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# The longevity in the yeast *Saccharomyces cerevisiae*: A comparison of two approaches for assessment the lifespan

Mateusz Molon*, Renata Zadrag-Tecza, Tomasz Bilinski

*Department of Biochemistry and Cell Biology, University of Rzeszow, Zelwerowicza 4, 35-601 Rzeszow, Poland*

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ABSTRACT

Longevity of the selected "longevity mutants" of yeast was studied using two methods. The standard method was based on counting the number of daughter cells produced. Modification of that method allowed for establishing the length of life expressed in units of time. It appeared that all the studied "deletion longevity mutants" showed a statistically meaningful increase in the number of daughters produced (replicative lifespan), whereas only one of the mutants, previously regarded as "short lived", showed a meaningful increase in the time of life. The analysis of the available data shows that the time of life of most yeast strains is similar irrespective of their genetic background and mutations, which suggests a quasi-programmed nature of yeast death.



## 1. Introduction

A single cell of the budding yeast *Saccharomyces cerevisiae* can only perform a limited number of mitotic cycles (buddings) [1]. That limit is considered to be a consequence of the aging process; therefore, the number of the daughter cells produced has been called replicative lifespan (RLS) and treated as a measure of age and longevity [2].

Studies concerning on the RLS indicate that the number of daughter produced by the "mother" cell can be modulated by a number of extracellular and intracellular factors. In particular, it describes a number of genes whose deletion leads to an increased the number of generations, hence they are referred to as "longevity genes". One of the first described "longevity genes" in yeast was *LAG1* (***L**ongevity **A**ssurance **G**ene*). Initial reports showed that the *lag1Δ* strain boosted reproductive potential by approx. 50% compared to the wild type strain [3], but later studies disproved significant impact of that mutation [4]. Since then, a number of genes were identified, the deletion of which resulted in increasing the number of cells produced [4–6]. These genes relate to various aspects of cell function. One of the most famous "longevity gene" is the *FOB1* gene. This gene is specific for yeast, but the effects of the mutation are always significant and independent of genetic background. Mutants lacking *FOB1* gene have a reduced rDNA recombination and therefore low ERCs levels [5], which are proposed as a one of the "senescence factor" in yeast. It was also demonstrated that the deletion of a number of genes involved in nutrient sensing also leads to an increase in the number of generations like *SCH9*, *TOR1*, *HXK2*. Numerous of these genes have a counterparts in animals and are potentially connected with their longevity. The budding yeast Sch9p is a functional ortholog of the mammalian S6 protein kinase 1 (S6K1) [7], whereas *TOR1* encodes one of two closely related factors that regulate cell growth in response to nutrient availability and cellular stresses [8,9], and the *HXK2* gene encodes the glycolytic enzyme hexokinase II (Hxk2) [10]. Many studies were shown that the mutation in the *SCH9*, *TOR1* and *HXK2* genes leads to extension of reproductive potential in yeast [11–15]. Another group are the genes associated with ribosome biogenesis or the process of translation. The *rpl20bΔ* mutant as a representative of mutations in large ribosomal subunit proteins. It has been known that deletion of genes encoding the 60S subunit proteins or processing the factors that inhibit the 60S subunit biogenesis is sufficient to ensure a significant increase in replicative lifespan [6]. The analysis of the yeast replicative lifespan and longevity is based on the widely used procedure, which involves only determining the number of daughter cells produced by an individual mother cell. Measuring longevity on the basis of the number of progeny was criticized earlier [16], since in other organisms longevity is expressed in units of time. Therefore, a paper published in 2005 strongly suggested modification of the basic protocol of

*Abbreviations:* RLS, replicative lifespan; PRLS, postreproductive lifespan; TLS, total lifespan.

* Corresponding author.
*E-mail address:* mateuszmolon@univ.rzeszow.pl (M. Molon).

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determining RLS [17]. The authors changed the original procedure by adding a non-toxic dye Phloxine B to the medium, which enabled them to detect the moment when a cell actually dies. According to the previous protocol, the experimenter was only able to establish the moment when the cell ceased reproduction (budding); hence, it was erroneously assumed that such a cell was dead. The authors of the modified method demonstrate clearly that in most cases such a cell is still alive and can live for a long time. This method allow express the yeast lifespan in time unit, what is especially important because, for the sake of comparison, the results should be presented in those same units also in the case yeast.

