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Review

# The budding yeast, *Saccharomyces cerevisiae*, as a model for aging research: a critical review

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**Abstract**

In this review we discuss the yeast as a paradigm for the study of aging. The budding yeast *Saccharomyces cerevisiae*, which can proliferate in both haploid and diploid states, has been used extensively in aging research. The budding yeast divides asymmetrically to form a 'mother' cell and a bud. Two major approaches, 'budding life span' and 'stationary phase' have been used to determine 'senescence' and 'life span' in yeast. Discrepancies observed in metabolic behavior and longevity between cells studied by these two systems raise questions of how 'life span' in yeast is defined and measured. Added to this variability in experimental approach and results is the variety of yeast strains with different genetic make up used as 'wild type' and experimental organisms. Another problematic genetic point in the published studies on yeast is the use of both diploid and haploid strains. We discuss the inherent, advantageous attributes that make the yeast an attractive choice for modern biological research as well as certain pitfalls in the choice of this model for the study of aging. The significance of the purported roles of the Sir2 gene, histone deacetylases, gene silencing, rDNA circles and stress genes in determination of yeast 'life span' and aging is evaluated. The relationship between cultivation conditions and longevity are assessed. Discrepancies between the yeast and mammalian systems with regard to aging are pointed out. We discuss unresolved problems concerning the suitability of the budding yeast for the study of basic aging phenomena. 

*Keywords:* *Saccharomyces cerevisiae*; Budding life span; Stationary phase; Aging

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"There is no place for dogma in science. The scientist is free to ask any question, to doubt any assertion, to seek any evidence, to correct any error". (J. Robert Oppenheimer, 1904–1967)

The choice of the budding yeast as a model organism for studying complex processes is understandable. It has inherent, advantageous attributes that make it very attractive for modern biological research. There are, however, certain pitfalls in the choice of this model for the study of aging.

## 1. General considerations that favor the budding yeast as an experimental model

1. It is a unicellular eukaryotic organism with a relatively uncomplicated and short life cycle.
2. It has a small genome comprising about 6000 genes, which has been completely sequenced (Goffeau et al., 1996) and extensively mapped.
3. Special characteristics of this organism have enabled the development of essential molecular genetic tools that contribute significantly to the understanding of some of the major processes in cell biology. These processes include signal transduction (e.g. Whitmarsh and Davis, 1998), control of cell cycle progression (e.g. Nasmyth, 1996), the basis of the switch from mitosis to meiosis (Nasmyth, 1996), genetic recombination (Stahl, 1996), intracellular trafficking of proteins (Kim et al., 1998), response to stress (e.g. heat shock genes; Glover and Lindquist, 1998), and protein degradation (e.g. Xie and Varshavsky, 1999).
4. Many yeast genes have been shown to have orthologs in the human genome, including some disease-causing genes. Several human proteins can functionally substitute for their yeast analogs following transfection of human genes into yeast. A large number of custom-made plasmids and mutant yeast strains have been constructed for this purpose. This observation makes the budding yeast an enticing model for studies of phenomena and processes encountered in multi-cellular organisms. The latter are much more difficult experimental systems because of their anatomical complexity, much longer life spans, longer cell cycles and expensive maintenance and husbandry.
5. For the experimentalist, yeast is a desirable organism since it is relatively cheap to grow in large quantities on simple medium. It can be plated on agar plates as single cells and observed for clonal growth. They can be separated from progeny cells by micromanipulation. They can be manipulated genetically with ease. Yeast has a well-developed system of homologous recombination and lends itself to relatively simple knockout of individual genes. They can also be maintained and studied in the haploid state.

## 2. The biology of the yeast cell and its relevance to aging

### *2.1. Cell cycle and spore formation*

Yeast are unicellular eukaryotic fungi. They are highly suitable for the study of cell biology because they propagate very rapidly and their genome size is about 1% that of mammalian genomes. The two species of yeast that have been very extensively studied are the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe*. The former has also been used extensively in aging research. Both species can proliferate in both haploid and diploid states. As long as nutrients are abundant, proliferation in the diploid state takes place, with a cell cycle of about 2 h. Upon starvation, the cells are driven into meiosis and spore formation. These haploid progeny germinate and start propagating when the nutritional conditions improve. For obvious reasons, haploid cell lines facilitate the isolation and study of gene mutations. This is an outstanding characteristic that has been cleverly used to extensively study the molecular genetics of yeast cell biology. Sexual reproduction, which is very important in the normal biology of the yeast, is achieved by the fusion of **a** and **α** type spores in $G_1$ phase resulting in the formation of diploid cells. The mechanism of maintenance of these mating types has been well elaborated. Under conditions of deprivation, spores can survive for enormously long periods of time in proportion to the length of the cell cycle (months versus hours). A few hundred genes are involved in sporulation in addition to those engaged in control and progression of meiosis (Chu et al., 1998). Sporulation is essential for survival of this unicellular organism that interacts directly with extreme environmental challenges and is not protected by extracellular homeostatic mechanisms. Needless to say, no analogous developmental program is found in mammalian cells.

The fission yeast has a typical eukaryotic cell cycle (including $G_1$, S, $G_2$ and M). However, unlike metazoa, the nuclear envelope does not disappear during meiosis and the spindle forms inside the nucleus. In the budding yeast $G_1$ resembles that of higher eukaryotes; however, the spindle starts forming during S phase, there is no $G_2$, and the nuclear envelope remains intact during mitosis. The cell divides asymmetrically. The two products of division being the 'mother' cell and a diminutive cell, the bud, which usually grows to the full size of the 'mother' cell after the completion of cell division.

