic acid (Büttner et al. 2007). Thus, EndoG regulates yeast cell death in dependency of the metabolic state (respiration versus fermentation) and fulfills vital and lethal functions (Büttner et al. 2007).

Benefits of Apoptosis

Taking together all reports on the impact of apoptosis factors on the aging process, on first glance, an inhomogeneous picture arises. On the one hand, deletion of apoptosis factors like metacaspases and AIF results in lifespan extension, on the other hand apoptosis has repeatedly been established to be an important line of defence against overwhelming cellular damage. This contradiction can be easily solved by looking in more detail on the results. For example, deletion of the yeast metacaspase is beneficial with respect to a single culture but is detrimental in co-cultivation experiments. Herker et al. (2004) demonstrated that the *Yca1* deletion strain is outgrown by wild-type cells in a mixed culture. The increased replicative lifespan of metacaspase deletion strains of *P. anserina* might be the result of a lacking response to increased cellular ROS levels. The deletion strains still die and the increased lifespan seems to be paid by a reduced fertility. In yeast, the population of single cells might profit by the release of substances from dying cells, while in the filamentous ascomycete damage spreading can be prevented by the programmed cell death of senescent hyphae. Especially in filamentous fungi like *P. anserina* it is in principle possible that healthy hyphae from the center of the colony can fuse with hyphae from the periphery resulting in the propagation of damaged material, e.g. deficient mitochondria. To overcome this problem, apoptosis of the peripheral damaged hyphae is a suitable method to eliminate harmful material.

It is now more and more clear that apoptosis factors have vital as well as lethal functions, the role of which depends on the cellular context (Galluzzi et al. 2008). In addition, even in fungi, different homologs of apoptosis factors seem to adopt different functions with respect to cell death promoting and cell death preventing functions. For example, deletion of the gene encoding the AIF-like protein of *Neurospora crassa* leads to an increased resistance against phytosphingosine- and hydrogen peroxide-induced apoptosis, while deletion of the *N. crassa Amid* gene (encoding the homolog of the AIF-homologous mitochondrion-associated inducer of death) results in increased sensitivity against these compounds (Castro et al. 2008). Also in *Aspergillus nidulans* a vital function of a member of the AIF family of proteins has recently been demonstrated: Deletion of *AifA* encoding an AIF-like protein dramatically increases sensitivity against farnesol, an inducer of fungal apoptosis (Savoldi et al. 2008). These data suggest that AIFA is involved in preventing detrimental effects of the oxidative stress induced by farnesol. In fact, in mammals, AIF has repeatedly been demonstrated to fulfill vital functions via its importance in the process of oxidative phosphorylation, although it is still a matter of debate whether it acts as assembly factor or as a maintenance factor (reviewed in Modjtahedi et al. 2006).

In addition to vital roles of AIF homologs, recently, a few examples demonstrate vital functions of fungal metacaspases. In *A. fumigatus*, these enzymes facilitate growth under ER stress conditions, deletion results in severe growth defects upon disruption of ER homeostasis (Richie et al. 2007). In addition, the yeast metacaspase YCA1 accelerates G1/S and slows the G2/M transition, demonstrating a clear involvement in cell cycle progression (Lee et al. 2008).

In microorganisms like yeast and filamentous fungi it thus appears that apoptosis is the last line of defence when all other surveillance systems are not able to cope with excessive molecular damage to biomolecules and pathways. In contrast to higher multicellular organisms in which apoptosis is effective to keep a single individual functional for longer, in fungi the beneficial effect seems not to work on the individual but rather on population level.

Appendix

A short description of recent important papers related to the topic of this chapter.

Although the understanding of the mechanisms regulating mitophagy has progressed in the last years, there remain still some important questions, e.g. how the molecular autophagy machinery is able to detect energetically compromised mitochondria. A possible key factor in this regard could be the protein *Atg32* which has been recently identified in two independent screens for genes involved in *S. cerevisiae* mitophagy (Okamoto et al. 2009; Kanki et al. 2010). ATG32 was shown to be a protein residing in the outer mitochondrial membrane. Interestingly, ATG32 is neither needed for bulk autophagy nor pexophagy but is essential for the removal of mitochondria when yeast cells are grown under respiratory conditions (Kanki et al. 2009). So far, it is not clear whether ATG32 may act as a signal for compromised mitochondria, because *Atg32* deletion mutants show no obvious mitochondrial defects. Therefore it is certainly possible that there are further, hitherto unknown factors that might act as signals or effectors for maintaining quality control of mitochondria by mitophagy.

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Chapter 12
Genome-Wide Analysis of Yeast Aging

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

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

Abbreviations and Accronyms

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

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

Introduction

The budding yeast *Saccharomyces cerevisiae* has been used as a model of cellular aging for more than 6 decades (Fabrizio and Longo 2007; Jazwinski 2005; Kaeberlein et al. 2007; Steinkraus et al. 2008). *S. cerevisiae* has several features that make it useful as a model organism for aging research, including short life span, well-characterized genetic and molecular methods, low relative cost, cell type homogeneity, and a vast organismal information base. These advantages have facilitated unbiased screens for genes that influence life span in yeast, as well as candidate gene approaches. Several dozen genetic determinants of yeast longevity have been identified from these studies, at least some of which appear to play a conserved role in the aging of multicellular eukaryotes.

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

One tool that has greatly facilitated studies of longevity and other processes in yeast is a collection of isogenic single-gene deletion strains encompassing a majority of non-essential yeast open reading frames (ORFs). The yeast ORF deletion collection contains more than 20,000 unique strains (6061 single ORF

deletions in one or more strains), with full-genome collections of homozygous and heterozygous diploids as well as haploid deletions in both mating types (Winzeler et al. 1999). Screens have been carried out across this deletion collection for many different phenotypes, including sensitivity to a variety of stresses, metabolism of different carbon sources, and growth rate (Que and Winzeler 2002; Scherens and Goffeau 2004). Essentially any yeast-based assay that can be modified for moderate- or high-throughput capacity can, in principle, be used in conjunction with the deletion collection to perform genome-wide queries of the process under study. This chapter describes large-scale studies of both replicative and chronological life span using the yeast ORF deletion collections in detail and touches briefly on alternative strategies for studying aging in yeast.

Yeast Replicative Life Span

A Brief History of Yeast Replicative Aging

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

Since these early morphology-based studies, yeast replicative aging has become a prominent model for aging genetics and has been instrumental in the discovery and characterization of several of the best studied genetic pathways involved in life span determination. These pathways include dietary restriction (DR), sirtuins, TOR signaling, and mitochondrial metabolism (Table 12.1).

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

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

The data in this table was compiled from numerous sources (Kaeberlein et al. 1999, 2004, 2005b, c; Lamming et al. 2005; Lin et al. 2000; Tsuchiya et al. 2006)
Table entries indicate effect of each intervention on replicative life span in each genetic background (N.E. = no effect).

Sirtuins and the Ribosomal DNA

The role of sirtuins in life span determination was first discovered using the yeast replicative model of aging and is closely linked to the influence of extrachromosomal ribosomal DNA (rDNA) circles, or ERCs, on yeast aging. The silent information regulator (SIR) complex was first identified in a screen for stress resistance and maintenance of viability at 4°C, both phenotypes that correlate with longevity (Kennedy et al. 1995). The SIR complex includes Sir2, Sir3, and Sir4 and acts to repress transcription at telomeres, rDNA, and the silent mating-type locus (Rusche et al. 2003). The screen specifically identified a semi-dominant mutation in *SIR4*, *sir4-42*, that resulted in a redirection of the SIR complex from the telomeres and silent mating-type locus to rDNA (Kennedy et al. 1995, 1997). Sir2, a conserved NAD-dependent histone deacetylase, has since emerged as the vital component of the SIR complex with respect to aging. In addition to its role in the SIR complex, Sir2 has a Sir3- and Sir4-indepdendent role in preventing rDNA recombination and has been shown to silence a Pol II gene artificially inserted into the rDNA (Table 12.2) (Bryk et al. 1997; Defossez et al. 1999; Gottlieb and Esposito 1989; Smith and Boeke 1997). Mutants lacking *SIR2* have a life span that is roughly 50% shorter than wild type, while overexpressing *SIR2* extends replicative life span by 30–40% (Kaeberlein et al. 1999, 2004; Kennedy et al. 1995).

Eglimez and Jazwinski (1989) first suggested that deleterious factors may accumulate with age in yeast cells and contribute to replicative senescence based on changes in generation time with replicative age in yeast. Yeast cell division is asymmetric, with the mother retaining a larger portion of the cell contents than the daughter. This phenomenon gives the mother cell the potential to preferentially retain the majority of deleterious factors that accumulate with age resulting in daughter cells with renewed replicative capacity (Egilmez and Jazwinski 1989;

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

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

The data in this table was compiled from numerous sources (Bonawitz et al. 2007; Bryk et al. 1997; Defossez et al. 1999; Fabrizio et al. 2005; Fritze et al. 1997; Gottlieb and Esposito 1989; Huang and Moazed 2003; Kaeberlein et al. 1999, 2004 2005a, b; Kaeberlein and Powers 2007; Lin et al. 2000, 2002; Longo et al. 1997; Riesen and Morgan 2009; Rusche et al. 2003; Smith et al. 2009; Smith and Boeke 1997; Wang et al. 2008; Wei et al. 2008, 2009)

Kennedy et al. 1994). ERCs represent the first such aging factor identified (Sinclair and Guarente 1997). Yeast rDNA consists of a tandem repeat of a 9.1 kb sequence coding for the ribosomal RNA (Petes and Botstein 1977; Philippsen et al. 1978; Rustchenko and Sherman 1994). ERCs form through homologous recombination between rDNA repeats and accumulate with age in mother cells as a consequence of two factors: (1) a replication of origin within the rDNA that allows ERCs to self-replicate, and (2) the lack of a CEN element, causing biased segregation toward the mother cell during asymmetric division (Murray and Szostak 1983). Cell senescence is thought to occur when ERCs accumulate past an unknown threshold level.

Several lines of evidence support a model in which Sir2 promotes longevity by preventing rDNA recombination and thus inhibiting ERC formation. First, deletion of *SIR2* increases rDNA recombination by 6–10 fold, increases ERC accumulation, and reduces replicative life span (Table 12.2), while overexpression of *SIR2* extends life span (Kaeberlein et al. 1999). Second, deletion of *FOB1*, a replication fork barrier protein with rDNA-specific activity that increases rDNA recombination, extends replicative life span, dramatically reduces ERC levels, and prevents the short replicative life span caused by deletion of *SIR2* (Table 12.2) (Defossez et al. 1999; Kaeberlein et al. 1999).

The life span characteristics of *sir2Δ* and *fob1Δ* strains suggest that promotion of ERC formation is not the only mechanism by which Sir2 influences longevity. Similar ERC levels are observed in both *sir2Δ fob1Δ* and *fob1Δ* strains (Kaeberlein et al. 1999); however, *FOB1* deletion alone results in extension of replicative life span relative to wild type, while deletion of both *SIR2* and *FOB1* together results in

a replicative life span similar to wild type (Table 12.2). This suggests that Sir2 has a pro-longevity function independent of both Fob1 and ERC accumulation. Further support for this idea comes from the recent finding that life span extension by *SIR2* overexpression is largely dependent on *SIR3*, while inhibition of rDNA recombination is *SIR3*-independent (Dang et al. 2009). This study also shows that Sir2 protein levels decline with increasing age, resulting in enhanced histone H4K16 acetylation at a variety of subtelomeric sites (and potentially others). Together these data suggest a model whereby increased Sir2 activity leads to altered transcription at key non-nucleolar loci resulting in activation of a second pathway influencing life span in yeast. A second possibility for an ERC-independent role for Sir2 in aging is increased oxidative stress resistance, which stems from the finding that *SIR2* overexpression suppresses the short life span of yeast exposed to H_2O_2 (Oberdoerffer et al. 2008). This model is supported by the finding that yeast lacking Sir2 are unable to maintain asymmetric segregation of hydrogen peroxide and carbonylated proteins to the mother cells during division (Aguilaniu et al. 2003; Erjavec and Nystrom 2007). Erjavec and Nystrom (2007) found that the reduction in hydrogen peroxide results from a Sir2-dependent segregation of the cytosolic catalase Ctt1 toward the daughter cell during division. Sirtuin-associated life span extension has also been linked to oxidative damage in nematodes (Hekimi and Guarente 2003). Another possible mechanism is highlighted by several prior studies implicating Sir2 in mediating repair of DNA damage (Lee et al. 1999; Martin et al. 1999; McAinsh et al. 1999; Mills et al. 1999; Tamburini and Tyler 2005). One dilemma arising from a DNA damage model is that aging wild-type yeast do not appear to pass heritable mutations to daughter cells, which is based on the observation that daughter cells from aged mothers, although short-lived themselves, eventually produce progeny will full life span potential (Kennedy et al. 1994).

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

Dietary Restriction

DR, which refers to a reduction in nutrient intake without malnutrition, is the most universally effective intervention to extend life span across a wide range of eukaryotic species. Yeast replicative aging has been used extensively to study the molecular and genetic factors involved in the life span extension resulting from DR. In yeast, DR is typically performed by limiting the availability of glucose to cells by reducing the glucose concentration in the media from 2% to either 0.5% or 0.05% (Lin et al. 2000), with optimal life span extension achieved at 0.05% glucose in the strain background of the yeast ORF deletion collection (Kaeberlein et al. 2004; Lin et al.

2000). A less commonly used form of DR involving restriction of amino acids has also been shown to extend life span (Jiang et al. 2000). Genetic models of DR are also available, including deletion of *HXK2*, which encodes a hexokinase responsible for converting glucose into glucose-6-phosphate for entry into the glycolytic pathway (Walsh et al. 1983). Deletion of *HXK2* extends replicative life span (Lin et al. 2000), although it remains unclear whether this is attributable to reduced cellular hexokinase activity (Rodriguez et al. 2001; Walsh et al. 1991).

The precise molecular mechanisms through which DR acts to extend life span in yeast are not yet known; however, it is commonly thought that DR manipulates these mechanisms, at least in part, by influencing several partially redundant nutrient-responsive signaling kinases, including target of rapamycin (TOR), cyclic AMP-dependent protein kinase (PKA), and Sch9. Mutants with reduced activity for any of these kinases have long replicative life spans that cannot be further extended by DR (Fabrizio et al. 2004; Kaeberlein et al. 2005c; Lin et al. 2000). TOR signaling is a nitrogen sensitive pathway regulated by glutamine levels and carbohydrate levels that controls a variety of cellular processes, including mRNA translation. TOR is discussed in greater detail later in the chapter. Yeast PKA is an essential complex consisting of three catalytic subunits and regulated by two upstream sensing pathways, one involving RAS and the other a G protein-coupled receptor system. Two genes, *GPA2* and *GPR1*, encode subunits of the G protein-coupled receptor. Mutants lacking either *GPA2* or *GPR1* are replicatively long-lived relative to wild type and are commonly used as models of reduced PKA activity (Lin et al. 2000). The third kinase, Sch9, shows sequence homology to Akt kinase, a component of insulin/IGF-1-like signaling (Burgering and Coffer 1995; Paradis and Ruvkun 1998), but also functions as a ribosomal S6 kinase, a substrate of TOR and regulator of translation in multicellular eukaryotes (Powers 2007; Urban et al. 2007). While yeast does not possess a formal insulin/IGF-1-like signaling pathway, Sch9 may fulfill an equivalent role in yeast to both Akt and S6 kinases in multicellular eukaryotes.

A conclusive answer has not yet been reached to the question of what downstream mechanisms mediate replicative life span extension by DR. Two nonmutually exclusive models have been proposed: increased sirtuin activity and altered mRNA translation (Kaeberlein et al. 2005c; Medvedik et al. 2007; Steffen et al. 2008). DR may activate Sir2 by either elevating NAD levels through increased respiration (Lin et al. 2002) or by increasing transcription of *PCN1* in an Msn2/4 dependent manner. *PNC1* is necessary for the full life span extension from DR (Anderson et al. 2003; Lin et al. 2004) and encodes an enzyme that deaminates nicotinimide, which otherwise inhibits Sir2. Contrary to the idea of sirtuins as mediators of DR, *SIR2* is not required for the replicative life span extension caused by DR (Table 12.1). Specifically, DR does not increase replicative life span in the short-lived *sir2Δ* background (Kaeberlein et al. 2004; Lin et al. 2000), but when the short life span of *sir2Δ* is repressed by deletion of *FOB1*, DR robustly extends replicative life span (Kaeberlein et al. 2004; Lamming et al. 2005). One proposed explanation is that in the absence of Fob1, other sirtuins (such as Hst2) are activated by DR to repress ERC formation (Lamming et al. 2005), though this result has not yet been

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

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

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

Asymmetric Segregation, Oxidative Damage, and Mitochondria

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

Near the end of a mother cell's replicative life span the division asymmetry between mother and daughter breaks down, resulting in daughters with reduced replicative potential (Jazwinski et al. 1989; Johnston 1966; Kennedy et al. 1994). This change is not caused by late-life heritable mutations, as subsequent asymmetric division results in progeny with renewed replicative potential (Kennedy et al. 1994). Lai et al. (2002) performed a screen for temperature sensitive mutants lacking

division asymmetry and identified mutants that exhibited clonal senescence at the restrictive temperature. One of these mutations was identified as a point mutation in *ATP2*, encoding the β-subunit of the mitochondrial ATP synthase. The *ATP2* mutants showed a time-dependent loss in mitochondrial membrane potential followed by a loss of mitochondrial mass, particularly in younger cells. They also found that older mother cells tended to segregate dysfunctional mitochondria to their daughters and propose dysfunctional mitochondria as an asymmetrically segregated aging factor in normal replicative aging. A later study found that the abnormal segregation of mitochondria in *ATP2* mutants can be rescued by overexpression of Pex6, a peroxin protein, and suggested that Pex6 may promote mitochondrial biogenesis (Seo et al. 2007).

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

The Retrograde Response and Mitochondrial Back-Signaling

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

Interestingly, the induction of the retrograde response is also associated with an increase in ERC production (Conrad-Webb and Butow 1995). In addition to its role in retrograde response signaling, Rtg2 is a suppressor of ERCs (Borghouts et al. 2004). The two roles apparently cannot be performed simultaneously, as Rtg2 ERC

suppression is reduced while the retrograde response is active (Borghouts et al. 2004). An aging cell may therefore have to balance the benefits of activating the retrograde response against the deleterious effects of ERC accumulation.