Many basic features of the control mechanisms of the cell cycle have been preserved between the yeast and higher metazoa. This fact has enabled the use of yeast cells to study this most fundamental function of the cell and has resulted in significant progress in the understanding of its complex features. It must be stressed, however, that control of the cell cycle is considerably more elaborate in metazoa than in yeast. Gene duplication and divergence have created several cell cycle gene variants that exist in the same mammalian cell albeit with specialized functions. This has provided metazoan cells with more sophisticated controls of cell cycle progression that afford improved adaptation to varying situations.

The yeast cell adapts well to extreme environmental conditions. For example, when deprived of sugar, yeast cells undergo an extensive change in metabolism including shut down of transcription by RNA polymerases I and II and drastic reduction in the rate of degradation of mRNA (Jona et al., 2000). The cell with the drastically down-regulated metabolism retains viability for extended periods of time and recommences growth when conditions improve.

The individual yeast cell, which is an entire organism, is designed to control cell growth and cell division with little if any interaction with other yeast cells. While its functions are determined by direct interaction of the single cell with its environment (nutrition, temperature, oxygen supply etc.), this is very different from higher metazoa, which maintain a homeostatic internal environment that protects individual cells from extreme environmental changes. The interaction of the yeast cell with other cells in a population is of importance primarily when the two types of spores (**a** and **α**) release chemotactic pheromones to attract each other in order to attain fusion.

### *2.2. Budding*

The budding yeast [and perhaps also the fission yeast (Barker and Walmsley, 1999)] divides asymmetrically to form a 'mother' cell and a bud. The asymmetric division entails programmed unequal distribution of a transcription repressor that is localized specifically in the bud (O'Shea and Herskowitz, 2000). This mechanism bears a certain resemblance to the asymmetric distribution of transcription regulators involved in the determination of the fate of differentiating daughter cells during embryonic development in *C. elegans*, and *D. melanogaster*. However, in multicellular organisms, which require the formation of many specialized cell types through asymmetric cell division, additional essential factors are involved (Jan and Jan, 2000).

Mortimer and Johnston (1959) were the first to measure the rate of bud production from single cells. This necessitated both observation of individual cells and separation of the 'mothers' from progeny buds. They determined that the number of buds produced by a cell is finite. The last 2–3 rounds of budding are of longer duration after which the cell ceases to divide. Non-dividing cells eventually die (vide infra for a discussion of the validity of this evidence). The number of buds produced by a cell varies considerably depending on genotype, nutritional conditions and perhaps temperature.

There are contradictory findings regarding the number of progeny produced by early and late buds removed from the same 'mother' cell. Kennedy et al. (1994) claim that 'maternal' age affects the 'longevity' (perhaps fecundity is more appropriate) of daughter cells. On the other hand, Egilmez and Jazwinski (1989) report that daughter cells of older 'mother' cells have the same reproductive capacity as those from younger 'mothers'. The former claimed that their data could be interpreted as an accumulation of 'senescence substance' in 'mother' cells as a function of time that is transmitted to the progeny buds. This is not supported by

Egmilez and Jazwinski's observation (vide infra for a discussion of the questionable assertion equating cessation of cell division to cell death).

## 3. Experimental protocols used to measure yeast 'life span'

Two major approaches have been used to determine 'senescence' and 'life span' in yeast. The first, initiated by Mortimer and Johnston uses individual cells to observe budding capacity as a function of time in culture (Mortimer and Johnston, 1959). This methodology has been employed extensively by Jazwinski and his group (e.g. Kim et al., 1999a) and by Guarente and coworkers (e.g. Kennedy et al., 1994). In this approach, the finite number of buds produced by a 'mother' cell is determined and is designated **budding life span** (akin to the 'proliferative life span' of mammalian cells in vitro). After the cells undergo a certain number of buddings they cease to divide. A longer cell cycle (slower bud production) is observed in late buddings prior to the complete cessation of cell division (Mortimer and Johnston, 1959). It should be noted that Mortimer and Johnston cautioned that local nutrient depletion around the individually cloned cells should be avoided, a fact that has not been given enough attention in recent work. In addition to depletion by cellular consumption, medium that is kept in the incubator at 25°C for a number of days, and at times several weeks, undergoes a decline in nutritional value. In order to prevent overcrowding, which may drive the cells into stationary state (Werner-Washburne et al., 1996), each daughter cell must be separated from the 'mother' cell by micro-manipulation throughout the 'budding life span'. In this system, there are strain differences and even individual variation and the fate of the post-mitotic cell is variable and not well defined. In such studies, death verification is not always the endpoint, but rather it is implicitly accepted that post-mitotic cells are dead cells. The basic questions that remain unanswered are: what are the pathologies of these cells, and more importantly, what is the state of their viability, as determined directly and not by their capacity to produce buds (see below in the discussion of chronological aging in the yeast). It is quite possible that a fraction of these cells are quiescent rather than dead. A similar problem of equating a post-mitotic cell to a dead cell prevails in the studies of in vitro cell cultures of vertebrates (to be discussed in a subsequent review in this series). In order to formulate valid conclusions, these questions must be seriously addressed.

The second experimental model for studying aging in yeast is the **stationary phase** system in which the cells are not grown individually on nutrient agar plates but rather whole populations are maintained in liquid medium until cell number reaches a plateau (generally at about $10^8$/ml) (Longo, 1999). The cells are then maintained for an additional period on either the expired medium or distilled water (Liou et al., 2000). Viability is determined by the ability of individual cells plated from aliquots that are removed periodically from the cultures to form colonies on agar plates. Since the number of cells grown in suspension in this system is vast, viability can and is also verified by other techniques including

estimation of the percentage of cells that are metabolically active (Longo, 1999). Taken together, these essential criteria that are not considered in the budding system (see above), are more relevant to the determination of life span than just the determination of the budding capacity used in the individually plated cells of the 'budding life span' method.

In the 'stationary phase' approach, the cells initially grow rapidly in glucose-rich medium. Energy production under these conditions is primarily derived from fermentation rather than respiration. Growth slows down and the cells enter a stationary phase when the glucose in the medium is depleted. At this stage the mitochondria begin to use stored substrates and there is a switch from fermentation to mitochondrial respiration. The population of cells maintained in the expired medium with very limited growth shows increased mortality after about 5 days (Longo, 1999). This has been attributed primarily to the production of reactive oxygen species generated by the increasingly active mitochondria (Longo, 1999). In an alternative protocol, after depletion of the glucose in the medium the population is transferred to water in which it can be kept for weeks in a non-dividing but viable state presumably due to a 100-fold reduction in the rate of metabolism with no switch to oxidative respiration.

A variation of the 'stationary phase system' for the study of chronological, rather than budding 'life span' involves growing the cells to a stationary phase on minimal glucose medium. The mean survival time, with the particular strains used in this system, studied from aliquots serially withdrawn from the culture, is 7–8 days (Longo et al., 1997). In this experimental protocol, the cells maintain high rates of metabolism, as determined by oxygen consumption, virtually throughout the life span (Liou et al., 2000). In either variation of this approach the disturbing point is that the major observation recorded is actually the effect of nutrient deprivation and possibly of accumulation of toxic compounds in the medium. It is highly questionable whether this is relevant to understanding the underlying mechanisms of aging.

The discrepancies observed in metabolic behavior and longevity between cells in rich medium, minimal medium and water, in 'stationary phase' liquid culture and the 'budding life span' methods raise the fundamental question: How do we define and what is the meaning of 'life span' in yeast? Longo (1999) suggests that the stationary phase system reveals 'chronological age' rather than 'budding life span'. The relationship between the two forms of 'life span' is not very clear, although recently Ashrafi et al. (1999) have made an attempt to reconcile the two systems. They showed that the mean replicative 'life span' gradually decreases with the length of time the cells are maintained in stationary phase. This is in contrast to the observations of Jazwinski (2000) that the progeny of any budding cycle have the capacity for full 'life span' regardless of whether they are derived from early or late buds.

Another puzzling difference between the two systems emerges from studies on the Ras2 gene. This gene has been claimed to be cardinal in the modulation of the 'life span' of yeast in both culture systems (e.g. Jazwinski, 1999a; Liou et al., 2000).

(Examination of the protocols and data suggest that survival might be a more appropriate term than 'life span'.) Disturbingly, Ras2 has been shown to have opposite effects on survival in cells in the 'budding life span' system and in the stationary phase system (see discussion on the Ras2 gene below).

The results obtained with the two methodologies to determine 'life span' in yeast are thus often difficult to reconcile. The 'life spans' determined under the different culture conditions differ. Survival under minimal medium conditions which is closer to what the yeast cell encounters in its natural habitat may be more meaningful. In any case, in both systems it is very difficult to distinguish the difference between the ability to survive under various environmental conditions and true 'life span' determinants.

Added to this variability in experimental approach and results is the variety of yeast strains with different genetic make up that are utilized in the various studies. In his chapter on methodology in yeast aging studies, Jazwinski recommends the following: ''The strain chosen as the 'wild type' control may depend upon the experiments you wish to perform; however, several things should be considered. One consideration that is very important in choosing a strain is the ease with which the buds can be removed from the individual cells. … the life span of your control strain is also an important consideration. A strain with a life span that is too short makes it hard to examine variables that shorten the life span, while a strain with a life span that is too long may make it difficult to detect extensions and take too long to assay. We have chosen a strain with a mean life span of about 19 generations and a maximum of around 30 generations, for many of our studies. As with any strain that you want to use for genetic manipulations, it is a good idea to choose a strain containing a variety of nonreverting selectable markers'' (Kim et al., 1999a). In other words, as a primary consideration it is suggested that one should select the strains that are easy to work with and that will a priori give the appropriate results without rigorously examining their direct suitability to the basic process under study, namely, senescence. In our view this approach is seriously flawed. Extension of the 'life span' of a short-lived strain or shortening of the 'life span' of a long lived strain are meaningless to the study of the fundamental processes of senescence. Mutation in non-redundant genes borne by a strain or inflicted experimentally are expected to exert a deleterious effect on the survival capacity. Such findings would have no direct relationship to senescence but rather to adaptation to the environmental conditions.

Another problematic genetic point in the published studies on yeast is the use of both diploid and haploid strains. Some laboratories use a diploid strain chosen according to the above criteria for the 'proper life span', while others use a haploid 'wild type' strain (Kennedy et al., 1994). Using a haploid strain, it has been claimed that genome instability is a major determinant of the 'life span' (Park et al., 1999). It is obvious that a genomic instability in a haploid strain cannot effectively represent the situation in diploid yeast nor in higher eukaryotes. Thus the use of a haploid strain may lead to problems of interpretation and its usefulness for studying the complex phenotype of aging is questionable.

The study of limited budding (Mortimer and Johnston, 1959), received an impetus by a seeming similarity to the limited number of population doublings that mammalian fibroblasts undergo in vitro (Hayflick, 1965). The relevance of the latter phenomenon to in vivo aging has recently been refuted (Cristofalo et al., 1998). (This will be discussed in detail in a future review in this series.) In a modification of previous claims (Jazwinski, 1990), it has been pointed out that there is a basic difference between the finite budding capacity in yeast and replication of cultured mammalian cells (Jazwinski, 2000). While mammalian cells lose the capacity to replicate and undergo clonal senescence, yeast cells are immortal at the population level because individual cells bud only a limited number of times, yet their progeny produce the same number of buds as do the 'mother' cells. There is no obvious relevance of these findings to aging in multicellular organisms. One might suggest a similarity to germ cells of multi-cellular organisms in which there is 'infinite' linearity (immortality being a misnomer) and yet unfertilized ova become effete. Elimination of effete unfertilized ova is more comparable to the fate of yeast 'mother' cells that stop dividing. However, removal of unfertilized ova, which occurs in fertile females of all ages, has no bearing on organismic aging. Cessation of production of mature ova at menopause is of hormonal origin due to the termination of the production of estrogen. No such hormonal phenomenon can be invoked in yeast where isolated cells show cessation of budding. Moreover, oogonia exist for many years in the ovary as quiescent cells and retain the capacity to differentiate into oocytes and mature ova when instructed to do so by the appropriate hormonal signals.