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

Loss of Heterozygosity

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

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

authors observed a marked increase in loss of heterozygosity with age (McMurray and Gottschling 2003).

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

Apoptosis

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

The known causative role for oxidative damage in apoptosis combined with the increased ROS in aged yeast cells, the role of mitochondria in producing ROS, and the asymmetric distribution of dysfunctional mitochondria to mother cells during division suggest that apoptosis may play a role in yeast aging through changes in mitochondrial function. Mitochondria and oxidative stress have been connected with both forms of yeast aging. In the replicative paradigm, overexpression of *NDE1* or *NDE2*, which encodes components of the yeast electron transport chain, extends life span (Lin et al. 2004), consistent with a model where increasing electron transport chain efficiency inhibits aging via decreased ROS production (Korshunov et al. 1997; Starkov 1997). In support of this model, enhancing respiration through mitochondrial uncoupling leads to a decrease in ROS production and an increase in both replicative and chronological life span (Barros et al. 2004; Starkov 1997). In the chronological paradigm, overexpression of superoxide dismutase Sod1 or Sod2 extends life span and deletion of *SOD2* prevents the life span extension resulting from deletion of *CYR1*, which encodes an adenylate cyclase required for production of cyclic AMP that controls a variety of downstream processes including metabolism and stress resistance, or *SCH9* (Fabrizio et al. 2003). Acetic acid, which is known to induce apoptosis (Ludovico et al. 2001), has also recently been identified as a primary mechanism of chronological aging in yeast (Burtner et al. 2009).

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

Genome-Wide Analysis of Replicative Life Span

True high-throughput methods for quantitatively measuring replicative life span in yeast have yet to be described. Replicative life span determination currently requires the relatively time consuming microdissection of daughter cells away from mother cells every 1–2 generations. At least 50 cells are typically necessary to obtain reliable replicative life span data for a single strain, with the experiment preferably performed in triplicate. The *MATα* deletion set contains ~4800 strains with an average life span for the parental strain (BY4742) of approximately 26 generations (daughter cells produced) (Kaeberlein et al. 2005b). In order to screen the entire *MATα* deletion collection for replicative life span, standard methodology requires microdissection of approximately 19 million daughter cells. These factors have limited large-scale attempts at replicative life span determination and caused investigators to focus primarily on hypothesis driven or candidate gene studies of replicative aging.

In 2005, we developed an iterative strategy for identifying replicatively long-lived single gene deletions from the haploid yeast ORF deletion collection in order to bring large-scale screens for replicative life span in to the realm of practicality. This approach uses the standard microdissection method for determining replicative life span, but focuses on using smaller set sizes for each single gene deletion mutant available in the *MATα* deletion set. In order to minimize the effort required per strain, statistical methods were used to identify the minimum number of mother cells that needed to be assayed in order to identify 95% of mutants with a 30% or greater increase in replicative life span. The result is an iterative method in which 5 cells are initially assayed for each deletion mutant (Fig. 12.1) (Kaeberlein et al. 2005c). Based on the average replicative life span of these 5 cells relative to the wild type parental strain, each mutant is given a putative longevity classification. Additional cells are assayed for strains that show potential for long life span until a definitive classification can be made. Once a deletion mutant has been definitively classified as long-lived in the *MATα* background, the corresponding deletion from the *MAT***a** deletion collection is examined for replicative life span. Those deletions that are found to be long-lived in both haploid mating types are considered to be high-confidence modifiers of replicative life span.

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

The replicative life span data for wild-type mother cells (N > 500) were pooled into one set (WT) and replicative life span data for the mother cells of long-lived mutants (LLMs) *hxk2Δ*, *gpa2Δ*, *gpr1Δ*, and *fob1Δ* (N > 500) were pooled into a second set. Probability distributions were then generated for mean life span as

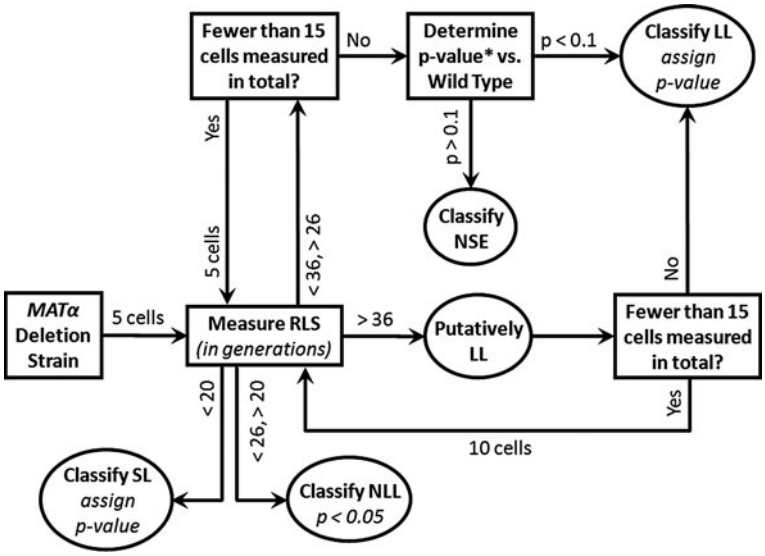

Fig. 12.1 Flow diagram illustrating the iterative approach for identifying long-lived strains from the yeast MATα deletion collection. LL – Long-Lived; NSE – No Significant Extension; NLL – Not Long-Lived; SL – Short-Lived. Mutations classified as LL by this process are subsequently verified as replicatively long-lived in the *MAT***a** background. P-values are for comparison of mean replicative life span for the deletion strain in question to the cumulative probability distribution for BY4742 wild type with n = 5, except *, which indicates a p-value for a Wilcoxon Rank-Sum test comparing replicative life span of deletion mutant to experiment matched BY4742 wild type

a function of the number of cells examined (n), when n = 3, 5, 10, 15, and 20 for WT and LLM, respectively. For example, the n = 3 distribution for wild-type was generated by randomly choosing 3 data points from the pooled wild-type life span set, calculating the mean of the 3 values, and repeating the process 100,000 times. A histogram was then generated for the probability that a particular mean life span is obtained for a set size n = 3, with bins of width 0.1 generations. From this numerical analysis, we were able to establish that an iterative strategy initially analyzing the replicative life span of 5 cells per deletion strain should allow us to identify a large fraction of strains with mean life span greater than 30% longer than wild type.

In the final iterative method (Fig. 12.1), if the 5 cell mean replicative life span is less than 26 generations, the strain is classified as not long-lived (NLL). From the cumulative probability distribution for known long-lived strains with n = 5, this is predicted to result in misclassification of a long-lived strain less than 5% of the time (false negative rate, FNR < 0.05). If the mean life span is less than 20, the strain is classified as short-lived (SL) and a p-value is assigned based on the cumulative probability distribution for wild-type cells with n = 5. If the mean life span for 5 cells is greater than 36, the strain is putatively classified as long-lived (LL) and an additional 10 cells are examined. From the cumulative probability distribution for wild-type cells with n = 5, this is predicted to result in misclassification of a strain

with wild-type life span less than 2% of the time (false positive rate, FPR < 0.02). For the remaining strains with a 5-cell mean life span between 26 and 36, an additional 5 cells are analyzed (1 iteration) and the same criteria for classification are applied. This process is repeated until every strain is either classified as SL, NLL, or LL, or until replicative life span has been determined for a total of at least 15 cells for each unclassified strain. The replicative life span data for strains from which at least 15 mother cells have been assayed are compared against experiment-matched wild type replicative life span data using a Wilcoxon Rank-Sum test and a p-value is generated. Strains with $p \leq 0.1$ are classified as LL, and strains with $p > 0.1$ are classified as having no significant life span extension (NSE). All strains classified as LL are subsequently analyzed in the *MAT***a** background by determining the replicative life span for the corresponding deletion strain contained in the *MAT***a** deletion collection.

In practice, replicative life span analysis of the deletion set is carried out in 95-strain sets (94 deletion strains and wild-type). The ORF deletion collection is packaged in 96-well plates, each plate containing 94 strains. Replicative life span is determined for 5 cells per strain, with 12 strains (one row of the 96-well plate) analyzed per 100 mm YPD plate. All replicative life span experiments are carried out "blind", with each strain coded in a manner such that the researcher performing the microdissection has no knowledge of the identity of any strain until after the experiment is completed.

The iterative strategy described substantially reduces the manual labor necessary to identify gene deletions that confer increased replicative life span. Even with this advantage, screening the entire *MATα* deletion collection requires a significant investment of time and effort and is still in progress. An initial screen was performed to characterize the replicative aging properties for 564 single-gene deletion strains (Kaeberlein et al. 2005c). Of the 564 deletion mutants, 13 were verified to have extended replicative life span relative to the parental strain (Kaeberlein et al. 2005c). The expected false positive rate is low due to validation of long-lived strains by testing of an independently derived deletion allele from the *MAT***a** deletion collection. The false negative rate is not known. For comparison, it is worth noting that the percent of deletions conferring increased replicative life span for the initial 564 strains examined (∼2%) is about twice the percentage of genes that increase life span when expression is reduced via RNAi in *C. elegans* (∼1% for pooled data from all reported RNAi screen) (Smith et al. 2007). This may represent an intrinsic difference in the fraction of genes involved in aging in the two organisms, though there are also two possible method-based explanations for the discrepancy. First, the yeast genome was screened using deletion mutants and therefore excludes essential genes, while the nematode screens used RNAi. Differences in the fraction of genes that influence longevity among essential and nonessential genes would therefore bias the yeast screens. Second, numerous independent worm RNAi longevity screens have shown a remarkable lack of overlap in the genes identified as long-lived (Curran and Ruvkun 2007; Dillin et al. 2002; Hamilton et al. 2005; Hansen et al. 2005; Lee et al. 2003). This is indicative of a high false-negative rate for *C. elegans* RNAi longevity screens and suggests that many worm longevity genes have yet to be discovered.

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

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

Yeast ORF	Yeast gene	Nematode ORF	Nematode gene	Function
YNR051C	BRE5			Molecular function unknown
YBL087C	RPL23A			Structural constituent of ribosome
YBRO84C-A	RPL19A	C09D4.5	Rpl-19	Structural constituent of ribosome
YBR238C				Molecular function unknown
YBR255W	MTC4			Molecular function unknown
YBR266C	SLM6			Molecular function unknown
YBR267W	REI1			Sequence-specific DNA binding
YCR028C-A	RIM1			Single-stranded DNA binding
YDL035C	GPR1			G-protein coupled receptor activity
YDL075W	RPL31A			Structural constituent of ribosome
YDL082W	RPL13A			Structural constituent of ribosome
YDR006C	SOK1			Molecular function unknown
YDR110W	FOB1			Ribosomal DNA (rDNA) binding
YDR268W	MSW1			Tryptophan tRNA ligase activity
YDR382W	RPP2B			Structural constituent of ribosome
YDR500C	RPL37B			Structural constituent of ribosome
YDR523C	SPS1			Protein serine/threonine kinase activity
YER017C	AFG3	Y47G6A.10	spg-7	ATPase activity

Table 12.3 (continued)

Yeast ORF	Yeast gene	Nematode ORF	Nematode gene	Function
YFR032C-A	RPL29			Structural constituent of ribosome
YGL076C	RPL7A			Structural constituent of ribosome
YGL078C	DBP3	B0511.6		ATP dependent RNA helicase activity
YGL147C	RPL9A	R13A5.8	rpl-9	Structural constituent of ribosome
YGL167C	PMR1	B0365.3	eat-6	Calcium-transporting ATPase activity
YGL208W	SIP2			SNF1A/AMP-activated protein kinase activity
YGR063C	SPT4	F54C4.2	spt-4	Pol II transcription elongation factor activity
YGR162W	TIF4631	M110.4	ifg-1	Translation initiation factor activity
YHL002W	HSE1	C14F5.5	sem-5	Protein binding
YIL002C	INP51	JC8.10	unc-26	Inositol-polyphosphate 5-phosphatase activity
YIL052C	RPL34B			Structural constituent of ribosome
YJL138C	TIF2	F57B9.6	inf-1	Translation initiation factor activity
YJR066W	TOR1	B0261.2	let-363	Protein binding
YJR094W-A	RPL43B			Structural constituent of ribosome
YKL056C	TMA19			Molecular function unknown
YKR059W	TIF1	F57B9.6	inf-1	Translation initiation factor activity
YKR072C	SIS2	Y46H3C.6		Phosphopantothenoylcysteine decarboxylase activity
YLR061W	RPL22A			Structural constituent of ribosome
YLR136C	TIS11	F52E1.1	pos-1	mRNA binding
YLR180W	SAM1	C06E7.1	sams-3	Methionine adenosyltransferase activity
YLR262C	YPT6	T23H2.5	rab-10	GTPase activity
YLR371W	ROM2			Signal transducer activity
YLR448W	RPL6B	R151.3	rpl-6	Structural constituent of ribosome
YNL037C	IDH1	F43G9.1		Isocitrate dehydrogenase (NAB) activity
YNL229C	URE2			Transcription co-repressor activity

Table 12.3 (continued)

Yeast ORF	Yeast gene	Nematode ORF	Nematode gene	Function
YNR030W	ALG12	T27F7.3		Alpha-1,6-mannosyltransferase activity
YOL086C	ADH1	W09H1.5		Alcohol dehydrogenase activity
YOL100W	PKH2	H42K12.1	pdk-1	Protein kinase activity
YOR109W	INP53	JC8.10	unc-26	Inositol-polyphosphate 5-phosphatase activity
YOR136W	IDH2	F43G9.1		Isocitrate dehydrogenase (NAD) activity
YOR312C	RPL20B			Structural constituent of ribosome
YPL079W	RPL21B			Structural constituent of ribosome
YPL101W	ELP4			Pol II transcription elongation factor activity
YGR130C[a]		C01G8.5	erm-1	Molecular function unknown
YHR205W[a]	SCH9	Y47D3A.16	rsks-1	Protein serine/threonine kinase activity

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

Insights into Mechanisms of Replicative Aging from Genome-Wide Screens

TOR Signaling Links Nutrient Availability and Replicative Life Span

The 51 long-lived gene deletion strains reported to date from the deletion collection screen for increased replicative life span have led to surprising advances in our understanding of the pathways modulating replicative longevity in yeast. For example, initial analysis of 564 randomly selected deletion strains led to the hypothesis that DR is mediated primary via reduced signaling through the target of rapamycin (TOR) kinase (Kaeberlein et al. 2005c). This was based on the observation that among the 13 replicatively long-lived single-gene deletion strains identified from the original 564, at least 5 are known to function in the TOR pathway (Kaeberlein et al. 2005c). TOR kinases are evolutionarily conserved proteins that function to mediate mRNA translation, cell growth, metabolism, degradation, and stress resistance (among other processes) in response to nutrient and growth factor cues (Table 12.2) (Arsham and Neufeld 2006; Martin and Hall 2005). Mutations that decrease TOR activity have also been reported to increase life span in both *C. elegans* (Jia et al. 2004; Vellai et al. 2003) and *D. melanogaster* (Kapahi et al. 2004), suggesting an evolutionarily conserved link between TOR signaling and aging.

Unlike most multicellular eukaryotes, yeast has two TOR paralogs: *TOR1* and *TOR2*. Tor1 is believed to function specifically in the rapamycin-sensitive TOR complex 1 (TORC1), while Tor2 functions in both TORC1 and TOR complex 2 (TORC2). While Tor2 is essential, strains lacking *TOR1* are viable and have increased replicative life span (Table 12.1), suggesting that TORC1 is an important player in replicative aging. Epistasis analysis with respect to replicative life span places *TOR1* in a genetic pathway that includes DR, but is independent of *SIR2* and *FOB1* (Table 12.1) (Kaeberlein et al. 2005c). This relationship between DR and TOR is supported by studies carried out in both worms and flies (Hansen et al. 2008; Kapahi et al. 2004), indicating that TOR signaling may represent an evolutionarily conserved nutrient response pathway important for mediating the longevity effects of DR.

TORC1 regulates several downstream processes that may contribute to its role in aging, including protein degradation via autophagy, mitochondrial metabolism, stress response, and mRNA translation (Stanfel et al. 2009). Autophagy, which literally means "self eating", is a degradative process through which cellular components are engulfed by cytoplasmic vesicles and transported to the lysosome/vacuole for degradation (Klionsky 2007). Autophagy is repressed by TOR signaling and is induced in response to starvation or treatment with TOR inhibitors, such as rapamycin (Noda and Ohsumi 1998). A decline in the autophagic response has been reported in aging mammals (Cuervo and Dice 2000), and increased autophagy is required for life span extension in long-lived *C. elegans* mutants with reduced insulin/IGF-1-like signaling (Melendez et al. 2003). Several recent studies have also uncovered an important role for autophagy in the response to DR. DR induces autophagy in yeast, worms, and flies (Juhasz et al. 2007; Morck and Pilon 2006; Takeshige et al. 1992) and is reported to be required for life span extension from DR or TOR-inhibition in both worms and flies (Hansen et al. 2008; Jia and Levine 2007; Juhasz et al. 2007). Recently, up-regulation of autophagy by spermidine has also been shown to be associated with increased life span in yeast, nematodes, and flies (Eisenberg et al. 2009).

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

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

Regulation of mRNA Translation Modulates Replicative Life Span

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

In the initial analysis of 564 deletion strains, strains lacking two different genes coding for ribosomal large subunit proteins (*rpl31aΔ* and *rpl6bΔ*) were among the long-lived mutants (Kaeberlein et al. 2005c). While most ribosomal proteins are thought to be essential in yeast, the majority of genes encoding ribosomal proteins are present in the yeast genome in duplicate, often allowing for viable deletion of either paralog (Komili et al. 2007; McIntosh and Warner 2007). Since this study, deletion of genes encoding 13 additional large subunit ribosomal proteins and 3 translation initiation factors (*tif1Δ*, *tif2Δ*, and *tif4631Δ*) have been found to increase replicative life span from the deletion set analysis (Table 12.3) (Steffen et al. 2008).

A recent study identified Gcn4 as a potential mediator of reduced mRNA translation with respect to life span (Steffen et al. 2008). Gcn4 is a transcription factor that induces expression of amino acid biosynthetic genes in response to amino acid starvation (Hinnebusch 2005) and plays a role in a variety of cellular processes including autophagy, ER stress response, organelle biosynthesis, and induction of mitochondria transport carrier proteins (Jia et al. 2000; Natarajan et al. 2001; Patil et al. 2004). Steffen et al. (2008) found that expression of a Gcn4-luciferase reporter was upregulated in strains lacking *RPL20B* and *RPL31A*, and that deletion of *GCN4* partially blocked the life span extension resulting from DR, deletion of *TOR1*, deletion of *SCH9*, or deletion of an *RPL*.

Cellular levels of Gcn4 are translationally regulated by four small inhibitory upstream open reading frames (uORFs) in the 5' leader region of the *GCN4*

gene (Hinnebusch 2005). The mechanism of regulation is thought to involve relative availability of the large and small ribosome subunits. Specifically, when 60S ribosomal subunit levels are low, ternary complexes containing initiation factors and 40S ribosomal subunits are proposed to more frequently scan through the inhibitory uORFs before interacting with 60S subunits, increasing translation of *GCN4* (Steffen et al. 2008). Thus, while reducing availability of 60S ribosomal subunits reduces overall translation, translation of the *GCN4* transcript specifically increases, supporting a model where reduced mRNA translation influences longevity by differentially modifying translation of specific mRNA targets. This mechanism for translation inhibition may extend to other genes involved in controlling life span and several yeast genes are known to contain inhibitory uORFs, including *HAP4* and *CLN3* (Vilela and McCarthy 2003; Zhang and Dietrich 2005), which are involved in nutrient response. As previously noted, overexpression of *HAP4* increases replicative life span (Lin et al. 2002).

Yeast Chronological Life Span

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

Genome-Wide Analysis of Chronological Life Span

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

Powers et al. (2006) described a high-throughput method for qualitatively measuring chronological life span of cells aged in 96-well microtiter plates. Rather than monitoring survival by determining colony forming units, Powers et al. (2006) estimated relative cell viability of the population by diluting the aging culture into rich liquid media and measuring the optical density at 600 nm (OD) following a fixed outgrowth period. All cell and liquid transfers were automated using a high-density replica pinning robot. While less quantitative than the traditional methodologies, this method offers the ability to monitor survival for several thousand strains simultaneously. As proof-of-principle, Powers et al. (2006) screened the homozygous diploid ORF deletion collection for long-lived mutants and identified five deletion strains with increased chronological life span: *gln3Δ*, *lys12Δ*, *mep3Δ*, *mep2Δ*, and *apg1Δ*.

More recently, we modified the method described by Powers et al. (2006) to create a high-throughput assay for measuring chronological life span that allows quantitative analysis of chronological life span across the entire deletion collection (Murakami et al. 2008). This redesigned method improves the quantitative resolution by using an entire outgrowth curve to calculate residual survival rather than a single outgrowth time-point. Our studies use a Bioscreen C MBR (Growth Curves USA) machine to obtain outgrowth curves from aged cells, though any shaker/incubator/plate reader combination will suffice. To monitor viability at each age-point, 5 μL of the aging culture is inoculated into 145 μL of YPD in one well of a Bioscreen Honeycomb plate. Outgrowth of the inoculated cells takes place in the Bioscreen C MBR machine at 30°C with continuous shaking. OD is determined every 30 min for 24 h yielding highly reproducible outgrowth curves from which relative survival can be calculated. Outgrowth curves of aging cells show a distinct age-dependent rightward shift, such that the length of time required to achieve a given OD value increases with age (Fig. 12.2a). A survival curve can be generated from the Bioscreen growth data based on the estimated fraction of cells retaining viability at each time point (Fig. 12.2b). The viable fraction is calculated relative to the initial time point (typically day 2) based on the rightward time shift required for outgrowth to reach a fixed OD value of 0.3 using the formula:

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

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

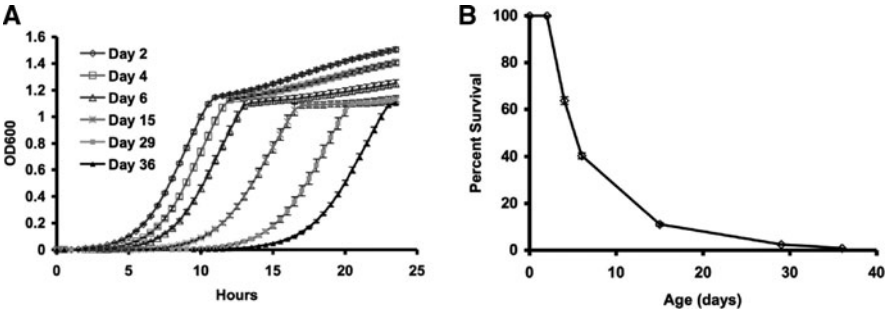

Fig. 12.2 Chronological life span survival curves are calculated based on time delay for strain specific outgrowth. **a** Outgrowth curves shift rightward as stationary phase cells age. **b** Survival curves are calculated from the time shift between outgrowth curves

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

TOR Signaling Promotes Chronological Aging

As mentioned previously, the nutrient sensitive TOR pathway appears to play an important role in both replicative and chronological life span. In both yeast aging paradigms, genetic or pharmacological inhibition of TOR signaling increases life span and is believed to mediate the life span extending benefits of DR (Table 12.2) (Fabrizio et al. 2001; Kaeberlein et al. 2005c; Powers 3rd et al. 2006). DR can be accomplished in the chronological aging assay in a manner similar to the replicative aging assay, by reducing the glucose concentration of the growth media, or by an alternative method in which aging stationary phase cells are transferred to water. Similar to the case for replicative life span, chronological life span extension from DR is believed to be independent of Sir2. In contrast to replicative life span, the mechanism by which reduced TOR signaling and DR promote chronological life span appears to be mediated primarily by regulation of carbon metabolism, which is discussed in detail below (Bonawitz et al. 2007).

The chronological life span screen of the homozygous diploid ORF deletion collection performed by Powers et al. (2006) identified 16 genes implicated in the TOR pathway. Of these, 5 were found to consistently extend chronological life span when subject to more stringent analysis: the nitrogen-responsive transcription factor *GLN3*, the lysine biosynthetic homo-isocitrate dehydrogenase *LYS12*, the nitrogen-responsive ammonium permeases *MEP2* and *MEP3*, and amino acid permease *AGP1*. Each of these deletion mutants shows increased glycogen accumulation characteristic of starvation, and each mutation inhibits TOR signaling by limiting amino acid uptake or synthesis (Powers 3rd et al. 2006). Powers et al. (2006) also demonstrate that pharmacological inhibitors of TOR signaling (methionine sulfoximine and rapamycin) increase chronological life span.

Acetic Acid Is a Molecular Cause of Chronological Aging

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

While exploring the possible mechanisms by which DR might increase chronological life span, it was observed that cells cultured in low glucose media do not acidify their cultures to the same extent as cells grown in 2% glucose (Burtner et al. 2009). Standard growth media for chronological aging experiments initially has a pH of about 4.5. Within a few days, the expired media reaches a pH of approximately 3.0 when the starting glucose concentration is 2%, does not change significantly when the starting glucose concentration is 0.5%, and becomes alkalinized to about pH 6.0 when the starting glucose concentration is 0.05%. Interestingly, buffering the pH of cells grown in 2% glucose media at 6.0 is sufficient to extend chronological life span in a manner comparable to cells grown in un-buffered 0.05% media. Further experiments demonstrated that the causative factor underlying these observations is acetic acid, which is produced by chronologically aging cells during back-fermentation of ethanol and is known to induce an apoptotic-like response in yeast cells (Herker et al. 2004). An important additional conclusion from the studies of Burtner et al. (2009) is that many of the previously known mutations that increase chronological life span can be explained by either (1) reduced production of acetic acid during growth into stationary phase or (2) increased resistance to acetic acid (Table 12.4). By modifying the chronological life span procedure it may be possible to minimize the cell non-autonomous effects of organic acid secretion during fermentation.

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

Table 12.4 Increase in chronological life span related to acetic acid can be caused either by reduced acetic acid production or increased acetic acid resistance

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

aging under conditions commonly used in chronological life span assay and is consistent with the idea that acetic acid is a primary molecular cause of chronological aging.

Recent Genome-Scale Screens Identify New Potential Chronological Aging Pathways

The past 2 years have seen the emergence of a competitive-survival strategy for identifying genes involved in chronological aging. In this strategy, the ~4800 strains from the yeast deletion collection are pooled in a common chronological aging culture. Portions of the culture are taken at different time points and allowed to grow for a specified amount of time before harvesting DNA. Microarray or deep sequencing is used to determine the relative abundance of each deletion strain in the aged culture using two unique sequences built into each deletion mutant. Strains enriched in the chronologically aged culture are considered putatively chronologically long-lived and confirmed using standard single-strain chronological life span techniques. Novel chronological longevity genes were reported based on this approach in three recent publications (Fabrizio et al. 2010; Gresham et al. 2011; Matecic et al. 2010).

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

Gresham et al. (2011), and Matecic et al. (2010) also all identified multiple short-lived autophagy mutants, suggesting that the ability of a cell to degrade cellular components is important for long-term survival in a non-dividing state.

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

Alternative High-Throughput Methods for Studying Aging

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

Emerging High-Throughput Strategies for Studying Replicative Aging

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

generations to be used for microarrays or other large-scale approaches. Two additional methods have been developed to produce populations of replicatively aged cells from dividing cell populations in yeast.

One technique for extracting replicatively aged cells from a mixed population is termed counter flow centrifugation elutriation (CCE). CCE, originally invented in 1948, uses the balance between centrifugal force and counter flow drag in a spinning buffer media to separate cells of different sizes in a mixed population (Sloot et al. 1988). As yeast cells divide they also increase in size (Mortimer and Johnston 1959; Nestelbacher et al. 2000), resulting in a population of small, young cells and large, old cells. Using CCE to select large cells from a dividing population thus results in a subset enriched for cells with advanced replicative age. This technique was employed to separate mixed yeast cell populations and compare young cells (2–3 generations) to old cells (16–18 generations) and to "old" cells lacking *DNA2-1*, a model of premature aging (8 generations) (Lesur and Campbell 2004). Lesur and Campbell (2004) found transcription upregulation of a variety of genes involved in energy storage and environmental stress response in the aged and prematurely aged cells relative to young cells. Laun et al. (2001) used CCE to produce produced cell populations with 30% terminally senescent cells, indicating that the population was substantially enriched for cells near the end of their replicative life span. This technique was used in a transcriptome study comparing replicatively aged cells to a population of cells driven to apoptosis by mutation of *CDC38*, which identified *MRPL25/AFO1* (Laun et al. 2005), a gene that encodes a mitochondrial large subunit ribosomal protein that influences longevity by interacting with TOR1 through mitochondrial back signaling (Heeren et al. 2009). A related method combining growth synchronization and rate-zonal sedimentation in density gradients was used to produced cell populations highly enriched for cells aged up to 20 generations (Egilmez et al. 1990). This technique was used in a transcriptome study to identify the long-lived *LAG1* deletion strain (D'Mello et al. 1994). While an improvement on fluorescence-based cell sorting, CCE is still only able to enrich for cells aged to around 20 generations, which is still well below the median replicative life span of many of the common strains in yeast aging (typically in the low to mid 20s).

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

In addition to applications in young-vs.-old comparisons, the MEP has been proposed as a high-throughput method to measure replicative life span (Lindstrom and Gottschling 2009). Since daughter cells are produced but do not continue dividing, the viability of MEP cultures is determined specifically by the replicative life span

of the mother cells. The use of a plate reader system, such as that used to measure chronological life span (Murakami et al. 2008) can allow rapid, high-throughput measurement of replicative life span for strains carrying the MEP biological machinery in liquid media. While there are still technical challenges yet to overcome before MEP becomes widely used, this technique shows particular promise for the application of replicative life span drug screening, where current labor-intensive methods for measuring replicative life span limit the number of drugs that can be easily tested, and where further genetic manipulation of the strains is not necessary.

Microarrays, Proteomics, and Metabolomics

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

An alternative microarray approach to comparing yeast of different ages is to compare the expression profiles of different age-matched long-lived mutants and look for pattern similarities that may identify genes that are generally important in the aging process. One study used this approach to compare the expression profiles

of wild type yeast to three chronologically long-lived strains with mutations in *TOR1*, *SCH9*, and *RAS2* (Cheng et al. 2007a, b). The expression patterns implied an overall reduction in transcription in the three mutant strains as compared to wild type, as well as a downregulation in genes involved in the TCA cycle and oxidative phosphorylation relative to genes involved in glycolysis. The *ras2Δ* strain also showed a reduced expression of genes involved in mitosis, distinguishing it from the other two long-lived mutants.

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

Microarrays and related technologies that measure the “transcriptome” of a tissue or organism can provide valuable insight into the genes that are involved in phenotypes associated with a given genetic background or biological intervention. A limitation of these techniques is that they only give indirect information about the content of proteins, metabolites, and other molecules directly involved in an organisms interaction with its environment. The detection of these molecules is the focus of up and coming fields such as proteomics, which studies the protein complement of a cell or organism, and metabolomics, which studies the array of small molecule metabolites present in an organism. One group has recently taken the first steps toward establishing these methods in yeast aging by comparing the metabolic histories of chronologically aging yeast with and without DR using a variety of techniques to measure phenotypes ranging from protein and metabolite levels to ROS, mutation rates, and stress resistance (Goldberg et al. 2009). They conclude that yeast set up a metabolic profile prior to entering a non-proliferative state which depends on the contents of the original media and present a model suggesting how this profile might contribute to chronological aging. While proteomic and metabolomic methods have yet to be widely applied to the study of yeast aging, both fields are growing and hold promise to provide valuable insight in the future.

Comparison of Yeast Aging to a Multicellular Eukaryote

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

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

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

are substantially enriched for genes that code for proteins involved in regulating mRNA translation. Among the 25 homolog pairs, only two were previously known to modulate aging in both yeast and worms: *TOR1*/ *let-363* and *SCH9*/*rsks-1*. *SCH9* and *rsks-1* are functional orthologs of mammalian ribosomal S6 kinase, which functions downstream of TOR signaling to modulate mRNA translation initiation (Pan et al. 2007; Urban et al. 2007). Excluding *TOR1*/*let-363* itself, 6 of the 10 remaining ortholog pairs in the high-stringency set can be definitively assigned functions related to mRNA translation: three ribosomal proteins of the large subunit (*RPL19A*/*rpl-19*, *RPL6B*/*rpl-6*, *RPL9A*/*rpl-9*) and three translation initiation factors (*TIF1*/*inf-1*, *TIF2*/*inf-1*, and *TIF4631*/*ifg-1*). Given that TOR and S6K are known to negatively regulate both ribosome biogenesis and translation initiation factor activity, it is reasonable to speculate that all of these factors act in a single conserved longevity pathway.

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

Conclusion

A great deal of progress has been made in advancing our understanding of yeast aging through genetic and, of late, genomic studies. Through these studies a large number of genes involved in the aging process have been identified. Collectively, the field has been quite successful at extending both chronological and replicative life span. Most of the aging genes identified thus far are regulatory components and include genes involved in signal transduction, transcription, or translation. A subset of homologs of these genes are likely to have similar effects in mammals. Regulatory factors are often pleiotropic in function and it remains unclear which downstream targets drive aging in yeast. The next challenge facing aging researchers is to use the available knowledge of these regulatory factors to work downstream and uncover the spectrum of molecular events that lead to age-associated deterioration in yeast and other organisms. ERCs and acetic acid represent the first steps down this path in the replicative and chronological aging paradigms, respectively, but these factors are only part of the story. What else is involved? Reactive oxygen species and DNA damage? Mitochondrial degeneration? Loss of protein

homeostasis? Epigenetic drift? Some as yet unidentified molecular mechanism? The answer will likely involve some or all of these possibilities. We anticipate that research in the coming years, driven in combination by unbiased genome-scale longevity studies and focused hypothesis-driven experiments, will provide the answers.

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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.