## 4. Reservations about the suitability of the budding yeast for the study of basic aging phenomena

When one considers the suitability of yeast as an experimental model for aging research all the above-mentioned attributes hold. The well studied genetic system of the organism; its short 'life span' and ease of handling are attractive to the experimentalist (Kim et al., 1999a). However, in considering the yeast model, one of the major questions that must be addressed is whether or not a unicellular life form can serve as a relevant model for aging of multi-cellular organisms. We suggest that for certain purposes a unicellular organism cannot serve as an adequate model for the study of the aging of multi-cellular systems. There are some problems with the adequacy of this model that stem from the following:

1. In considering the universality of findings in the yeast model it is critical to consider that this system lacks one of the major features of aging in multi-cellular organisms: the differentiation and specialization of cells to perform specific functions with very high efficiency. This specialization is at the expense of the ability of the individual cell to perform basic functions executed by a unicellular organism. Most differentiated cell types such as neurons, muscle cells and many glandular cells in multi-cellular organisms forgo the capacity to divide and remain in $G_0$. These non-dividing cells survive for long periods of time,

sometimes decades, with a very slow loss of functional capacity. Because of the programmed, limited repertoire of cellular functions, their survival is obligatorily dependent on communication with and support of other cells of the same type or cells with different specialization over short or long distances. Failure of this communication is a major factor in aging of the multicellular organism. An example of this situation is skeletal muscle deterioration due to loss of innervation derived from damage and reduced regenerative capacity of cholinergic synapses (Herscovich and Gershon, 1987). Another example is the reduced ability to respond to hormones due to alterations in structure or control of synthesis of appropriate receptors (e.g. Supakar and Roy, 1996) that lead to loss of function.

2. Dysfunction or death of individual yeast cells (unicellular organisms) has limited or no effect on the function of the whole community of cells (with the exception of overcrowding). Dysfunction or death of cells within an organized, non-regenerating tissue of multi-cellular organisms may exert a significant effect on the function of many cells or even a whole tissue. An example of this is the loss and/or reduced function of cardiac and skeletal muscle cells and neurons that occurs with age.
3. Multi-cellular organisms, composed of many types of highly specialized cells, must establish a systemic homeostatic environment. In these organisms, very few cells are directly exposed to external environmental conditions. Thus at least the initial response to stress is dealt with in parallel in the individual cell and the systemic levels, i.e. individual cells are at least partially protected by the organismal system. For example, in mammals the capacity to respond to a reduced oxygen supply is monitored by sensor cells in the kidney, which are induced to produce erythropoietin (EPO). EPO induces an increased production of erythrocytes from progenitor cells in the bone marrow that results in increased supply of oxygen to the tissues. Induction of expression of the gene encoding for erythropoietin under hypoxia is mediated by the activation of the universal transcription factor HIF-1 (hypoxia inducible factor-1) (Semenza, 2000). The capacity to induce active HIF-1 in the kidney is considerably reduced with age (Frenkel-Denkberg et al., 1999). This affects the capacity of the whole organism to withstand hypoxia. In the mammal there are auxiliary mechanisms that counter the effect of hypoxia such as increased production of NO (also via HIF-1) in the blood vessels (Semenza, 2000) which in turn increases vasodilation and, consequently, blood supply to hypoxic tissues. Hypoxic cells also produce vesicular endothelial growth factor (VEGF) that induces blood vessel growth thus increasing oxygen supply to the deprived tissue (Semenza, 2000). This example serves to indicate that as a rule the multi-cellular organism has a diversity of system-based functions involving inter-cellular communication to counteract many forms of stress. This essential inter-cellular communication system with its alternative fail-safe mechanisms are absent in unicellular organisms like the yeast.

It has been repeatedly suggested that the budding yeast may serve as a model for replicative cell senescence, particularly for those metazoan cellular systems that

divide throughout the life span of a multi-cellular organism (Sinclair et al., 1998; Kim et al., 1999a). Contrary to what we have developed above, it has been suggested that yeast is a highly appropriate model for the study of aging because: "Yeasts present a simple, stripped-down version of aging. Extracellular factors, such as hormones, and interactions with other cells can largely be ignored in analyzing the intrinsic features of aging. Cellular and organismal aging are one. Indeed the germ line and the soma are contained in the individual cell" (Kim et al., 1999a). Implicit in this assertion is the concept that dividing individual cells in metazoa undergo replicative senescence. As will be discussed below, it has been claimed that yeast 'mother' cells, which undergo a finite number of mitoses (buddings), then perish. The legitimacy of this assertion is based on Hayflick's theory (Hayflick, 1965) that normal, diploid human cells can undergo a finite number of divisions in culture after which cell death ensues. This theory implies that the number of divisions constitutes an aging clock which determines the proliferative life span of the cell and consequently of the organism. This theory and its relevance to organismal aging will be assessed at length in a later review in this series. Suffice it to mention at this point that extensive evidence from in vivo systems does not substantiate this theory. In this regard the reader is referred to the article by Cristofalo et al. (1998) in which it has been critically shown that there is no correlation between donor age and the number of population doublings attained by fibroblasts in culture. Thus, the doubtful validity of the contention that individual dividing cells undergo replicative senescence in vivo opens to question one of the major incentives for considering yeast as a good model for aging research.

## 5. Studies on individual yeast genes as 'life span' or longevity modulators

1. A few introductory remarks must be made in order to facilitate the development of the following discussion.
   1.1. Yeast is an ancient organism that has acquired genetically determined means of survival and efficient reproduction in its own variable natural environment. Evolutionary forces select for the establishment of an optimal combination of genotypes adapted to a specific habitat. In general mutations exert detrimental effects on an organism under the variable conditions that prevail in the natural habitat of any given species. Evolutionarily selected genotypes are presumably those which are most successfully competitive at the population level.
   1.2. Most mutations in essential genes have a detrimental effect. Thus, most, if not all, so-called 'life-shortening' mutations bear very low relevance to the aging process. Mutations in many yeast regulatory, or even house keeping genes, will exert a range of effects that include lethality, lower fecundity and declining adaptability to the environment. Interference with the delicate and intricate intra-genomic balance of an organism is likely to be

detrimental. Therefore, claims that certain individual genes have an essential role in the determination of senescence because when mutated they shorten the life span are unwarranted.

1.3. For the purpose of facilitating laboratory research, yeast strains have been adapted, by selection of mutants or by genetic engineering, to efficient growth under the artificial conditions of constant nutrition and temperature (as opposed to their being variable in nature). These derived strains, therefore, contain seemingly 'redundant' genes, which are not essential for viability under laboratory conditions. This is an indication of the difficulties in studying a complex phenotype such as senescence with such artificial constructs as laboratory strains. An interesting example is aquaporin, the water channel protein, which has a significant physiological function in wild type isolates and none in laboratory strains of *S. cerevisiae* (Bonhivers et al., 1998). Also, Goffeau et al. (1996), in their review of the completion of the sequencing of the yeast genome, emphasize that "the example of the citrate synthase genes suggests that much of the redundancy in the yeast genome may be more apparent than real. In this case, it was our knowledge of the rules of protein targeting in yeast that allowed us to discern that these genes play different physiological roles. It is likely that a large number of apparently redundant yeast genes are required to deal with physiological challenges that are not encountered in the laboratory environment but that yeast commonly encounters in the natural habitat of the rotting fig or grape" (Goffeau et al., 1996). Most of the mutations that affect longevity under laboratory growth conditions are part of the process of transition from genotypes that are adapted to natural habitat to those that are better adapted to the artificial laboratory environment. This may be a fascinating 'mini-evolution' in the making but the role of the mutated genes in determining life span is questionable. Many genes become unessential under these conditions because the environment is rich and supplies all the necessities of the cell. All the strains used as 'wild type' in laboratory models are auxotrophs (see section 1.5.) that survive well in the laboratory. These strains would be feeble, if at all viable, under the much more demanding conditions in nature. It should be noted that the laboratory environment also eliminates many of the conditions of stress that prevail in nature such as extreme temperatures, deprivation of nutrients, and large fluctuations in humidity.

1.4. In the 'budding life span' experimental protocol, the yeast cells are maintained and observed as solitary cells in agar plates (Mortimer and Johnston, 1959). It is a rare situation in nature that yeast grow as solitary clones rather than non-clonal populations composed of presumably mixed genotypes. In non-clonal 'suspension culture' populations, new mutant genotypes that have better adaptation for artificial laboratory conditions will have selective advantage over genotypes that are more adapted to the natural habitat. These latter will be eliminated or very much reduced in occurrence. Some of the new genotypes may show extended longevity in

single cell cultures where they are not required to compete with other genotypes. It is likely that 'longevity' mutations carry pleiotropic effects on metabolic functions, cell cycle progression and fecundity. In the laboratory under clonal budding conditions where the cells are solitary these mutations are sustained. It can thus be reasonably assumed (but this assumption should be tested) that in mixed-genotype suspension culture, cells bearing these mutations would be at a selective disadvantage. Such mutations, as will be mentioned below, are perhaps interesting for other purposes but are not very useful in the search for the underlying mechanisms of aging. They are not 'life extending' mutations but rather genotypes adapted to artificial environments.

1.5. Artificial choice of so-called 'wild type' strains for 'life span' studies is very worrisome. The suggestion by Jazwinski, cited above, that one chooses a wild type that is neither too short living nor too long living by itself almost completely abolishes the validity of the model. There has been no attempt to go back and study one of the progenitor strains mentioned by Mortimer and Johnston (1986). The 'wild type' strains currently under investigation carry various mutant alleles such as the W303 strain which bears ade2-1, his3-11-15, trp1-1, leu2-3, 112 and can1-100 (Austriaco, Jr. and Guarente, 1997); the EG103 strain which bears DBY 746; MATα, leu2-3,112, his3Δ1, trp1-289, ura3-52 $GAL^{+}$ genes (Longo et al., 1997); the YPK4.7 strain which bears MATα, ura3-52, lys2-$801^{amber}$, ade 2-$101^{ochre}$, trp1-Δ63, his3-Δ200 and leu2-Δ1 (Kim et al., 1999b). These are but some of the multitude of varied engineered strains used in various laboratories. All of these genetic variants are auxotrophs for several nutrients. Under various conditions these mutations may have a significant effect on the expression of other genes. A good example of such an effect is described in a study of SNZ genes by Padilla et al. (1998). Under nitrogen-limiting conditions, SNZ1 mRNAs accumulate in tryptophan, adenine and uracil auxtrophs but not in prototroph counterparts. This demonstrates that genotypic modifications can have complex phenotypic ramifications. Allelic differences between strain backgrounds can crucially affect experimental outcome. This is emphasized even in the instructions given to novices in the introduction to the Cold Spring Harbor Laboratory Course Manual on Methods in Yeast Genetics (Adams et al., 1997). The basis for the comparative evaluation of 'life span' studies carried out by the different research groups who use different strains is very difficult at best, and made more difficult when results obtained with haploid strains are compared to those from diploid strains (e.g. Kim et al., 1999b).