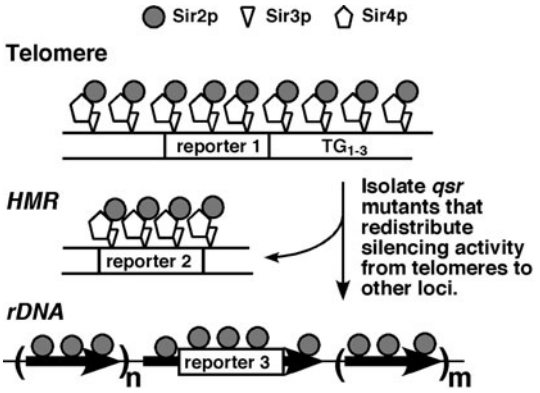

Fig. 13.1 The triple silencer strain concept and selection for *quirky redistribution of silencing* (*qsr*) mutants. The triple silencer strain has three different reporter genes at three different loci whose expression is partially silenced; allowing both increases and decreases in silencing to be monitored. 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

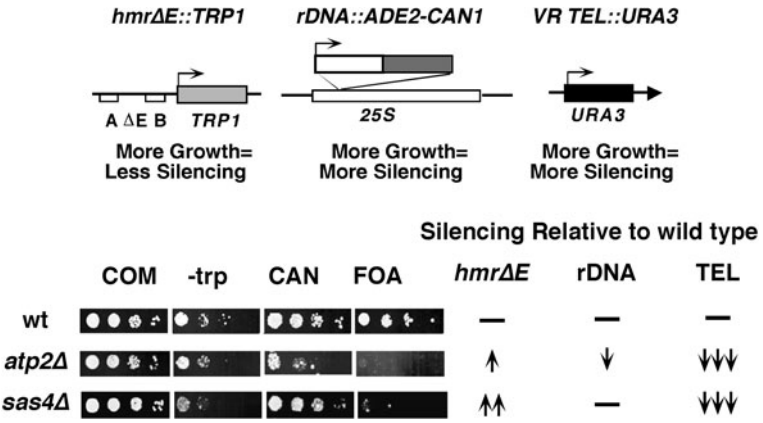

Fig. 13.2 The *qsr* mutants *atp2Δ* and *sas4Δ* have similar silencing phenotypes. A spot test assay of the three reporter genes, using the amount of growth as a measure of the level of gene expression. Ten-fold serial dilutions of single yeast colonies bearing the indicated mutations were spotted onto 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

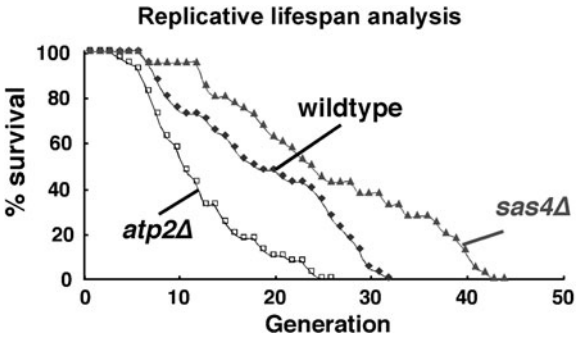

Fig. 13.3 The *atp2Δ* and *sas4Δ* mutants with the same silencing phenotypes (Fig. 13.2) have different replicative lifespan. Replicative lifespan assays (the number of times a cell can divide) were performed as described (Ray et al. 2003)

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

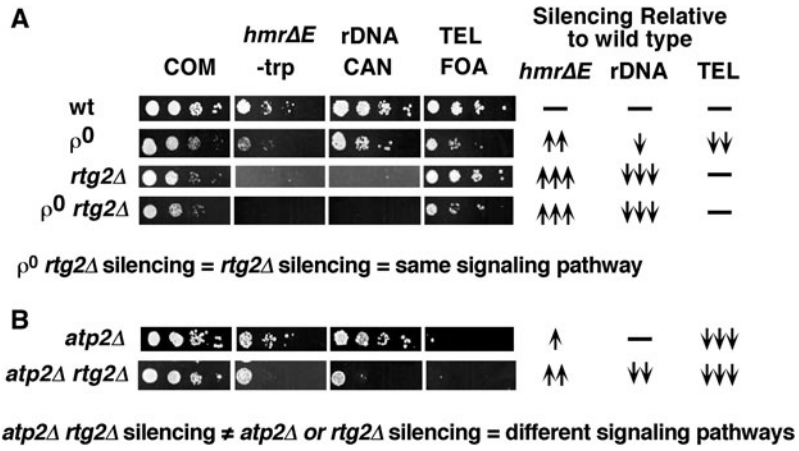

Fig. 13.4 The redistribution of silencing phenotype responds to mitochondrial state, and shows that the *atp2Δ* defect signals through a new pathway. **a** The silencing redistribution assay responds to the known *RTG2* control of cellular response to loss of the mitochondrial genome. Cells that have 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%

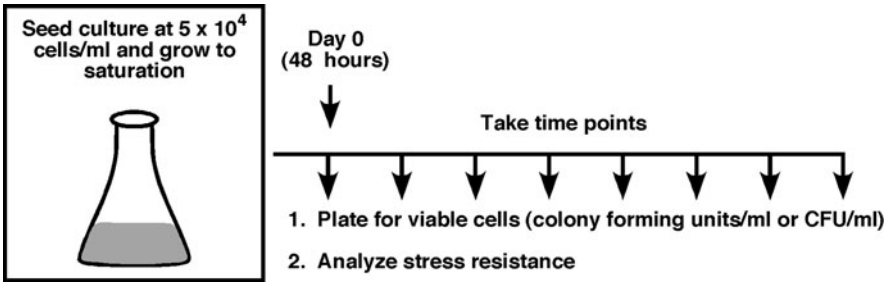

Fig. 13.5 Basics of the *S. pombe* CLS assay. Recently grown *S. pombe* cells are resuspended in 30 ml of medium at a low density (5×10^4 cells/ml) in a 125 ml flask and grown at 30°C under rotation for the course of the experiment. After 2 days of growth, cells reach their maximum density, which is 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.

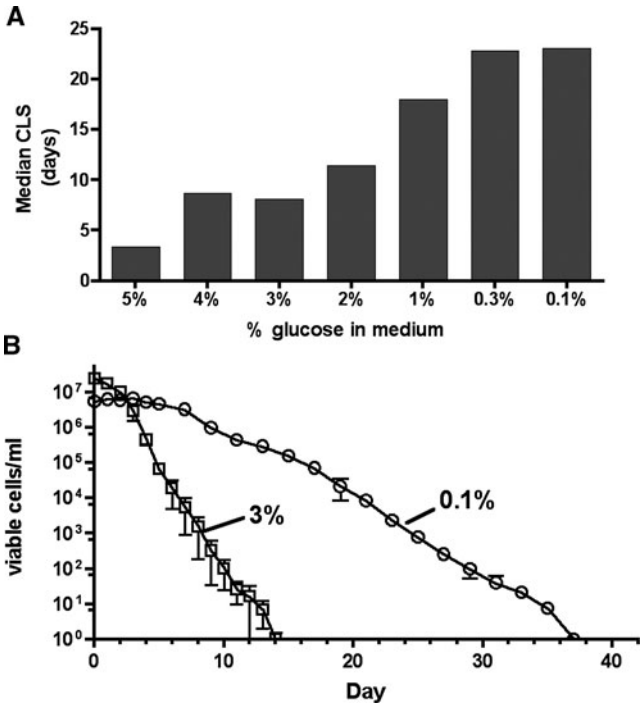

Fig. 13.6 The *S. pombe* CLS assay shows lifespan shortening under overnutrition and lifespan extension under caloric restriction. **a** The median CLS of wild type *S. pombe* in SD media with different glucose concentrations as described in Chen and Runge (2009). The standard 3% glucose condition does 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

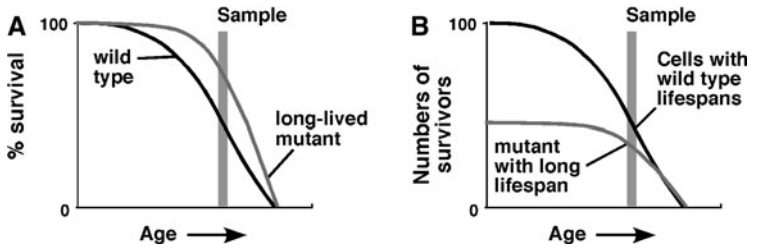

Fig. 13.7 Hypothetical survival curves of a long-lived mutant whose median lifespan is extended and whose maximum lifespan is equal to wild type. The *gray bar* labeled "Sample" indicates a point in the CLS when viability is monitored and colonies of surviving cells are isolated. **a** Examples of wild 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

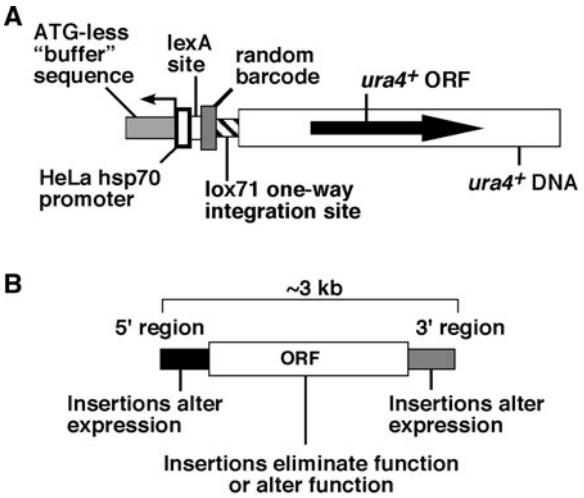

Fig. 13.8 Construction and rationale for the *S. pombe* DNA insertion library. **a** A map of the DNA insertion vector. The insertion vector contains the *ura4*$^+$ gene for selection in *S. pombe*, a random barcode containing 27 random nucleotides, a *lexA* binding site for tethering transcriptional 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

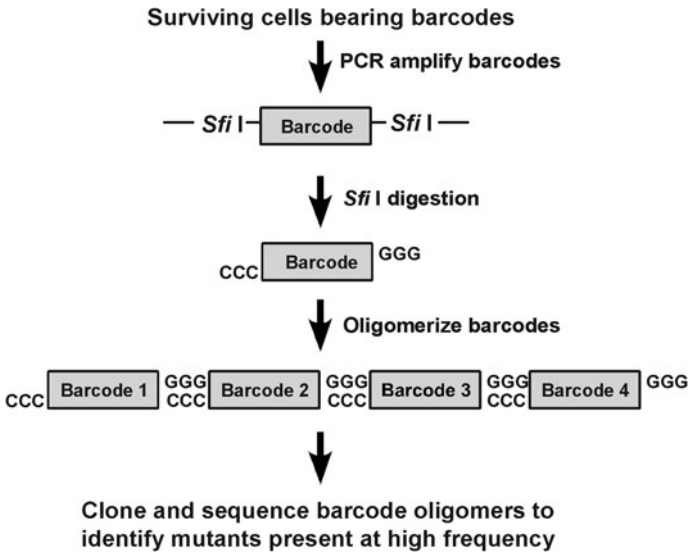

Fig. 13.9 Using barcodes to identify long-lived mutants. Each barcode is bordered by two *Sfi* I sites. *Sfi* I recognizes GGCCAGGGxAGGCC, where "x" shows the cleavage site that leaves a 3 nt overhang. The *Sfi* I sites in the insertion vector yield fragments that can be ligated into head-to-tail oligomers, 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

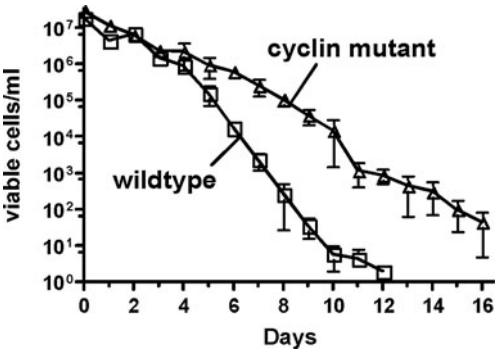

Fig. 13.10 The CLS of a long-lived *S. pombe* mutant that was isolated by direct selection using the insertion mutant library and barcode screening strategy. The mutation was later verified by recreating it in a wild type strain and showing that two independently recreated mutants also had longer lifespan. 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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Chapter 14
Evolution of Asymmetric Damage Segregation: A Modelling Approach

Armin Rashidi, Thomas B.L. Kirkwood, and Daryl P. Shanley

Abstract Mother cell-specific ageing is a well-known phenomenon in budding yeast *Saccharomyces cerevisiae*. Asymmetric segregation of damage and its accumulation in the mother cell has been proposed as one important mechanism. There are, however, unicellular organisms such as the fission yeast *Schizosaccharomyces pombe*, which replicates with almost no asymmetry of segregation of damage and the pathogenic yeast *Candida albicans*, which falls around the middle of the segregation spectrum far from both complete symmetry and complete asymmetry. The ultimate evolutionary cause that determines the way damage segregates in a given organism is not known. Here we develop a mathematical model to examine the selective forces that drive the evolution of asymmetry and discover the conditions in which symmetry is the optimal strategy. Three main processes are included in the model: protein synthesis (growth), protein damage, and degradation of damage. We consider, for the first time, the costs to the cell that might accompany the evolution of asymmetry and incorporate them into the model along with known trade-offs between reproductive and maintenance investments and their energy requirements. The model provides insight into the relationship between ecology and cellular trade-off physiology in the context of unicellular ageing, and applications of the model may extend to multicellular organisms.

Keywords Ageing · Asymmetry · Evolution · Damage · Segregation

Introduction

The ability of all forms of life, from simple unicellular organisms to complex eukaryotes, to continue to survive is closely linked to their ability to detect errors that occur in their macromolecules and then repair them. From an evolutionary perspective, the primary function of the maintenance systems is to provide a sound internal state as the organism grows and reproduces. The external environment,

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however, is not under control of the organism. Indeed, the environment is typically highly variable, harsh and stressful. One inevitable product of such environments for the organism is cellular damage which, if not properly countered, threatens the survival of the lineage in unicellular forms of life and the integrity of tissues in multicellular organisms. Unrepaired genetic damage is particularly dangerous because of its direct flow to future generations. Non-genetic damage (e.g. protein damage) is important too. When a cell divides at mitosis, all of its cytoplasmic materials, including damaged macromolecules are distributed during cytokinesis between the two daughter cells. Depending on its reparability, all or parts of damage may be further transferred to the next generation.

How can a cell deal with damage and maximise its Darwinian fitness in environments with various sources of stress? Irreversible protein damage is a serious problem because the multitude of costly repair mechanisms that exist in the cell is of no use in this regard. The way this damage is inherited by the daughter cells is also important. Maximal dilution occurs with symmetric distribution of damage at division. In order to be effective, symmetry needs to be accompanied by sufficiently rapid proliferation (Rashidi 2008). Otherwise, both daughter cells (i.e. the lineage) would suffer the same risk of accumulated damage rising eventually to a lethal level. Thus, symmetry is expected to be an efficient strategy to cope with irreversible damage when the environment is not too harsh, the cell is sufficiently rich in repair mechanisms, and/or proliferation is sufficiently fast. The advantage of making do with symmetry is that it does not require a separate and potentially costly mechanism to evolve. The disadvantage concerns the requirement for sufficient investment in maintenance (i.e. efficient repair) and/or reproduction (i.e. rapid proliferation). As an example, damage inheritance in the unicellular fission yeast *Schizosaccharomyces pombe* (*S. pombe*) is strikingly symmetric. The "old" daughter cell in this organism receives on average only 55% of maternal carbonylated proteins (Minois et al. 2006).

In contrast, asymmetric segregation of damage favours the survival of a lineage by specifically promoting the survival of the daughter cell that inherits less damage. Sufficient asymmetry (one daughter cell is born with no damage in the extreme case) may guarantee lineage survival and compensate for slow reproduction and/or inefficient maintenance systems. Not surprisingly, asymmetric inheritance is prevalent in all kingdoms of life, from yeast to higher eukaryotes (Aguilaniu et al. 2003; Macara and Mili 2008; Rujano et al. 2006). The main issue with asymmetry is a potential need for specific mechanisms including new genes, molecules or interactions to evolve. It is not trivial to see what proportion of resources the cell is best to invest in maintenance, reproduction, and possibly asymmetry when resources are limited. Resource limitation causes a multitude of trade-offs at different levels (subcellular, cellular, organismal) to emerge (Fischer et al. 2009; Stearns 1976). Mathematical modelling is an alternative to the classical experimental approach in the study of these trade-offs. Here we investigate and model the evolution of non-genetic damage segregation in unicellular organisms.

The Model

Basic Assumptions

The model is based on a single haploid asexual cell which founds a genetically homogenous colony. Regarding damage accumulation and degradation, we study 3 genes, each with one quantitatively indentified locus. The quantitative value (s, Δ, g) of the genes (e.g. corresponding to their expression level) determines the strength of the particular traits they produce. The traits are protein synthesis rate, protein damage rate, and protein damage degradation rate, respectively. Since colonies are assumed genetically homogenous, we ignore rare mutations that might occur to the three genes of our interest. We are interested in damage only to the non-genetic materials inside the cell (e.g. proteins) and only irreversible types of damage (e.g. protein carbonylation (Stadtman 2006)). Proteins are synthesised at a constant rate s. A constant proportion of the existing proteins are assumed to become irreversibly damaged at rate Δ. While repair is the principal way by which cells handle reversible forms of damage, there are generally three ways to cope with irreversible damage: (i) degradation (e.g. by the ubiquitin/proteasome system), (ii) exocytosis, and (iii) simply living with damage. With the latter, and if the cell survives to reproduce, damage may be diluted between the two daughter cells such that each inherits only part of the damage. The proportion of damage received by each daughter cell affects their chances for survival and thus their fitness (Ackermann et al. 2007; Evans and Steinsaltz 2007; Fredriksson and Nyström 2006; Watve et al. 2006). This proportion may also influence the survival of the colony (Erjavec et al. 2008). For example, with sufficient asymmetry in segregation of damage, the daughter cells in each generation which are born with relatively little damage can guarantee the survival of the lineage. The level of segregation asymmetry is assumed to be a heritable trait and rare mutations are not considered. We combine strategies (i) and (ii), collectively referred to as "degradation". Reproduction takes place by cell division during which damaged particles in the mother cell are distributed between the two daughter cells. The timing of cell division depends on the amount of undamaged materials (in this model, native proteins) in the cell (Erjavec et al. 2008). When the number of native protein molecules reaches a certain threshold, the cell divides. If damage (D) increases beyond a fixed threshold D^*, the cell dies. Death occurs by an apoptosis-like process and does not affect neighbouring cells.

Fitness (of a cell) is defined as the number of descendants it produces per unit of time. The unit of time is arbitrary (as long as it is constant for comparison purposes) and can be defined large enough for cells to reproduce. We assume that the most primitive unicellular organisms had more symmetric damage segregation strategies than the more recently evolved cells. Therefore, the default strategy is assumed to be symmetric and we try to find triplets of the form (s, Δ, g) for which asymmetry pays. As a specific case, if all members of a colony that segregate damage symmetrically and descend from a given triplet survive, asymmetry will be associated with no fitness advantage and will thus not evolve. We develop the model in three successive steps. First, we assume that degradation mechanisms have not yet evolved. Next,

we relax this assumption. In these two steps, the cell does not have to pay any costs (metabolic, energetic) to evolve asymmetry. In the third step, asymmetry is associated with a fixed cost detracted from resources invested in maintenance and/or reproduction.

Structure of the Model

There are 3 continuous-time processes (protein synthesis, protein damage accumulation, and damage degradation) and 3 instantaneous events (cell division, damage segregation, and cell death) in the model. The continuous-time processes are embedded in and modelled as the following 2 ordinary differential equations:

$$\begin{aligned} dp/dt &= s - \Delta p \\ dD/dt &= \Delta p - gD \end{aligned} \tag{14.1}$$

Proteins (p) are synthesised at a constant rate (s) and are irreversibly damaged by a first-degree kinetic process with constant Δ. Damaged proteins (D) are also degraded by the same kind of process with constant g. The cell divides when normal proteins reach a threshold p^* unless damage has already reached a threshold D^* in which case the cell dies before division. Without loss of generality, we let $p^* = 1$. Time (t) is measured from the time the cell is born. In order to give our cells a chance to reproduce, we assume $s > \Delta$. Without this assumption, the cell dies before it can divide. Segregation of damage at division is assumed to occur according to a segregation coefficient σ, which is a heritable trait. A proportion σ of damage segregates to one of the daughter cells and the rest of the damage goes to the other cell. To set the initial conditions, we note that $D(0)$ is the amount of damage that a daughter cell inherits from its mother. Since aggregation of damaged proteins makes them more stable (and so makes their turn-over slower than that of normal proteins), we do not consider the inheritance of normal proteins (Carrio and Villaverde 2003; Maisonneuve et al. 2008). In other words, we let $p(0) = 0$.

Results

No Degradation

In this case, corresponding to $g = 0$, the degradation mechanisms have not yet evolved or are negligibly primitive. The system has no fixed points (i.e. stable or unstable steady states) and the amount of accumulated damage increases in an explosive manner. The only chance for survival is rapid reproduction such that before damage reaches fatal levels, the cell has already divided.

To examine the fate of the population and the advantages of asymmetry, let us look closely into the behaviour of the model. It can be shown that the necessary and sufficient condition for survival of a cell (until division) is

$$D(0) < 1 + D^* + \Delta^{-1} sLn(1 - \Delta/s) \tag{14.2}$$

The accumulated damage during a cell cycle is constant:

$$x = -1 - \Delta^{-1} sLn(1 - \Delta/s)$$

and (14.2) can be rewritten as:

$$D(0) + x < D^*$$

We define $d(0)$ to be the initial amount of damage in the founder cell. With that, three possibilities can be considered:

(i) $x < d(0)$: In this case, and as long as the cells follow a symmetric segregation strategy, the accumulated damage at the time of cell division declines with advancing generations and there is no death in the population. Therefore, asymmetry cannot be associated with any fitness advantage in this situation.
(ii) $d(0) < x < D^*/2$: In this case, and as long as the cells follow a symmetric segregation strategy, the accumulated damage at the time of cell division grows with advancing generations to $2x < D^*$. Again, symmetry offers the highest possible fitness and there is no selection pressure for asymmetry.
(iii) $x > \max\{d(0), D^*/2\}$: In this case, and with symmetry, the accumulated damage at the time of cell division grows with advancing generations to $2x > D^*$. If the younger daughter cells receive more than a proportion $\max\{(D^* - x)/D^*, d(0)/(d(0) + x)\}$ of maternal damage, the population will disappear at some point. Sufficient asymmetry enhances fitness and prevents the population from vanishing.

With Degradation

Now we evaluate the case where cells already possess degradation mechanisms and hence $g > 0$. The cell cycle duration and total damage accumulated in the cell at time of division, if the accumulated damage is not high enough to have already killed the cell are given by

$$T = -\Delta^{-1} Ln(1 - \Delta/s)$$
$$D_T = \Delta(g - s)/g(g - \Delta) + (D(0) + s/(g - \Delta) - s/g)(1 - \Delta/s)^{g/\Delta}$$

The analysis of the system shows that with sufficiently long cell cycle duration, the dynamics of the accumulated damage in a cell follows one of the following 3 patterns:

(i) Damage increases to a finite maximum. This case occurs when $D(0) = 0$.
(ii) Damage decreases to a minimum. This case occurs when $D(0) > s\Delta/g(\Delta - g) > 0$.

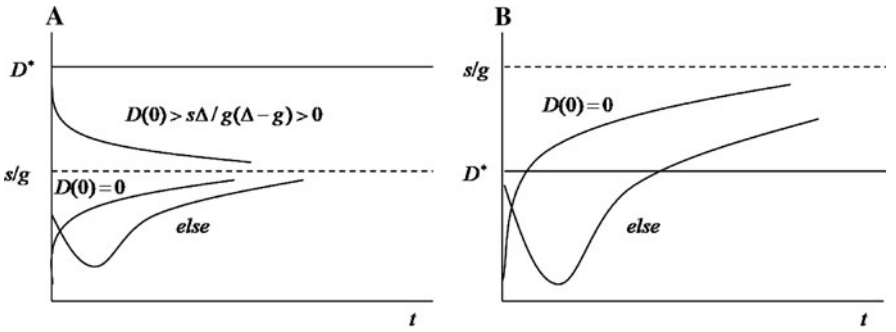

Fig. 14.1 The stable steady state of the system with three typical trajectories. All trajectories are attracted to the steady state, shown by the *dashed line*. **a** The ratio of maintenance to growth investment is sufficiently high ($g/s > 1/D^*$). **b** The maintenance/growth ratio is low ($g/s < 1/D^*$)

(iii) There is an initial decline in damage after which damage increases to a finite maximum. This is the case for any condition that does not satisfy (i) and (ii).

The system has one steady state$(p, D) = (s/\Delta, s/g)$, which is stable (Fig. 14.1). Symmetry offers the highest possible fitness (all cells survive) when $s < gD^*$. Also, symmetry is the best strategy (all cells survive) if both of the following two conditions hold (see Appendix for detailed analysis):

$$gD^* < s < \Delta\left(1 + D^*\right)/4$$
$$\left(\Delta - \sqrt{\Delta^2 - 4s\Delta/\left(1 + D^*\right)}\right)/2 < g < \min\left\{\Delta, \left(\Delta + \sqrt{\Delta^2 - 4s\Delta/\left(1 + D^*\right)}\right)/2\right\} \tag{14.3}$$

Costs of Asymmetry

In the previous two sections, we have implicitly assumed that asymmetry has no cost (in terms of available resources or energy budget of the cell) for the cell, that is, the molecules that may provide an asymmetry-generating mechanism have already evolved in the cell for other purposes and asymmetry is either a costless by-product of their existence or the costs to the cell of the new interactions that need to be created between those molecules are negligibly small. Now, we relax this simplistic assumption and analyse a case in which the total energy budget of the cell is allocated in an optimal fashion to three categories of physiological functions: maintenance (required budget: e_m), reproduction (required budget: e_r), and asymmetry (required budget: e_a). There is no explicit mathematical formula to deal with this in a general way, so for tractability we only compare symmetry with complete asymmetry ($\sigma = 1$). In other words, we do not calculate the fitness conferred by partially asymmetric segregation strategies (e.g. $\sigma = 0.8$).

We make two further assumptions: (i) the rate of damage accumulation is inversely related to maintenance investments (under constant environmental

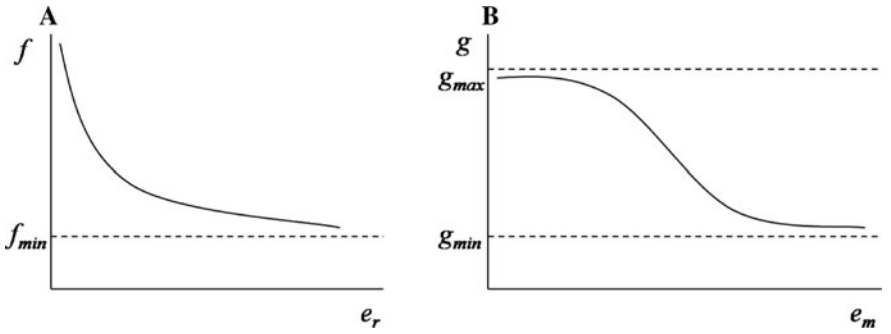

Fig. 14.2 Fundamental relationships stemming from physiological trade-offs. A cell invests in reproduction and maintenance. **a** The proliferation rate increases and thus the cell cycle duration decreases with increasing reproductive investment. The cell cycle can become infinitely long (with little investment in reproduction), but due to physicochemical constraints it cannot be shorter than a certain minimum. **b** The rate of damage accumulation decreases with increasing maintenance investment. The environment is assumed as a finite source of stress such that damage accumulation cannot be infinitely fast, even when maintenance investment is minimal. A minimum rate of damage accumulation is inevitable, irrespective of the level of maintenance investment

conditions), and (ii) the cell cycle duration is inversely related to reproductive investments (Fig. 14.2). Let us represent these two relationships with $g\,(e_m)$ and $f\,(e_r)$, respectively. Therefore, the amount of damage accumulated (and added to the initial damage the cell is born with) during a cell cycle will be $g\,(e_m) f\,(e_r)$.

It can be shown that with a symmetric segregation strategy, every cell in the population survives to reproduce if

$$g\,(e_m) f\,(e_r) < \min\left\{D^*/2, D^* - D\,(0)\right\}$$

Consequently, the highest possible fitness with symmetry is achieved by

$$e_m\,(opt) = \min\left\{e_m : g\,(e_m) f\,(e_r) < \min\left\{D^*/2, D^* - D\,(0)\right\}\right\}$$

Figure 14.3 and the fitness (population size at time t) associated with this optimal strategy will be

$$\text{fitness}_{max} = 2^{t/f(e-e_m(opt))} \tag{14.4}$$

With (complete) asymmetry, the amount of damage in a given cell increases to the time of division and then completely segregates to one of the daughter cells. As a result, one can trace the original cell through generations. When damage reaches the fatal threshold D^*, the cell dies. Therefore, a cell lives for a certain number, l, of cycles that depend on its initial damage, the fatal damage threshold, and the amount of damage accumulated in each cell cycle. We have

$$l = \left[\left(D^* - D\,(0)\right)/g\,(e_m) f\,(e_r)\right],$$

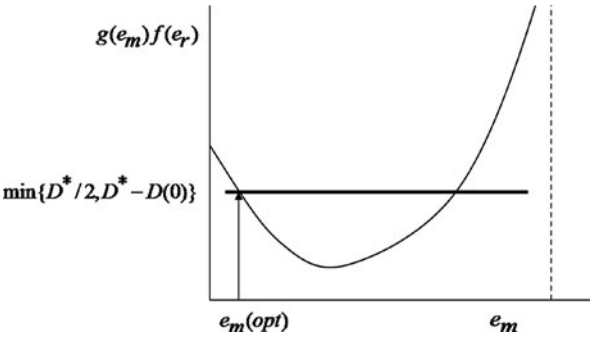

Fig. 14.3 The condition in which symmetry offers its highest possible fitness advantage. Maintenance investment needs to be set at the lowest value for which the amount of damage accumulated during a cell cycle is less than a certain minimum

where $[u]$ is the largest integer smaller than or equal to u. For example, if the accumulating damage kills an initially damage-free cell (i.e. its old daughter cell in this example) during its second cell cycle, l will be 1 and the population size at any time (after the first cell cycle is completed) will be 2. Compared to the symmetric case discussed above, this is a considerably low fitness value. When $l = 2$ (i.e. damage kills a granddaughter of an initially damage-free cell), it can be shown that

$$N(n+1) = 2\left(N(n) - N_d(n)\right), \tag{14.5}$$

where $N(n)$ and $N_d(n)$ denote the total number of living cells and the number of cells that die in generation n, respectively. Further analysis shows that $N_d(n)$ is a Fibonacci number and hence

$$N_d(n) = \left(\phi^{n-1} - (1-\phi)^{n-1}\right)/\sqrt{5}, \tag{14.6}$$

where $\varphi = \left(1+\sqrt{5}\right)/2$. Substituting (14.6) into (14.5) and with some algebraic calculations we have

$$N(n) = 2\left(\varphi^{n+1} - (1-\varphi)^{n+1}\right)/\sqrt{5}$$
$$N(0) = 1, N(1) = 2$$

$N(n)$ in this formula generates delayed (i.e. without the first term in the classical sequence) Fibonacci numbers. There is no general formula for $l > 2$. The sequences corresponding to $l > 2$ are Fibonacci-like sequences in which each term is the sum of its previous l terms, and the first l terms of the sequence are increasing non-negative powers of 2. The growth rate of such sequences is initially higher than that of the simple power sequence derived in (14.4), which will eventually overtake the Fibonacci-like sequences. Therefore, asymmetry pays at small carrying capacities. When asymmetry is rare, it remains rare if the carrying capacity of the population is sufficiently large. It should be emphasised that we have only considered $\sigma = 1$ as asymmetry. Our results might be different with intermediate levels of asymmetry. This is important because although there is currently no known mechanism that generates a well-regulated submaximal level of asymmetry, the

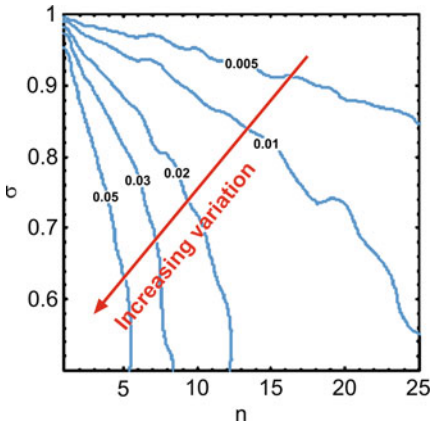

Fig. 14.4 The effects of particle number and segregation fidelity on damage variation. Variance of the proportion of premitotic maternal damage received by d_1, the daughter cell into which damaged particles segregate with a higher probability, is higher for smaller values of n (the number of independently behaving damaged particles in the mother cell immediately before mitosis) and σ, the segregation coefficient. Points located on a given curve exhibit the same level of variance in damage, represented by the number on the curve

fidelity of damaged macromolecules in following complete segregation asymmetry may not be complete. This imperfectness becomes particularly important when the number of independently behaving damaged molecules is small (e.g. aggregation of damaged proteins) and the infidelity of segregation is considerable. The effects of this type of stochasticity are shown in Fig. 14.4. In such conditions, a mechanism which is meant to produce complete asymmetry will actually lead to intermediate levels of asymmetry.

Figure 14.5 compares symmetric cases with asymmetric ones for the relationship between e_m and fundamental properties of the system (i.e. cell cycle

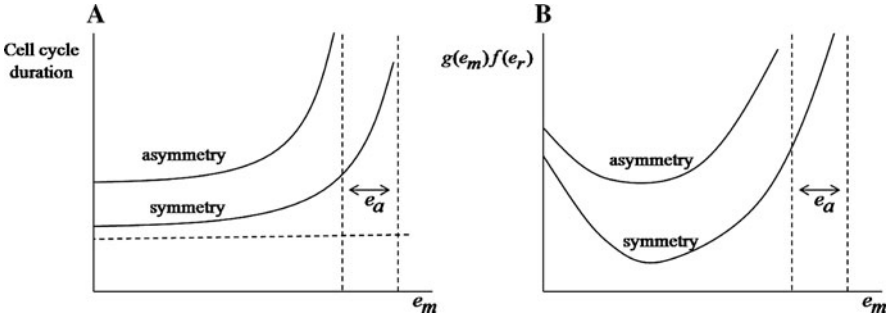

Fig. 14.5 Comparison between symmetry and asymmetry for the relation between maintenance investments and fundamental properties of the system (i.e. cell cycle duration and the accumulated damage per cell cycle). With asymmetry, there are lower amounts of resources available for maintenance/reproduction investment. With asymmetry, the cell cycle duration curve is shifted to the left (**a**) and the damage accumulation curve is transformed and shifted to the left (**b**)

duration and accumulated damage per cell cycle). The costs of asymmetry are inevitably detracted from resources that could otherwise be maximally allocated to reproductive and/or maintenance functions.

Discussion

General Implications of the Results

In the first section of the model, we showed that before degradation evolves, symmetry is an optimal strategy unless damage accumulation during a cell cycle is significantly high. This case is possible with small investments in reproduction, which prolong the cell cycle, and in stressful environments. If resources in such environments are low, cells will not be able to increase the energy allocated to reproduction and the damage caused by stress will accumulate during the (relatively) long cell cycle to dangerous levels. Asymmetry is the only solution in these conditions. In the second section of the model, cells had already evolved degradation mechanisms. As expected, asymmetry evolves less readily in this case. In particular, symmetry is an optimal strategy when the ratio of maintenance to growth investment is sufficiently high (i.e. $g/s > 1/D^*$). When this is not true, the condition derived in (14.3) opposes the evolution of asymmetry. It should be noted that there might be other cases in which symmetry is the optimal strategy. We only derived the conditions leading to two major categories of such cases.

The third section of the model was the most general situation and considered the possibility of asymmetry being associated with certain costs. Potential costs of asymmetry have not yet been identified. Many components of the known asymmetry-generating mechanisms have other functions in the cell. For example, heat shock proteins, a large family of highly conserved and constitutively synthesised molecular chaperones, assist refolding and degradation of misfolded proteins, and may mediate the upstream part of the pathway leading to asymmetric segregation of damage (Bardwell and Craig 1984; Kültz 2003, 2005; Liang and MacRae 1997). Two well-known heat shock proteins that bind to irreversibly damaged proteins are Hsp70 (DnaK) and Hsp104 (ClpB) (Barnett et al. 2005; Erjavec et al. 2007; Hartl 1996; Mogk et al. 1999; Zimmerman et al. 2004). At least some elements required for asymmetry have therefore already evolved and hence natural selection could simply exploit them for a novel purpose without needing to pay additional costs. However, new molecular interactions might have been needed to be established and the costs associated with these inventions remain to be seen. Distal parts of the pathways that move damaged molecules (using the cytoskeleton) seem to be more specifically linked to the asymmetry mechanism. Examples of such components are certain J domain proteins (e.g. rsp1p in *S. pombe* and glsA in the simple multicellular organism *Volvox carteri*) that form a bridge between heat shock proteins and the cytoskeleton (Miller and Kirk 1999; Zimmerman et al. 2004). The costs associated with evolution of these molecules may have well been significant. It has been estimated that the average yeast protein can change its expression only by 0.5%

without a change in energy costs visible to natural selection (Wagner 2005, 2007). The constraint introduced by the limited available energy budget on changes in gene expression become particularly significant at large effective population sizes, where rapid proliferation is tightly coupled to an efficient energy metabolism (Fay et al. 2004; Townsend et al. 2003). Recent studies on budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) suggest that even single amino acid replacements (which might generate asymmetry here) can be subject to natural selection on the basis of their material costs (Bragg and Wagner 2009). We showed that the carrying capacity of the population is a critical determinant of early stages of evolution of asymmetry, i.e. when asymmetry was rare. With abundant resources in the environment, for example, we do not expect high selection pressures for evolution and spread of asymmetry when it is rare.

The condition derived for evolution of asymmetry when it is costly is not uncommon. It is reminiscent of a well-known evolutionary question on origin of life. In competition between a Malthusian replicator (capable of template-mediated self-replication) and a one-member hypercycle (capable of both template-mediated and enzyme-mediated self-replication), large carrying capacities oppose the evolution of Malthusian replicators when they are initially rare (Michod 1999).