1.6. An extensive number of publications in the last decade describing molecular investigations have neglected basic biological facts and are, therefore, replete with over-interpretation of results. This has inevitably led to the suggestion of models of aging in yeast that have claimed to be the cornerstone of our understanding of aging in general. These are described in a deluge of recent reviews (e.g. Jazwinski, 1996, 1999b; Johnson et al.,

1999; Sinclair, 1999). The above discussion on longevity-assuring genes in yeast, that resemble similar ones in other systems, particularly *C. elegans*, introduces doubts regarding such claims. A large number of genes are involved in the determination of life span. Many individual genes affect, to a greater or lesser extent, survival under a variety of environmental conditions. Survival under adverse conditions and life span are not synonymous. One is referred to the previous review in this series for all the additional considerations that must be made when aging hypotheses are seriously suggested (Gershon and Gershon, 2000).

2. Is Ras 2 a longevity modulator?
   2.1. Ras proteins perform very important functions in the response of cells to external signals. Ras is a GTP-binding switch protein that alternates between inactive GDP-bound and active GTP-bound forms. In higher eukaryotes, the binding of certain hormones or growth factors to specific cell surface receptors induces the activation of Ras. Guanine nucleotide releasing proteins (GNRPs) bind to Ras-GDP and cause GDP dissociation thus enabling the binding of GTP (which is found in much higher concentrations in the cell than GDP) to free Ras. Ras activates serine/threonine phosphorylation cascades that activate MAP-kinases. An example of an important Ras function via the MAP kinase cascade is the activation of Fos transcription, which in turn causes phosphorylation of the Jun protein. Jun forms a heterodimer with Fos to form the versatile transcription factor AP-1.
   2.2. In yeast, Ras-1 and Ras-2 are closely related genes with overlapping functions in normal growth (Werner-Washburne et al., 1993), invasive growth and filamentation (Mosch et al., 1999), spore germination (Herman and Rine, 1997) and stress resistance (Toda et al., 1985). Mutation in Ras-2 increases the 'life span' of stationary phase cells (Liou et al., 2000). Over-expression of Ras-2 causes death in stationary phase cells (Longo, 1999). In contrast to these findings it has been reported that over-expression of Ras-2 increases budding 'life span' (Sun et al., 1994). Reconciliation of these results in terms of 'life span' modifications would require the illogical assumption that there are two types of 'life span' in yeast. It is possible that what is observed is gene 'redundancy' due to artificial growth conditions. In other words, the activity of this gene is required under the solitary cell cultures in the budding system and is extraneous or even detrimental under the conditions of the cell suspension system. This would result in conflicting results between the budding and stationary stage systems. If this is the case it would make Ras mutations not relevant to aging, despite their anti-stress functions (e.g. Jazwinski, 2000; Liou et al., 2000).
3. The purported roles of Sir2, histone deacetylases, gene silencing and rDNA circles in determination of yeast 'life span' and aging.
   3.1. The silent information regulator (SIR) genes encode a group of proteins that are involved in silencing of transcription. There are four SIR genes in

yeast that are required for repression of the chromosomal regions encoding for mating type, telomeres and ribosomal DNA (rDNA). They may also be involved in the silencing of other chromosome domains. Sir2, Sir3 and Sir4 are involved in chromatin silencing at the mating type loci and telomeres whereas Sir2 is solely responsible for silencing of ribosomal DNA genes of yeast. Kennedy et al. (1995) described a mutant of Sir4 that 'delays aging' in yeast. In this review, we shall concentrate only on the SIR2 gene that has been thoroughly explored with regard to 'aging' in yeast. In a recent review Guarente (2000) offers an overview of the function of Sir2 associated with gene silencing and its relationship to yeast 'aging'. He also speculates on its relationship to the metabolic state of the cell derived from the very interesting finding that Sir2 is an NAD-dependent histone deacetylase (Imai et al., 2000). Of the four Sir genes, abrogation of Sir2 activity was sufficient to reduce the number of buds produced (Kaeberlein et al., 1999). Conversely, an extra copy of Sir2, increased the number of buds produced per cell (Kaeberlein et al., 1999) ('life span', according to the authors).

3.2. Guarente (2000) has made the point that rDNA circles accumulate in 'mother' cells with age. The NAD-dependent deacetylation of H3 and H4 histones silences active chromatin. This accumulation, due to loss of gene silencing in the ribosomal gene chromosomal domain, resulted in increased homologous recombination and thus formation of extra-chromosomal rDNA circles. It has been proposed that this accumulation of circles is a central cause of yeast 'aging' (Sinclair and Guarente, 1997; also see model in Guarente, 2000). Jazwinski and colleagues explored the function of the RPD3 deacetylase gene in relation to the effect of Sir2 on the number of buds produced per cell (Kim et al., 1999b). They found that the 'life span' shortening in Sir2 mutants is due to increased loss of rDNA silencing *without* increased presence of rDNA circles. One questions whether the difference is due to different methodology or to the different genetic background of the strains used in the two laboratories. Moreover, in experiments using the stationary stage protocol it has been found that rDNA circles play no role in 'aging' of cells (Longo, pers. commun.). Due to these conflicting findings the theory of rDNA circles as a cause of yeast 'aging' is at present hard to accept and no such phenomenon has been observed in cells of higher organisms (Guarente, 2000). Guarente does, however, speculate by analogy that, as a function of age, there may be a problem of loss of silencing in higher organisms in other loci. This, we suggest, can be tested on inactive X-chromosomes in aging female mammals. However, it must be emphasized that in higher organisms the number of chromatin acetylases and deacetylases and the complexity of the system is much greater than in yeast (Afshar and Murnane, 1999).

3.3. Afshar and Murnane have shown that the human Sir2 ortholog is cytoplasmic whereas it is nuclear in yeast (Afshar and Murnane, 1999). Unlike yeast, over-expression of Sir2 in human cells does not affect growth. These

observations indicate differences in structure and mode of action of the two orthologs. Roy and Runge (2000) have shown that the chromatin silencing mechanism in yeast entails more than just Sir gene activity. They showed that two paralogs ZDS1 and ZDS2, in combination with the Sir3 gene, antagonistically affect silencing at the mating-type cassette and rDNA. While ZDS1 causes an increase in silencing, ZDS2 causes a decrease in rDNA silencing. In other words, there are many more players involved in the control of chromatin silencing than just the Sir 2,3,4 genes. The interaction among those genes is important and the experimental results obtained depend to a great extent on the background genotype of the strains used. Taken together with the results of others it is difficult to accept the suggestion that silencing in the repetitive rDNA chromosomal region leads to the formation of rDNA circles which are "Vicious circles: a mechanism for yeast aging" (Defossez et al., 1998). Once again, a search for a single gene directly involved in the determination of the 'life span' seems unconvincing.

4. Stress genes

4.1. Stress genes have been explored extensively in yeast (e.g. reviewed in Longo, 1999; Jakubowski et al., 2000). The failure to distinguish between survival under adverse conditions and aging mechanisms has led to the establishment of misconceptions. Survival of yeast cells, as well as cells of multi-cellular organisms and bacteria, depends, amongst others, on protection against oxidative, thermal and osmotic stresses. Unicellular organisms such as bacteria and yeast have genetically determined protection mechanisms to ensure survival and proliferation under various adverse conditions. Interference with the genes that control these mechanisms will inevitably lead to changes in viability. The mechanism of stress response in yeast can be different in detail from that of higher organisms. A very distinct example is the fact that in yeast, Hsp 104 plays a critical role in induced thermotolerance (Lindquist and Kim, 1996). It functions in the reactivation of aggregated proteins and is dependent on its interaction with Hsp70 and Hsp 40 (Glover and Lindquist, 1998). There is no HSP104 ortholog in mammals.

## 6. Cultivation conditions and longevity

While there is a superficial resemblance to aging mechanisms in multi-cellular organisms, yeast, like bacteria, are basically immortal at the population level. Cell death in these unicellular organisms does occur and is inevitably organismal. At most it resembles death of effete metazoan cells (mostly by apoptosis, which is absent in yeast) or those damaged by accidents in metazoans, but has little bearing on the senescence of the complex organism that is capable of replacing the dead cells without losing organismal viability. Moreover, immediately after the death of the multi-cellular organism, individual cells maintain viability and can be cultured

in vivo albeit without the ability to re-form the total organism. There is no evidence from multi-cellular organisms that the number of cell divisions is a clock that determines the onset of programmed cell death. Barring environmental disasters, populations of unicellular organisms go on multiplying indefinitely even though a proportion of cells die. Chemostat studies with yeast show that controlled cultivation conditions are absolutely needed in order to compare the metabolic regulation of different strains (van Hoek et al., 2000). It has been shown that conclusions from chemostat physiological studies cannot necessarily be extrapolated from one strain to another (van Hoek et al., 2000), presumably due to genotype differences that are critical in such comparisons. We suggest that this is even more crucial when one tests longevity that is stress-dependent. Standardization of conditions such as constant replenishment of growth medium will circumvent the problem of the effect of nutrient depletion and accumulation of harmful breakdown compounds in the medium on the survival of yeast cells. Critical chemostat studies will yield a deeper insight on the rate and the causes of mortality in yeast cells.

## 7. Summary of some relevant discrepancies between the yeast and mammalian systems with regard to aging

References are given only in the cases where they are not given in the above text.

1. As discussed throughout this review, several significant basic differences in the biology of the yeast and multi-cellular organisms must be consistently taken into consideration with regard to the suitability of the yeast model and generalization of findings in aging research.
2. The yeast is a unicellular organism that interacts directly with the environment, whereas cells of multi-cellular organisms are at least partially insulated by strict homeostasis.
3. The yeast cell has very limited interaction with other cells whereas, because of their diversified specialization, cells of multi-cellular organisms have an obligatory dependence on intercellular communication with cells of the same and other types. This inter-dependence is absolutely necessary for the control of cellular proliferation, differentiation, protection against a variety of stressors and, for many types, their own programmed cell death. The fate of a cell in higher eukaryotes is, in most cases, not determined intrinsically but by interaction with other cells (at close proximity and long distances). The breakdown of communication between cells is a major factor that affects the fate of the cells in the aging multi-cellular organism. This failure is completely absent in unicellular organisms.
4. Gene silencing involves chromatin acetylation in both yeast and higher eukaryotes. In higher eukaryotes this is associated with DNA methylation. No DNA methylation occurs in yeast. This means that the mechanism of gene silencing differs in important details between the two systems. Thus simple extrapolation from yeast to higher metazoans is, at best, risky.