Links to Ageing

The intrinsic biology of cellular ageing is closely linked to asymmetric damage partitioning (Ackermann et al. 2003; Johnson and Mangel 2006; Kirkwood 2005). Damage segregation in unicellular organisms is a strategy with several potential advantages including rapid growth (Evans and Steinsaltz 2007; Watve et al. 2006), improved damage handling (Ackermann et al. 2007; Fredriksson and Nyström 2006; Johnson and Mangel 2006), and more resistance against clonal senescence (Erjavec et al. 2008). Interestingly, neither symmetry nor asymmetry is universal. *S. cerevisiae*, *Candida albicans*, and *S. pombe* use, on average, segregation strategies $\sigma = 0.75$, $\sigma = 0.65$, and $\sigma = 0.55$, respectively (Aguilaniu et al. 2003; Fu et al. 2008; Minois et al. 2006). We have recently developed a stochastic model to account for this range (manuscript under review). Three parameters, namely d (damage accumulation rate), μ_i (damage-induced death), and m (proliferation rate), were found to be correlated to asymmetry. The simplest model which was capable of explaining more than 90% variation of the outcome of evolution was composed of d, m, and the interaction term $d \times \mu_i$. Specifically, large values of d and $d \times \mu_i$ and small values of m were significant predictors of asymmetry. The model was stochastic and did not include costs of asymmetry. The following points summarise the results of the stochastic model:

1. The outcome of evolution depends both on organismal and ecological conditions. A significant proportion of individuals in mutation-prone populations do not follow the fitness-maximising level of asymmetry (as related to individual fitness) after the population has reached steady state distributions of segregation strategies.

2. High rates of damage accumulation and severe damage with sufficiently detrimental effects on survival promote the evolution of asymmetry. Mutations that promote asymmetry are particularly favoured in harsh environments.
3. Rapid proliferation reduces the force of selection for asymmetry.
4. Asymmetry might be an alternative strategy to heavy investments in maintenance functions.

Here we provided analytic proofs and considered asymmetry costs. The total energy budget available to the organism (the cell in unicellular organisms) is limited. Accordingly, the disposable soma theory of ageing is built on the trade-offs resulting from resource limitation and concerns the evolutionarily optimised balance between cellular investment in reproduction and maintenance/repair (Kirkwood and Holliday 1979). With high rates of extrinsic mortality in nature, it is not beneficial to put more resources into maintenance functions than are needed for the organism to survive to the time of reproduction. Due to this submaximal maintenance investment, the organism accumulates damage, declines in its physiological functions, and thus ages. While investment in growth, reproduction, maintenance, and repair directly affect the investor, investment in asymmetry does not. It only makes sense when one considers the investor's related kin. The offspring and next generations, rather than the same cell, benefit from asymmetry investment. Nevertheless, the costs (if any) of asymmetry have to be paid by the same cell and from its total energy budget. Perhaps the disposable soma theory needs to be expanded to include asymmetry costs.

Transition to Multicellularity

The results of the model developed here cannot be immediately extended to multicellular organisms. One important reason concerns the definition of fitness. In unicellular forms of life, the cell is the whole organism and so the fitness of the cell is the same as the fitness of the organism. The well-being of the cell is equal to the well-being of the organism and strategies that improve cell survival and/or reproduction are selected for in these simple organisms. This is not the case in multicellular organisms. All cellular strategies have to be tuned during the course of evolution and aligned with the benefit of the organism. Cell-level selfishness in a multicellular organism leads to disruption of cooperative behaviour and pathology (e.g. cancer (Michor et al. 2003)). Cells might even be sacrificed in order for the individual to survive and reproduce, as is thought to occur when intestinal stem cells preferentially undergo apoptosis following low-dose irradiation (Potten 2004). In spite of these issues, asymmetry has found its way into all kingdoms of life. Stem cell division is one example. Damaged proteins targeted for proteasomal degradation are asymmetrically distributed during mitosis in human embryonic stem cells (Fuentealba et al. 2008). Stem cell division in adulthood is the same. Irreversibly damaged proteins in *Drosophila melanogaster* neuroblasts and intestinal crypts of patients with protein folding disease are asymmetrically distributed to one of the daughter cells (Rujano et al. 2006).

Damage may act as a cell fate determinant by at least two ways. Firstly, it reduces the chances for survival. Secondly, it might attract signalling molecules. The generated signal may then activate certain metabolic pathways and eventually lead to altered cellular decisions such as growth and differentiation. Has damage and its asymmetric segregation been utilised by evolution at early stages of multicellularity to promote cellular differentiation and division of labour? Is the efficiency and activity of the maintenance/repair system regulated during embryogenesis in a way that damage levels change at specific times and at specific locations within the growing embryo? How much of the difference between somatic and germ-line protection against damage (e.g. oxidative) can be explained so? These are some of the questions that should be addressed in future research.

Conclusions

We developed a simple model for evolution of asymmetric non-genetic damage segregation in unicellular organisms and investigated the conditions in which asymmetry might be a beneficial strategy to evolve. The main components of the model were protein synthesis, damage accumulation, and damage degradation. The energy requirements of growth, maintenance, and possibly asymmetry were incorporated into the model. We suggest that asymmetry is a fundamental fitness modulator and if sufficiently costly, needs to be considered as part of the trade-offs that arise as a result of resource limitation. The most obvious application of our results concerns the evolutionary origin of ageing and yeast is the best known but only one target for testing the predictions of the present model. The model may be extended to include any form of non-genetic damage. Preferential segregation and accumulation of extrachromosomal rDNA circles (ERC) in the mother cell have been proposed to contribute to yeast mother cell-specific ageing (Sinclair and Guarente 1997). One can choose to think of ERCs as damaged molecules and then use the idea of mother cell bias in asymmetric segregation (Shcheprova et al. 1998) to apply the model. Changes that occur to the asymmetry-related mechanisms at the transition to multicellularity are another interesting direction for future research.

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Appendix

Here we show that with conditions introduced in equation (14.3) in the text, all cells survive under symmetry, that is to say, symmetry is the best strategy. By looking at Fig. 14.1b, we realize that the condition for survival of the cell is

$$D_T < D^*$$

Considering the equation for D_T derived in section "With Degradation", we will have

$$D(0) < s\Delta/g(\Delta - g) + \left[D^* + \Delta(g - s)/g(\Delta - g)\right]\left[s/(s - \Delta)\right]^{g/\Delta}$$

If the right-hand side of the above equation is larger than D^*, it can easily be seen that everybody will survive. For this to happen we need

$$\left[s\Delta/g(\Delta - g) - D^*\right]\left\{1 - \left[s/(s - \Delta)\right]^{g/\Delta}\right\} + \left[\Delta/(\Delta - g)\right]\left[s/(s - \Delta)\right]^{g/\Delta} > 0,$$

which then gives

$$s\Delta/g(\Delta - g) - D^* < \left[\Delta/(\Delta - g)\right]\left[s/(s - \Delta)\right]^{g/\Delta}\left\{\left[s/(s - \Delta)\right]^{g/\Delta} - 1\right\}^{-1} \tag{14.7}$$

The right-hand side of the above equation is larger than 1 for $g < \Delta$. For (7) to hold, we now only require

$$s\Delta/g(\Delta - g) - D^* < 1$$

Equivalently, we need

$$g^2 - \Delta g + s\Delta/\left(1 + D^*\right) < 0$$

This requires (for the above equation to have real roots)

$$s < \Delta\left(1 + D^*\right)/4 \tag{14.8}$$

and

$$\left(\Delta - \sqrt{\Delta^2 - 4s\Delta/(1 + D^*)}\right)/2 < g < \left(\Delta + \sqrt{\Delta^2 - 4s\Delta/(1 + D^*)}\right)/2 \tag{14.9}$$

Putting (14.8) and (14.9) together with $g < \Delta$, we have the conditions derived in (14.3). Note that these are sufficient, but not necessary, conditions.

Note

While this chapter was under review, an interesting article was published by Lindner et al. (2008). The authors demonstrated asymmetric segregation of protein aggregates between the offspring in *Escherichia coli*. Accumulation of the aggregates to the older pole of the cell results in a progressively ageing mother cell (i.e. old-pole progeny) and rejuvenated new-pole progeny. The authors showed that the segregation of protein aggregation is associated with significant loss of reproductive ability

in the old-pole progeny compared to the new-pole progeny. Also, Klinger et al. (2010) showed in another article that the oxidatively inactivated acotinase in budding yeast is distributed between the mother cell and the daughter cell according to volume asymmetry, but the still active part of the enzyme is preferentially segregated to the daughter cell. The authors suggested that this process aids the rejuvenation of the daughter cell.

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Chapter 15
Cellular Ageing and the Actin Cytoskeleton

David Amberg, Jane E. Leadsham, Vasillios Kotiadis, and Campbell W. Gourlay

Abstract For some time the view that the actin cytoskeleton acts primarily as a scaffold, to be assembled in response to a signaling cascade as an end point in the pathway, has prevailed. However, it is now clear that the dynamic nature of the cytoskeleton is linked to downstream signaling events that further modulate cellular activity, and which can determine cell fate. Examples of this lie within the regulation of programmed cell death, the maintenance of homeostasis and the process of cellular ageing. In yeast the actin cytoskeleton has been shown to interact directly with signaling pathways known to be important in the regulation of both ageing and cell death. For example it has been discovered that the level of damage sustained by the actin cytoskeleton under conditions of oxidative stress is directly linked to apoptosis. Further evidence comes from the finding that actin based propulsion mechanisms are required for the inheritance of mitochondria and anti-ageing factors into newly formed cells. In addition to this actin is known to directly influence the formation of protein aggregations. In this chapter we will discuss these points and postulate as to their significance with respect to the maintenance of cellular homeostasis.

Keywords Actin · Mitochondria · Protein aggregation · Oxidative stress · ROS · Cytoskeleton · Ras/cAMP/PKA · Autophagy · Mitophagy

Introduction

The actin cytoskeleton ActinCytoskeleton is a major eukaryotic cellular component that is involved in a variety of essential functions. It is also a crucial part of the signaling networks that link processes such as polarisation, organelle movement, motility and division to environmental signals. Actin has been shown to be a target of proteolytic cleavage by caspases during the process of apoptosis (Mashima et al. 1995), and endures oxidative damage under conditions of cellular stress (see section "Actin as a Target and Effecter of the Oxidative Stress Response" below). In fact recent studies have shown that the actin cytoskeleton has an important role to play in the regulation of cell death pathways in diverse organisms from plant, animal and

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fungal kingdoms (reviewed in Franklin-Tong and Gourlay (2008)). It is therefore of interest to consider the effects of actin damage within the context of cellular activity and the stochastic process of ageing. The budding yeast *Saccharomyces cerevisiae* has proven to be a useful tool in elucidating the role of actin in cell death and ageing, facilitated in part by the degree of conservation that exists with regards to the fundamental principles of actin dynamics. Additionally, as actin in yeast is encoded by a single gene, *ACT1* (Shortle et al. 1982) strains expressing mutant alleles as the sole source of G-actin for filament construction can be generated (Whitacre et al. 2001; Ayscough and Drubin 1996). This is not possible in many higher eukaryotic systems as multiple actin genes are present whose products are known to exhibit functional redundancy.

The Budding Yeast Actin Cytoskeleton

The monomeric (G) and filamentous (F) forms of actin co-exist in a dynamic equilibrium. Actin filaments possess a fast growing, or barbed, and a slower growing, or pointed, end which imparts polarity upon the structure. Actin filaments are assembled in a head to tail manner, with monomers of ATP-bound actin preferentially added to barbed ends of filaments (Pollard 1986). The subsequent hydrolysis of the ATP to ADP + P_i (inorganic Phosphate), promotes a conformational change in the actin filament structure which acts to promote instability. This is also aided by the release of P_i to yield ADP-bound actin at the filaments pointed end (Pollard et al. 2000). Upon dissociation from the pointed ends, resulting ADP-bound actin monomers are manipulated by proteins that co-operate in to replenish the filament assembly competent pool of ATP actin. This is done by promoting the exchange of ADP for ATP on actin monomers released from existing filaments (Moseley and Goode 2006; Wolven et al. 2000). The net addition and dissociation of actin subunits is termed treadmilling and can impart force in the direction of the barbed end. Because the initial step in filament assembly is intrinsically slow (Sept and McCammon 2001) – with the first stable structure being an actin trimer – there is a requirement for factors to promote, and initiate, nucleation. The Arp2/3 complex and members of the formin family (Pollard 2007), are the most well characterised of these nucleation factors (see below). There are also factors that further promote filament nucleation (**N**ucleation **P**romoting **F**actors) (Welch and Mullins 2002), for example Las17p, the yeast homologue to mammalian WASp, is required to stimulate Arp2/3 activity and actin branching (Naqvi et al. 1998). Furthermore, because steady state release of actin monomers from filaments is not sufficient to keep up with physiological requirements, ADP-actin subunits must be dissociated from filament pointed ends at a faster pace with the aid of severing and disassembly inducing factors such as cofilin, Actin Interacting Protein 1 (Aip1p), twinfillin, and Srv2/CAP (Moon et al. 1993; Nicholson-Dykstra et al. 2005). However, in order for cells to exploit actin filaments and their properties, they need to assemble them into higher order structures (Moseley and Goode 2006). In yeast the prominent higher order structures are cortical actin patches, actin cables and the cytokinetic contractile ring.

Cortical Actin Patches

Patches are highly dynamic structures that exhibit rapid movement and facilitate internalisation of early endosomes (Kaksonen et al. 2003) (Fig. 15.1). Studies using electron microscopy (EM) were able to demonstrate that actin-rich structures surrounded invaginations of the plasma membrane before internalisation (Mulholland et al. 1994). The polymerisation of actin to form the patch ultrastructure is considered to be the driving force for generating endocytic vesicles by pulling the membrane inwards whilst also serving as a structural scaffold (Kaksonen et al. 2003; Ayscough et al. 1997; Huckaba et al. 2004). Once formed, vesicles bound to actin patches are driven inwards from the cortex using the force generated by actin polymerisation (Kaksonen et al. 2003). As the vesicle is internalised, actin

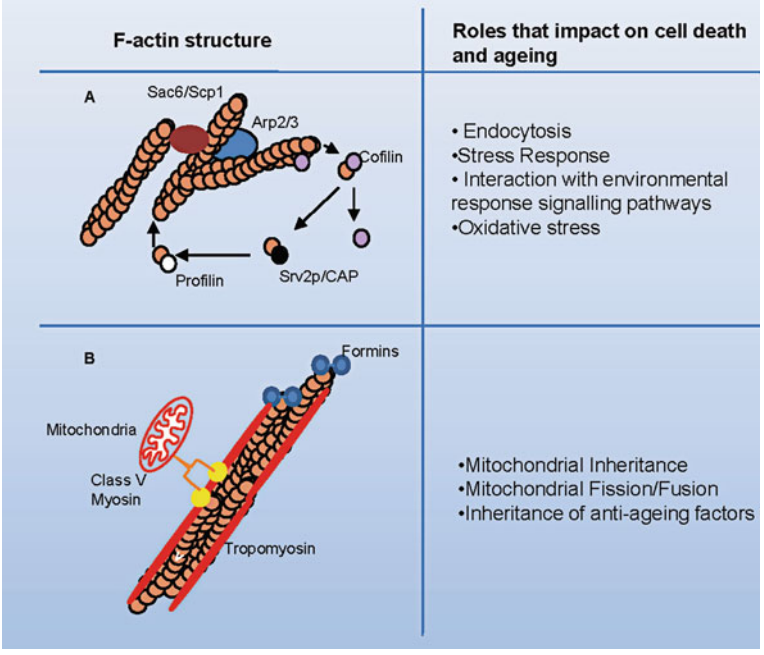

Fig. 15.1 Actin polymerization in cortical patches (**a**) and cables (**b**) and their impact on processes linked to ageing. Actin monomers exist in a dynamic equilibrium and are present in monomeric (G) or filamentous (F) form. F-actin is organized into higher order structures such as cortical patches and cables. These structures perform different tasks within the cell, a number of which contribute to cellular homeostasis and have an impact on ageing and cell death. In patches the Arp2/3 complex nucleates branched networks. The dynamic nature of patches is essential for the process of endocytosis and is facilitated by actin binding proteins such as cofilin, Srv2p/CAP and profilin. Stability in the patches is maintained by the bundling proteins Sac6p and Scp1p (**a**). Actin cables serve as tracks for the movement of organelles by the myosin motor proteins. They are nucleated by members of the formin family and stabilized by the tropomyosins (**b**). Inheritance of mitochondria into the newly forming cell, as well as the retention of damaged proteins within the mother cell relies on their function (Boldogh and Pon 2006)

polymerisation induced by the Arp2/3 complex is inhibited to help promote vesicle release (Smythe and Ayscough 2003, 2006). The release of vesicles requires the motor function of myosins Myo3p, Myo5p as well as additional proteins such as Rvs161p, and Rvs167p that interact with the Arp2/3 complex and F-actin. Mutants defective in these proteins exhibited an inability to achieve scission of endocytic vesicles from the cell cortex (Kaksonen et al. 2005). Once scission of the endocytic vesicles is achieved, the actin patch/vesicle structures are transported to endosomes. This is achieved by a passive transport system utilising the retrograde flow of actin cables from the cell cortex inwards (Huckaba et al. 2004).

Actin Cables

The actin cable network first described by Adams and Pringle (1984) is utilised by yeast cells for polarised growth, organelle segregation, and intracellular transport (Bretscher 2003) (Fig. 15.1). Cables are comprised of shorter actin filaments overlapping each other in a unidirectional conformation of barbed and pointed ends. Maintenance of cell polarity by actin was initially observed in temperature sensitive mutants of the major yeast tropomyosin isoform, *Tpm1* (Pruyne et al. 1998). This was further confirmed by temperature sensitive mutants of the type V myosin, Myo2p, which is required for the movement of cargo in to the growing bud (Johnston et al. 1991). Other factors involved in cable formation and stability such as capping protein (Amatruda et al. 1990), the formin Bni1p (Evangelista et al. 1997), and profilin (Haarer et al. 1990), also confer cell polarity defects when mutated.

The transport and segregation of organelles during bud formation and growth is achieved through the action of type V myosin coupled to actin cables. This has been shown by the GFP tagging of post-Golgi transport vesicles which can be visualised moving towards newly forming bud tips (Schott et al. 2002). Additionally organelles such as the vacuole, nucleus, cortical ER, and peroxisomes have been shown to be transported in a Type V myosin dependent manner to growing buds in *S. cerevisiae* (Pruyne et al. 2004). In addition mitochondria appear to be transported on cables, this phenomena is discussed in detail in section “The Role of Actin in Regulating Mitochondrial Function” of this chapter. The formation of actin cables, unlike patches, relies on the nucleation activity of formins (Evangelista et al. 2002; Sagot et al. 2002). The formin-based initiation of cable assembly was confirmed by studies in which fragments of the formin Bni1p were shown to nucleate actin in vitro (Pruyne et al. 2002). Formin assembly can be regulated by Rho1p, Rho3p, Rho4p, and cdc42p, which are members of the Rho-GTPase family (Dong et al. 2003). Rho3p and Rho4p have been shown to activate formins under normal growth, whereas Rho1p does so during stress conditions. Interestingly, although Cdc42p is not required to initiate cable filament assembly, its absence leads to improper cable assembly organisation during bud growth.

Once nucleated, actin filaments need to be organised in order to form stable cable ultrastructures. Actin cables are targeted specifically to polarity sites which are used to co-ordinate cables during bud formation and growth (Casamayor and Snyder 2002), as well as within other cellular events such as endocytosis and exocytosis (Pruyne and Bretscher 2000). Polarity sites are essentially assemblies of proteins that direct cell growth by and comprise components such as Spa2p, Pea2p, and Bud6p that can regulate both the actin cytoskeleton and signalling pathways involved in polarisation (Sheu et al. 1998).

The observation that actin cables are highly dynamic suggests that the usual factors involved in actin turnover should be present. Indeed studies implicate cofilin together with Aip1p in cable filament turnover (Okada et al. 2006). The current model suggested is a mechanism by which Aip1p, cofilin, and tropomyosin cooperate in order to appropriately regulate cable filament turnover, and steadily "prune" them along their length. In order to observe actin cable dynamics in real time, yeast cells with an Abp140p-GFP fusion were generated (Yang and Pon 2002). Studies utilising Abp140p-GFP have revealed that one end of each cable is associated with the budding tip or the mother cell neck region. Polymerisation then pushed filament growth into the mother or bud. The force driving this movement of cables maybe solely due to the polymerisation of actin, however recent studies have implicated myosins in actin cable dynamics (Huckaba et al. 2006). This study demonstrated reduced rate of cable transfer with the loss of the Myo1p motor activity.

Contractile Ring

During cytokinesis in *S. cerevisiae*, the bud is separated from the mother cell by the combined action of the contractile ring (which is principally comprised of actin and myosin) and the formation of a septum at the mother daughter cell interface. Although Adams and Pringle (1984) had observed the clustering of actin on the bud neck, it was not confirmed until much later that the actomyosin rings contracts specifically during cytokinesis to facilitate separation (Bi et al. 1998; Lippincott and Li 1998). Septins are the first structural components to be assembled, comprising Cdc3p, Cdc10p, Cdc11p, and Cdc12p and form a scaffold for the actin ring (Longtine et al. 1996). The septin ring recruits factors such as myo1p, formins and IQGAP (IQ motif containing GTPase activating protein) proteins which facilitate the formation of the actin ring (Lippincott and Li 1998). Once all of the essential components (e.g. organelles, genetic material, etc) are transported into the forming daughter cell, it must be separated from the mother through the concerted action of the actomyosin ring and septum formation (Bi et al. 1998). To achieve this, the actomyosin ring constricts driving the formation of the septum, which provides a physical diffusion barrier for both mother and daughter cell until membrane and cell wall synthesis is complete.

Actin Cytoskeletal Function and Toxicity

Nutritional Sensing and Stress Signaling

The actin cytoskeleton is highly responsive to environmental change. In yeast this can be visualized when cells are subjected to a range of stressful conditions such as glucose withdrawal, heat shock, osmotic shock or centrifugal force. Under these conditions dividing cells, which usually exhibit a polarized actin cytoskeleton (Fig. 15.2), rapidly de-polarise their actin patches throughout the cell. This phenomenon can also be seen under conditions of nutritional depletion when cells enter the diauxic phase of growth (Fig. 15.2). Recent studies have demonstrated that during the diauxic shift the dynamic status of the actin cytoskeleton is linked to the activity of the Ras/cAMP/PKA signaling pathway, which co-ordinates cell growth and proliferation with sensation of the nutritional environment (Rolland et al. 2002; Thevelein and de Winde 1999). Within this signaling cascade, production of the secondary messenger cAMP is carried out by the adenylate cyclase, Cyr1p, and can be stimulated by either the G protein coupled receptor GPR1-GPA2 system, or through binding of GTP-bound Ras and adenylyl cyclase-associated (Srv2p/CAP) proteins.

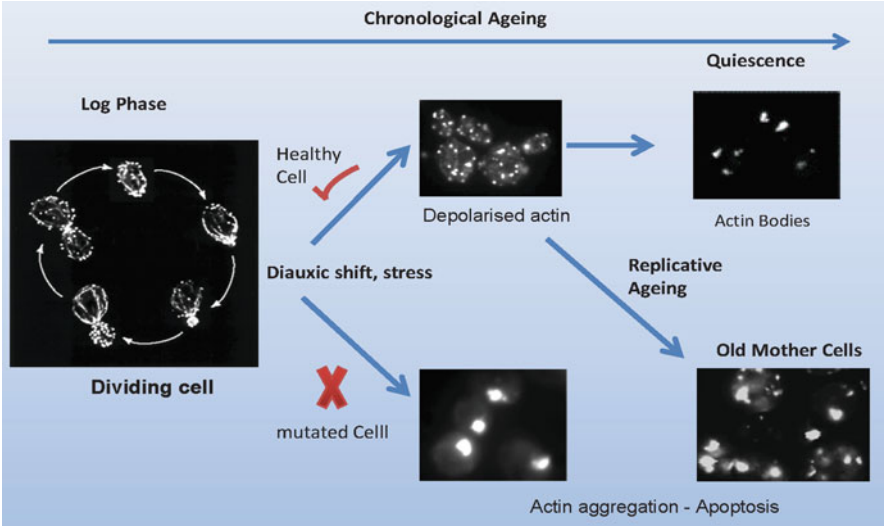

Fig. 15.2 F-actin re-arrangements in stressed, apoptotic and ageing cells. During the cell cycle actin structures are polarized to facilitate the movement of new cell material to the growing cell. Actin patches are found to accumulate in emerging buds and actin cables direct movement of organelles and vesicular cargo towards the new cell. The sensation of cellular stress, or nutritional depletion, is accompanied by depolarisation of actin patches and a reduction in the prominence of actin cables. As cells chronologically age actin forms into "bodies" which are aggregations of fully functional actin. These bodies act as reservoirs of actin that are used to rapidly engage cell growth when nutrition returns. In old cells, or in mutants with reduced actin dynamics, actin aggregates form spontaneously and can induce apoptosis

Elevation of cAMP levels leads to dissociation of the protein kinase A (PKA) regulator Bcy1p to yield active A kinases, which elicit alterations in processes such as cell cycle progression and stress responses. There are three A kinase catalytic subunits in yeast, encoded by *TPK1*, *-2*, and *-3*, which display overlapping and separable functions in response to activation by cAMP. The sensation of nutritional depletion leads to a reduction in Ras/cAMP/PKA pathway activity, allowing effective cell cycle exit and initiation of the cellular stress response. Published data suggests that the activity of the Ras/cAMP/PKA pathway is linked to mitochondrial activity as cells expressing the constitutively active $RAS2^{ala18val19}$ allele exhibit an increase in respiration rate and elevation of ROS levels (Heeren et al. 2004; Hlavata et al. 2003). Our work has shown that the dynamic status of the actin cytoskeleton can act to regulate the activity of Ras. We were able to demonstrate Ras hyperactivity in mutants that exhibit reduced actin dynamics, leading to the accumulation of aggregates of F-actin, mitochondrial dysfunction resulting in an apoptotic cell death. It was concluded that the inappropriate activation of Ras was indeed induced as a result of the formation of F-actin aggregations. This conclusion was drawn as the addition of an actin binding drug, Latrunculin A, at concentrations which prevented the formation of aggregates also prevented ROS accumulation and cell death (Gourlay and Ayscough 2005, 2006). F-actin aggregations were shown to require the activity of the protein Srv2p/CAP, a highly conserved actin regulatory protein that binds preferentially to ADP–G-actin via its C-terminal domain (Balcer et al. 2003; Mattila et al. 2004). Srv2p/CAP can also associate with actin filaments through an interaction between a proline region and the SH3 domain of actin binding protein 1 (Abp1p) (Freeman et al. 1996). This protein is a good candidate to link Ras signaling to actin reorganization as The N terminus of Srv2p/CAP can bind to adenylate cyclase (Cyr1p) and facilitate cAMP/PKA activation (Gerst et al. 1991; Mintzer and Field 1994). Actin aggregation that triggered Ras/cAMP/PKA pathway activity was shown to require only the C-terminal actin binding region of Srv2p/CAP, however presence of the N-terminal cyclase binding domain enhanced PKA stimulation leading to higher levels of ROS production. The accumulation of ROS induced damage and subsequent death in actin aggregating cells was demonstrated to require the activity of the PKA subunit, Tpk3p, an enzyme known to play an important role in mitochondrial function. Yeast lacking Tpk3p exhibit altered mitochondrial enzymatic content, including reduced levels and activity of cytochrome c, a constituent of the electron transport chain (Chevtzoff et al. 2005). Our unpublished data suggests that Tpk3 acts to suppress respiration during growth on fermentable carbon sources such as glucose, and that excessive Tpk3 activity leads to a dysfunctional electron transport chain that is prone to producing ROS in large amounts which in turn accelerates the ageing process and promotes cell death.

The appearance of aberrant actin formations in ageing and diseased cells, such as Hirano bodies and ADF/cofilin rods have been reported in neuronal populations from patients suffering from neurodegenerative diseases such as Huntington's and Alzheimer's. Hirano bodies are para-crystaline F-actin rich structures that contain a

number of associated proteins (Davis et al. 2008; Fechheimer et al. 2002; Maselli et al. 2002, 2003). Recent studies have concluded that the presence of Hirano bodies does not have an immediate toxic effect, however it may be the case that their presence or persistence leads to the disruption of actin-dependent processes. This in turn may have a detrimental effect on actin dependent signaling mechanisms under conditions of stress. In light of the evidence we propose that stabilization of actin structures can influence the apoptosis decision making process (Franklin-Tong and Gourlay 2008).

The budding yeast *S. cerevisiae* provides an interesting model system in which to study the effects of actin aggregation on cellular homestasis. Recent studies from Sagot and colleagues have investigated the nature of the F-actin cytoskeleton in chronologically aged, or in other words quiescent culture (Sagot et al. 2006). This research found that the actin cytoskeleton enters into what can be described as a holding state during quiescence (Fig. 15.2), presumably in order to provide a reservoir of actin that can be utilised by the cell when fresh nutrition becomes available. Indeed the re-introduction of fresh media was shown to lead to the breakdown of actin bodies in yeast cells and the rapid re-establishment of a dynamic cytoskeleton (Sagot et al. 2006). Although the mechanisms by which actin "bodies" form remains unclear, it appears to be largely a result of the reduction of the dynamic state of the cytoskeleton. It may be the case that efficient exit from quiescence requires the correct assembly of actin bodies, and that the accumulation of actin aggregates in ageing, or oxidatively stressed cells that abrogate signalling mechanisms represent a phenomenon that contributes to disease in higher organisms. Recent research from our group has tested this hypothesis using actin disrupting drugs, or mutant strains that modulate the dynamic status of the actin cytoskeleton. Actin aggregates can be induced to form in yeast through disturbance of an actin regulatory function, or by the treatment of cells with actin stabilizing drugs such as Jasplakinolide. It has recently been discovered that the stabilization of cortical actin structures induces apoptosis in yeast (Gourlay et al. 2004). A similar phenomena can also be observed in many mammalian and plant cells (Franklin-Tong and Gourlay 2008). Mutations in actin regulatory proteins that lead to the accumulation of aggregates of stabilized F-actin have been shown to trigger a process termed Actin mediated apoptosis (ActMAp) (Gourlay and Ayscough 2005, 2006; Gourlay et al. 2004). In actin aggregating cell lines, ActMAp leads to a loss of mitochondrial membrane potential and the production and release of ROS into the cell which results in an apoptotic cell death. Interestingly, mutations that lead to an increase in the dynamic nature of the actin cytoskeleton were shown to result in reduced levels of ROS (Gourlay et al. 2004). In addition, deletion of a gene encoding the actin bundling protein Scp1p, the yeast homologue of mammalian SM22/transgelin, which also destabilises cortical actin structures, reduced ROS levels and led to a significant increase in replicative lifespan (Gourlay et al. 2004). These data suggest that in yeast, control of actin dynamics is linked to processes that regulate mitochondrial function and in turn impact on lifespan.

Protein Aggregate Formation

A common observation in a variety of ageing related and neurodegenerative disorders is the accumulation of protein aggregates. Although there is widespread debate, it is generally thought that the formation, propagation and persistence of protein aggregations, such as those caused by the presence of expanded polyglutamine (Poly-Q) tracts, is important in the progression or onset of diseases such as Huntington's and prion associated encephalopathy. A current popular hypothesis is that the initial oligomers, or "seeds" harbour the highest potential for toxicity, while the consequential larger protein aggregations may be inert or even offer a protective role for the cell. It has long been the counter argument that aggregations may represent a method by which cells can safely store unfolded polypeptides and thus protect cells from potential disruption. Intriguingly, evidence derived from research on the budding yeast *S. cerevisiae* demonstrates a role for the actin cytoskeleton, and associated machinery important for endocytosis, in the formation of a variety of protein aggregates implicated in human disease (Bailleul et al. 1999; Meriin et al. 2003). A fragment of human Huntingtin followed by the normal 25-Q repeat failed to aggregate when expressed in yeast, but one containing a 103-Q region readily aggregated. The expression of 103-Q in yeast also proved to be toxic in yeast cells, while the presence of 25-Q had no effect. Therefore yeast cells form Poly-Q length dependent aggregates in vivo that are associated with an increase in cellular toxicity, analogous to that observed in higher eukaryotes. Interestingly the cell death associated with 103-Q aggregate formation was shown to exhibit a number of phenotypes associated with apoptosis (Sokolov et al. 2006). Research points to a conserved role for actin and the endocytosis machinery in the regulation of prion and poly-Q aggregate formation (Bailleul et al. 1999; Meriin et al. 2003). For example in yeast, the actin patch and endocytosis regulator Sla1p had been shown to interact with an extended Poly-Q sequence by yeast 2 hybrid studies (Bailleul et al. 1999). In addition the deletion of a large number of genes involved in the regulation of the actin cytoskeleton and endocytosis were found to promote an increase in sensitivity to the presence of Poly-Q aggregates (Meriin et al. 2003). The close relationship between these two processes is highlighted by the discovery that Poly-Q aggregate formation also leads to a severe disruption of endocytosis (Meriin et al. 2003). 103Poly-Q aggregates formed in yeast were shown to contain several proteins known to be involved in the regulation of endocytosis, namely Pan1p and the E3 ubiquitin ligase Rsp5p, as well as filamentous actin. The apparent interaction between Poly-Q aggregates and the actin/endocytosis machinery led to the suggestion that the loss of endocytic function may underlie the associated cellular toxicity. Interestingly the formation of 103-Q aggregates in a mammalian cell culture model also led to defects in endocytosis, suggesting some conservation of the underlying mechanism. As many of the proteins involved in endocytosis have been conserved, then this may well be the case, promoting yeast as a useful model in which to study the toxicity associated with PolyQ aggregation. This likelihood is further reinforced by the fact that huntingtin has been shown to localise to sights of endocytosis and

interact with components of the endocytic machinery (Velier et al. 1998; Metzler et al. 2001; Singaraja et al. 2002). Further evidence to support a common mechanism comes from experiments that show that in both yeast and human cells the disruption of actin with the monomer sequestering drug Latrunculin-A leads to an increase in the presence of poly-Q aggregates. Interestingly recent evidence suggests that the removal of aggregated proteins from the daughter cell into the mother relies on a functional actin cytoskeleton (Liu et al. 2010). It is therefore likely that studies in yeast will yield important information as to the role that actin dynamics and endocytosis plays in the regulation of protein aggregation in higher eukaryotes.

Yeast may also prove a good model for understanding the effects of toxic protein aggregates on the function of mitochondria, whose tightly regulated activity is essential to the well being of the cells (see below). Reduced movement of mitochondria is seen in injured neurons where damaged mitochondria cluster around the site of injury preventing the arrival of healthy mitochondria which can lead to neuronal degeneration. This is similar to what is seen in Alzheimer's disease where axonal swelling may block mitochondrial movement. The presence of huntington aggregates have also been shown to impair mitochondrial movement in neuronal cells (Chang et al. 2006). In yeast it has been shown when the actin cytoskeleton was disrupted by polyQ aggregate formation, reduced mitochondrial respiration and increased ROS production were observed (Solans et al. 2006). This demonstrates a link between protein aggregation, the integrity of the actin cytoskeleton and mitochondrial function. Further studies may lead to insights into the interplay between protein aggregation, actin integrity and mitochondrial function that impact upon diseases such as Huntington's, Parkinson's and ALS.

The Role of Actin in Regulating Mitochondrial Function

It has been speculated that the presence of mitochondria in eukaryotes originates from engulfment of aerobically respiring bacteria 1.5×10^9 years ago. The resulting symbiotic relationship gave eukaryotes efficient energy production in return for the use of its protein synthesis machinery (Gray 1993). Thus mitochondria have evolved from a common ancestor, and therefore, we may expect that some common mechanisms used by eukaryotic cells for maintaining, controlling and organising mitochondria may be conserved, despite the now large diversity of eukaryotic cell types from unicellular to multi-cellular organisms.

The primary function of mitochondria in cells is the production of ATP via oxidative phosphorylation. However other mitochondrial functions include synthesis of lipid, heme, some amino acids and nucleotides. Mitochondria are also clearly important organelles within programmed cell death pathways and have been broadly promoted as important factors in the process of cellular ageing. The maintenance and movement of mitochondria to facilitate function requires the presence of a transport network. The cytoskeleton is ideally placed to carry out this function. Good examples of this can be found within the transport and compartmentalization of mitochondria within nerve cells. Within neurons there is the need for elevated

ATP production at sights of high electrical signal transduction (Chang and Reynolds 2006). Mitochondria need to be concentrated in these areas due to the poor diffusion of ATP in the cytoplasm. Mitochondria must therefore be moved to and from the cell body effectively (Chang and Reynolds 2006; Davis and Clayton 1996). The microtubular molecular motors kinesin (plus end directed) and dynein (minus end directed) appear to be responsible for anterograde and retrograde transport of mitochondria respectively. However, there is some evidence of actin based transport in neurons which it is proposed may mediate transport over short distances (Morris and Hollenbeck 1995). Actin has also been implicated in responsive mitochondrial localisation (Morris and Hollenbeck 1995; Evans and Bridgman 1995). Chada and Hollenbeck (2004) showed that mitochondria localise in response to nerve growth factor (NGF) along an axon. In these experiments cells pre-treated with latrunculin-B, which abolishes F-actin, failed to accumulate mitochondria in response to NGF (Chada and Hollenbeck 2003, 2004).