5. Mitosis in yeast is asymmetric. For the most part, it is not so in higher eukaryotes. In the case of stem cells there is asymmetric distribution of cytoplasmic components that guides one daughter cell to differentiate and the other cell to retain stem cell characteristics. A major characteristic of stem cells is conditional proliferation that depends on intercellular signaling. The differentiating daughter cell when it reaches a state of complete specialization ultimately loses its proliferative capacity, but remains functional and viable in a post-mitotic state for long periods of time. The daughter cell that remains a stem cell retains its capacity to conditionally divide throughout the life span of the organism. There is no evidence of a finite number of divisions of stem cells in higher metazoans (a full discussion of this will be presented in a future review in this series). In the case of the yeast the 'mother' cell invariably ceases to bud and eventually dies. In higher eukaryotes, apoptosis is an important mechanism in the programmed removal of particular cells in development; it also plays a crucial role in the removal of effete and infected cells throughout the life span. There is no apoptotic mechanism (programmed cell death) in yeast that is analogous to that seen in metazoa (Fraser and James, 1998; Shaham et al., 1998). Death of the yeast cells seems to be sporadic, at least when they are observed in populations.
6. Yeast respond to unfavorable environmental conditions by radically reducing metabolism and by spore formation. Spores are highly viable cells with a very low metabolic rate ($\sim 1\%$ of the normal rate of proliferating cells). Such extreme adaptation is infrequent among higher eukaryotes and cannot be a basic underlying mechanism of aging, as is explained in our previous review (Gershon and Gershon, 2000). In lower metazoa similar, but not identical, adaptation occurs in many but not all species, e.g. dauer larvae in *C. elegans*; some mammals hibernate as a regularly programmed phenomenon. However this is not a general phenomenon and, therefore, cannot be a decisive underlying mechanism of longevity determination. The higher longevity of spores is not an extension of the life span. It should be considered as an unusual adaptive mechanism for survival under extreme adversity.
7. No time-dependent telomere shortening is observed in yeast (D'Mello and Jazwinski, 1991; Austriaco, Jr. and Guarente, 1997), whereas it is a common age-associated phenomenon in mammalian cells. Studies on artificial interference with telomere length in the yeast (Austriaco, Jr. and Guarente, 1997) are, therefore, not relevant to aging.

## 8. Unresolved problems in the yeast model for aging research

The following problems should be resolved in order to validate yeast as a model in aging research:

1. Do unicellular organisms truly have a life span or is the death of a 'mother' cell akin to removal of effete cells in higher eukaryotes? Since yeast cells do not possess an apoptotic pathway (Fraser and James, 1998), such 'mother' cell death

is not programmed as in higher organisms (Shaham et al., 1998; Li et al., 2000). The cellular changes that occur in post-budding yeast cells must be better characterized as should the mechanisms of cell death. Chemostat studies, which avoid starvation and lengthy deprivation of nutrients, should be helpful in this respect. Chemostat systems have been used for other purposes in the study of yeast for many years (Castrillo and Ugalde, 1994; van Hoek et al., 2000).

2. Which yeast strains should serve as wild type? Here consideration should be given to the problem of haploid versus diploid strains. It is deemed crucial to re-evaluate and eliminate the practice of selection of experimental strains for convenient life spans.
3. Are engineered strains that carry several mutations in various genes (e.g. causing auxotrophy) a tolerable choice as wild type for aging? Here one should return to original, un-engineered strains such as discussed by Mortimer and Johnston (1986). In order to standardize the results, wild type strains selected by consensus should be used in all laboratories that study longevity in yeast.
4. What does 'budding life span' mean in the context of 'chronological life span'? Does the finite number of buds a 'mother' cell produces have any relation to its chronological age? It has been both reported that daughter cells of old and young 'mother' cells have the same reproductive capacity (Egilmez and Jazwinski, 1989) and that 'maternal' age affects the fecundity of daughter cells (Kennedy et al., 1994). This controversial point is particularly relevant since it has become obvious that mammalian cells in culture do not exhibit a correlation between donor age and number of population doublings (Cristofalo et al., 1998).
5. What is the relationship between survival under adverse conditions and longevity in yeast? Are various cell culture conditions (individual cells on agar plates with micromanipulation of buds, or liquid cultures where the cells are actually starved for essential nutrients) equally valid for the possible study of aging? Does medium depletion occur only in confluent liquid cultures or also locally in agar cultures? What is the relationship between long-term survival under poor nutritional conditions (water only) in suspension cultures and the relatively short longevity in agar or minimal medium in suspension cultures (for the complex effect of nutritional conditions see, for instance, Granot and Snyder, 1991). Chemostat studies, which avoid lengthy deprivation of nutrients and possibly accumulation of toxic factors in the medium, should be helpful in this respect. Yeast chemostat systems have been used for many years (Castrillo and Ugalde, 1994; van Hoek et al., 2000).
6. Are the mutations (or deletions) of genes that appear to increase life span such as Ras2 really relevant to aging? It has to be demonstrated that yeast carrying mutations in these 'life span assuring genes' are not at a selective disadvantage during exponential growth when mixed in a population of wild type cells. If they are eliminated in a population consisting of various genotypes because of poor competitive capacity (and yeast in nature do not grow under solitary conditions), then the genes in question have highly questionable relevance to the determination of cellular life span.

7. Do orthologs in yeast perform the same functions as their counterparts in higher organisms? There are many cases in which they do not, or only partially do so. While genes derived from multi-cellular organisms can substitute for knocked out yeast genes, in many cases the 'rescued' cells are physiologically not as competent as the 'wild type'. Also in higher organisms the number of paralogs that participate in the control of many pathways is much greater thus creating better 'back up' systems and finer tuning of function. Thus, just finding a degree of homology between given yeast and mammalian genes is not sufficient to conclude that they carry out identical functions. Even partial rescue of 'knock out' genes is not sufficient in the study of longevity and viability of cells. Full rescue with complete restoration of cell growth, fecundity and stress response must be verified in each particular case.

## 9. Conclusions

The numerous problems listed above and misinterpretations for the yeast experimental 'aging' paradigm indicate that its relevance for aging of multi-cellular organisms, even at the cellular level, is, at best, highly questionable. This is very unfortunate, as the yeast provides a highly potent system for studying the genetic control of basic cellular functions. However, searching for answers under conditions where the generation of data is relatively simple and, therefore, rapid, rather than where the problems are really located, leads to the establishment of misconceptions and dogmas that are hard to weed out. This is unfortunate in the field of experimental gerontology that a priori suffers from a deluge of theories that are based on no experimental or on rudimentary experimental work. This tendency inevitably hampers seriously any progress in the unravelling of the underlying mechanisms of aging.

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