In yeast actin dependent movement of mitochondria has also been demonstrated. Initial evidence was achieved using temperature sensitive actin mutants which lose actin cables at the non-permissive temperature, concomitantly the mitochondria were seen to aggregate (Lazzarino et al. 1994). Live cell imaging of fluorescently labelled mitochondria and actin cables has also revealed that mitochondria are actively moved around the cell by the cytoskeleton (Fehrenbacher et al. 2004). In yeast binding of mitochondria to actin fibres requires a complex of linking proteins that can form a bridge between the actin cable and the mitochondrial outer membrane. The mitochore is a complex of three mitochondrial transmembrane proteins (Mdm10p, Mdm12p and Mmm1p) (Boldogh et al. 2003). Mdm10p/12p are located in the outer mitochondrial membrane while Mmm1p transcends both the inner and outer mitochondrial membranes, probably interacting with the mtDNA nucleoid in the matrix. A *Δmmm1* strain prevented binding of mitochondria to actin (Boldogh et al. 1998) and mutants in Mdm10p/12p also show loss of antero and retrograde mitochondrial movement. This structure has also been implicated in the stability of mtDNA. mtDNA nucleoids in a temperature sensitive *mmm1-1* mutant collapsed into aggregates and inheritance of mtDNA was compromised (Boldogh et al. 2003). The Arp2/3 complex, which binds to actin fibres and nucleates actin branching links actin to mitochondria via two accessory proteins Puf3p and Jsn1p. These proteins interact with the mitochore and loss of these genes effects mitochondrial morphology and movement, presumably leading to unequal energy distribution within the cell (Garcia-Rodriguez et al. 2007).

For some years a focus of research has been to determine the driving force which pushes mitochondria along the actin fibre during anterograde movement. In mammalian systems actin motor proteins, myosins, are involved. Until recently the class V myosin Myo2p had been implicated in mitochondrial inheritance into the bud tip and retention of mitochondria in the new daughter cell (Boldogh et al. 2004). Myo2p and its associated myosin light chain (Mlc1p) has now been shown to be required for mitochondrial binding to actin in vitro. A reduction of Myo2p or Mlc1p activity results in a loss of anterograde mitochondrial movement, inheritance in to the bud tip and reduced mtDNA content (Boldogh et al. 2004).

Inheritance of Mitochondria and Anti-ageing Factors

An important function of the actin-mitochondrial bridge is the inheritance of mitochondria into buds, which appears to be selective for healthy mitochondria (Klinger et al. 2010) and essential if the daughter cell is going to be born at a young age. Boldogh and Pon have suggested a model by which the actin cytoskeleton could be used to achieve inheritance of the fittest mitochondria (Boldogh and Pon 2006). Normally functioning mitochondria produce a membrane potential by pumping protons from the matrix to the intermembrane space. This proton motive force drives ATP generation. It also is required for the correct import of nuclear encoded and cytoplasmically synthesised mitochondrial proteins. A number of these proteins appear to be involved in the complex required for the interaction with the myosin motor required for anterograde transport into the daughter cell. Lack of these proteins, it is postulated, would leave the effected mitochondria either attached to the actin fibre but unable to be moved in the anterograde direction, or they would fall off the actin fibre thus the mitochondria would be unable to undergo retrograde transport and would remain in the mother cell. Thus the actin cytoskeleton may be able to function as a quality control mitochondrial sorter during cell division. Newly emerging cells have also been found to possess a greater capacity to reduce ROS levels than their progenitor mother cells. The mechanism of daughter cell ROS clearance was shown to utilise the actin cytoskeleton and the function of the Sir2 protein, a member of the Sirtuin family that have been shown to be a key regulators of aging in a variety of organisms (Longo and Kennedy 2006; Blander and Guarente 2004). Treatment of dividing cells with the actin disrupting drug Latrunculin-A prevented the protection of newly forming cells from ROS accumulation, implicating actin in this process. Therefore actin is involved in the segregation of mitochondria and ROS detoxification mechanisms into newly forming yeast cells, further strengthening the suggestion that this part of the cytoskeleton is important in the regulation of processes linked to cellular ageing.

Actin and Mitophagy

The distribution and quality control of mitochondria is important for the healthy lifespan of dividing cells. Despite inbuilt detoxification systems mitochondria are exposed to the danger of ROS induced damage, which in turn may lead to the accumulation of damaged mitochondria and threaten cell health. The maintenance of a healthy population of mitochondria is ensured by a specialized autophagic mechanism called mitophagy (Kanki and Klionsky 2008). A set of approximately 20 ATG (autophagy) genes have been identified in yeast, those involved in selective degradation of mitochondria ATG9 and ATG11 require a functional actin cytoskeleton. This has been observed in the *act1-159* mutant, which exhibits reduced ATPase activity and so a less dynamic cytoskeleton. The reduced dynamic status of this mutant leads to aberrant localisation of Atg11p, and to defects in Atg9p cycling and the Cytosol to vacuole targeting (Cvt) pathway (He et al. 2006). Atg9p localises

to the mitochondria and interacts with Atg11p which appears to target them in an actin dependent manner to the pre-autophagosomal structure, which then fuses with the vacuole (He et al. 2006). A further study observed the movement of Atg9 using real-time fluorescence microscopy in living cells (Monastyrska et al. 2008). A component of the Arp2/3 complex, Arp2p, was found to co-localise with Atg9 and to regulate the dynamics of Atg9 movement. The authors proposed that the Arp2/3 complex and actin are involved in the regulation of Atg9 transport for specific types of autophagy (Monastyrska et al. 2008).

Maintenance of mitochondria in peak condition and at optimal levels is vital to the cells well being. The research outlined above clearly suggests that the accumulation of damaged mitochondria and a failure to remove these aberrant organelles is linked to the integrity of the actin cytoskeleton. This relationship places actin at the centre of cellular homeostasis and processes that are tightly linked to the ageing process. In addition actin is directly influenced by the oxidative burden on a cell (discussed in the next section), so setting up a positive feedback loop that may contribute to cellular decline during the ageing process.

Actin as a Target and Effecter of the Oxidative Stress Response

The Relevance of Actin Oxidation in Mammalian Cells

It has been known for some years that in mammalian cells the actin cytoskeleton is regulated by oxidative stress and can be a target of oxidative damage, in particular through the oxidation of its cysteine residues (Dalle-Donne et al. 2001). Most of what we know about the cellular response of mammalian cells to oxidative stress come from studies of cell lines in culture treated with an exogenous source of reactive oxygen species (ROS), usually H_2O_2, menadione, or diamine. Despite the fairly diverse origins of these cells lines the responses are fairly common and include membrane blebbing, a rise in intracellular calcium levels, cell rounding with loss of cell adhesion, the formation of phalloidin stainable actin aggregates, and a general increase in cellular F-actin content (Mirabelli et al. 1988; Hinshaw et al. 1986, 1988; Omann et al. 1994). Although the term aggregates is commonly used, this is misleading as there is no reason to believe that these actin structures contain unfolded actin, quite the opposite: since they stain with phalloidin they must be constructed of actin filaments. In fact, in one case electron microscopy suggests that these filaments are well organized into parallel bundles of actin filaments (Hinshaw et al. 1988).

Examination of actin from oxidatively stressed cells on non-reducing gels shows the presence of molecular weight species consistent with the formation of dimers and even higher molecular weight forms (Mirabelli et al. 1989; Bellomo et al. 1990). These are presumed to reflect disulfide cross-links since in reducing gels the actin of the same samples run as a single species of monomer size. Interestingly, from menadione treated hepatocytes a faster migrating form of G-actin can be observed in non-reducing gels. We have made a similar observation for purified

yeast actin and have shown that the faster migrating form has a C285-C374 intramolecular disulfide bond (D. Amberg, unpublished observations). In addition to disulfide cross-links, several studies have reported the glutathionylation of actin on cysteine 374. In one case glutathionylation occurs in response to oxidative stress (Rokutan et al. 1994), in the second case de-glutathionylation occurs upon epidermal growth factor stimulation of quiescent human epidermal A431 cells (Wang et al. 2000) while in the third case it is induced by activation of the respiratory burst in human neutrophils (Chai et al. 1994). In general, it appears that glutathionylation of actin on Cys374 does not favor filament assembly or stability. Glutathionylated actin prepared in vitro has increased ATPase activity, an increased critical concentration and forms filaments that are sensitive to shear stress (Drewes and Faulstich 1990; Stournaras et al. 1990; Dalle-Donne et al. 2003). These results agree with in vivo observations of EGF-induced de-glutathionylation of actin that is followed by an increase in F-actin content (Wang et al. 2001). My laboratory has been unable to detect glutathionylation of yeast actin in vivo (D. Amberg, personal observations) and therefore it will not be discussed further in this review.

The properties of actin filaments assembled from oxidized actin subunits provide insight into ROS-induced alterations in the organization of the actin cytoskeleton. For example, treatment of actin in vitro with H_2O_2 renders 2 of the 5 cysteines nonreactive to maleimide and the resulting actin has a slower rate of polymerization, a longer lag phase, an increase in critical concentration and the resulting filaments do not cross-link well with proteins such as filamin (Dalle-Donne et al. 1995). Disulfide bonded actin dimers generated in vitro have been shown to assemble into F-actin and induce filament cross-links (Tang et al. 1999), while in other cases H_2O_2 treatment of actin was found to induce C374-C374 dimers that could not assemble into actin filaments (Faulstich et al. 1992), could not interact with profilin and induced the disassembly of existing actin filaments (Lassing et al. 2007).

Perhaps the best understood, and most relevant example of actin oxidation is the C285-C374 intra-molecular disulfide bond that has been found to accumulate in the actin of sickle red blood cells. In many ways, sickle cell anemia is a disease of oxidative stress (Hebbel et al. 1982); the mutant hemoglobin releases free Fe^{++} that in Fenton reactions can react with oxygen to generate high levels of ROS (Hebbel et al. 1988). For this reason, RBCs of sickle cell patients are in a state of chronic oxidative stress that is compounded by low levels of reduced glutathione (Lachant et al. 1983). In sickle cell patients that are in crisis, a form of the sickle RBC accumulates called the irreversibly sickle red cell (ISC) that is locked into the sickle shape (Kaul et al. 1983). The ISCs are of interest as they are believed to contribute to vaso-occlusion because of their unusual lack of morphological plasticity. This lack of plasticity was shown to be due to oxidative damage to the membrane cytoskeleton (Lux et al. 1976), specifically the accumulation of C285-C374 intra-molecular disulfide bonded actin (Shartava et al. 1995; Bencsath et al. 1996) such that it accounts for upwards of 90% of the actin in these cells. When purified, this form of oxidized actin was found to form filaments that disassemble unusually slowly and incompletely (Shartava et al. 1997) thereby explaining the lack of morphological plasticity of these cells. These studies are particularly relevant in that cysteines

285 and 374 are universally co-conserved in all actin isoforms, including yeast actin suggesting that this form of actin oxidation is likely to occur in all eukaryotes.

Actin Oxidation in the Yeast Model

Compared to work in mammalian cells, analysis of the roles of actin oxidation in yeast is in its infancy. However, the system has much to offer and that has allowed new insights to be made in a relatively short time frame. First of all, yeast (*S. cerevisiae*) has a single, essential ß-actin gene. This fact coupled with the ease of genetic manipulation means that oxidation resistant mutants can be rapidly made in the normal chromosomal locus and their phenotypic effects quickly analyzed. Furthermore, yeast actin is highly conserved with 87% identity and 100% similarity to human ß-actin. Yeast actin has 4 of the 6 cysteine residues found in human actin and these are the most highly conserved cysteine residues within the family of conventional actin isoforms: C17 and C217 are found in nearly all actin isoforms while C285 and C374 are universally co-conserved. Given the rather devastating effects of C285-C374 oxidation in sickle cell crisis, it begs the question as to why natural selection would favor retention of these cysteine residues. Mutation of either or both C285 and C374 to alanine in yeast is well tolerated under normal growth conditions (Haarer and Amberg 2004) and when these mutants are purified they display normal polymerization behavior (unpublished). Therefore, these cysteines do not appear to be involved in core actin functions but have perhaps been retained for the purpose of regulation.

Recently, we have found evidence that actin's cysteines function in part to help protect cells from acute exogenously applied oxidative stress. Treatment of yeast with H_2O_2 induces a rapid change in the organization of the actin cytoskeleton: actin cables rapidly disappear, cortical patches cease to move and begin to fuse to form large foci of F-actin that we refer to as oxidized actin bodies or OABs. In agreement with a protective role, mutation of actin's cysteines blocks the formation of the OABs and makes the cells very sensitive to exogenous oxidative stress. Therefore, it appears that a function of actin's cysteines is to protect the cells from severe oxidative stress by sequestering actin and associated proteins into OABs. We are currently unsure of the protective mechanism of the OABs but it may relate to the prevention of continued polarized cell growth and/or endocytosis until the cell is able to repair oxidative damage to its protein, lipid and nucleic acid constituents (D. Amberg, unpublished observations).

Lastly, although the data is preliminary at this time, an apparent actin redox cycle may act as a ROS buffer. Untreated cultures expressing either the C285A or C374A actin mutants have slightly increased numbers of ROS positive cells and elevation of endogenous ROS levels by up-regulation of respiration has a strong negative impact on the growth of C285A or C374A mutants versus wild type cells. This model is attractive as actin is a very abundant protein in most if not all cells and could therefore have a very high ROS buffering capacity. At this time no one knows what systems keep actin reduced but we have found some evidence that in yeast the

glutaredoxins may also contribute to actin redox regulation. The glutaredoxin system is well conserved and known to participate in the reversal of oxidation induced protein disulfide bonds (Herrero et al. 2010). Furthermore, reduced glutaredoxins are replenished by reduced glutathione which is scarce in oxidatively stressed sickle RBCs thereby possibly explaining the accumulation of C285-C374 actin in ISCs.

Actin is an abundant and arguably the most functionally diverse protein found within cells where it participates in a myriad of cytoplasmic processes as well as core nuclear functions (Bettinger et al. 2004). Therefore, it is not surprising to find that it may play an important role in the yeast oxidative stress response. Although it is yet to be determined if the same is true in mammalian cells, it seems likely to be so given the high amount of functional conservation within the cytoskeleton. Our current model for actin's roles in the oxidative stress response is that under normal stress conditions, the metabolic production of ROS can lead to actin oxidation but this is rapidly reversed by the action of cellular redox systems and this actin/redox system is partly responsible for the inactivation of ROS in these cells. Under extreme conditions of oxidative stress, most of the actin is trapped into a non-dynamic and protective pool of F-actin aggregates (the oxidized actin bodies). These OABs assist in allowing at least a subset of cells to survive the oxidative insult.

Future Perspectives

The importance of cytoskeletal damage and the downstream consequences for cell populations, or tissues, is likely to be of particular importance during the process of yeast ageing (summarized in Fig. 15.3). This conclusion is drawn from the fact that actin is sensitive to the oxidative damage that occurs as a consequence of cellular ageing and that the dynamic status/damage level of the cytoskeleton appears to modulate downstream signaling events. It also seems to be clear that cell fate is linked to the stability of the cytoskeleton, with actin's dynamic nature being closely linked to apoptosis in plant, fungal and animal kingdoms. The close relationship that appears to exist between actin and the function of mitochondria is likely to play a crucial role. As this relationship appears to have been maintained in divergent eukaryotic systems, further studies in yeast should help to further our understanding as to how actin and the mitochondria communicate. Perhaps, as rapid actin dynamics are reliant on high cellular levels of ATP, there exists energy based signaling between these systems. Additionally, actin offers structural and positional support to the mitochondria, which are both likely to be significant in maintaining cellular homeostasis. Actin has also been shown to play a role in the regulation of translational accuracy (Kandl et al. 2002) which may feed into the production of inaccurate, or aggregating, proteins as cells age and the cytoskeleton accumulates damage. Of particular interest for future studies will be to increase our understanding of how accumulated damage to the cytoskeleton is perceived within tissues and cell types linked to age-related disease, for example within post-mitotic populations, such as neurons, and what the consequences are for cell fate. Are there particular cells that are particularly sensitive to, or cannot easily repair, the actin

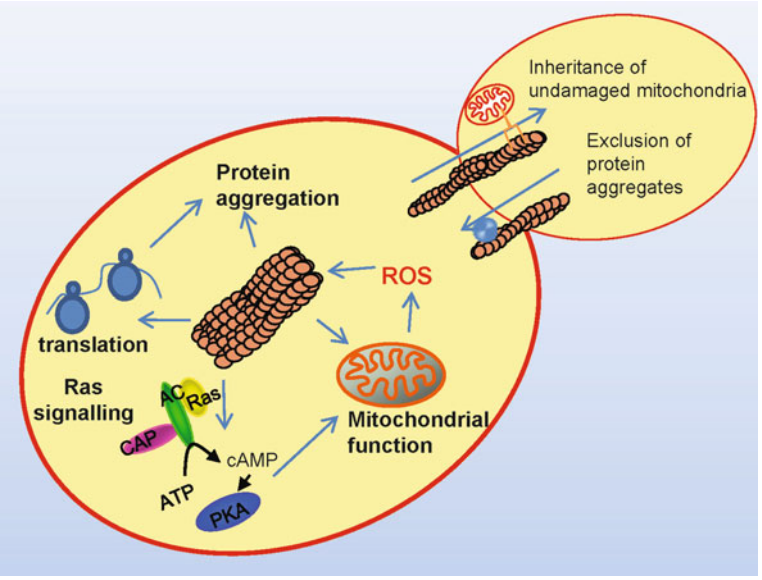

Fig. 15.3 Actin's role in the stochastic process of ageing. Actin has been shown to play a role in various cellular processes that have an impact on the ageing process. The formation of actin aggregates in aged or mutated cells is thought to trigger mitochondrial dysfunction and concomitant ROS production via inappropriate activation of the Ras/cAMP/PKA signaling cascade. Actin has also been shown to play a role in the regulation of translational accuracy and the formation of protein aggregations with an amyloid configuration such as prions and expanded poly-Q repeat proteins. The actin cytoskeleton also appears to be required for the inheritance of so called anti-ageing factors and for the inheritance of mitochondria into newly forming cells

damage incurred as cells age? If so, is the cytoskeletal dysfunction within such cells linked to increased cellular senescence, elevated levels of programmed cell death or disease pathologies associated with ageing?

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