We have already shown that the time of life of various mutants does not correlate with the value of RLS and is comparatively stable [18]. The only pattern noticed was negative correlation of the post-reproductive lifespan (PRLS) of yeast cells (the length of life of the cell after bearing the last daughter) and the number of daughters produced (RLS). Thus, the sum of the time when a yeast cell reproduces and the time it stays alive after producing its last daughter gives the total lifespan (TLS). The proposed procedure allows for the comparison of the time of life of yeast cells with longevity of animals.

One of major achievements of yeast gerontology was isolation of "longevity mutants" and discovering that deletion of homologous genes increased the longevity of *Caenorhabditis elegans* expressed in units of time [19]. However, lack of uniformity of the units in which both longevities are expressed suggests that an analysis of the time of life of various yeast "longevity mutants" is required.

The aim of the studies was to test whether the longevity phenotype of a selected yeast strain expressed as a number of daughters produced would be present if it were expressed in units of time.

## 2. Materials and methods

### 2.1. Yeast strains and growth conditions

All yeast strains used in this study are listed in Table 1. Yeast cells were grown in a standard liquid YPD medium (1% Difco Yeast Extract, 1% Yeast Bacto-Peptone, 2% glucose) on a rotary shaker at 150 rpm, or on a solid YPD medium containing 2% agar (reproductive potential), or on a solid YPD medium containing 2% agar containing Phloxine B (total lifespan). The experiments were carried out at a temperature of 28 °C.

### 2.2. SCH9 gene knockout

In order to perform the *SCH9* gene disruption the standard method was used. Deletion cassette was amplified by PCR. DNA for the reaction was isolated from the deletion mutants of the SP-4 *sch9Δ* strain (*mutant perform by Mateusz Molon in Doctoral thesis*). For amplification were used primers: kanMX4 (inside deletion cassete): GGATGTATGGGCTAAATGTACG; sch9-forward: CAGCTCTCTTCTACTTTATATACC; sch9-reverse: CTTTGGGAAATCACAGCGAAGC; sch9-verification (above to forward primer): AGGCTTACTTATTCACATTACGGG.

Mutants were selected on YPD medium containing geneticin (G-418) at a final concentration 200 μg/ml. DNA was isolated from colonies that grew on the medium with geneticin. Then the mutants were verified by PCR method using kanMX4 and sch9-verification primers.

### 2.3. Determination of reproductive potential

The replicative lifespan of individual yeast cells was determined microscopically by a routine procedure using a micromanipulator [20]. The number of buds formed by each cell signifies its reproductive potential.

### 2.4. Determination of total lifespan

The lifespan of the yeast *S. cerevisiae* was determined as described previously [17,21]. Five microliters of an overnight grown culture of yeast was collected and transferred on YPD plates with solid medium containing Phloxine B at the concentration of 10 μg/ml. In each experiment, forty single cells were analyzed. The total lifespan was calculated as the sum of the reproductive and post-reproductive lifespans [21]. During the manipulation, the plates were kept at 28 °C for 16 h and at 4 °C during the night. The data represent the mean values from two separate experiments.

### 2.5. Statistical analysis

The results represent the mean ± SD values for all cells tested in two independent experiments (80 cells). The differences between the mutant strain compared to the wild-type strain were estimated using a one-way ANOVA and Dunnett's *post-hoc* test. The values were considered significant if $p < 0.01$. Statistical analysis was performed using the Statistica 10 software.

## 3. Results

For the longevity analysis, we selected mutants: *fob1Δ, sch9Δ, rpl20bΔ, tor1Δ, hxk2Δ* representing major functional groups listed in the yeastgenome.org database. To the group of analyzed yeast strains has also been added the *sfp1Δ* mutant, as a representative of mutations regulating the translational system level. Sfp1p is a transcription factor that controls the expression of at least 60 genes involved in ribosome assembly [22].

Fig. 1 presents the data concerning two most important parameters of yeast cells describing the phenomenon known as "replicative aging" of yeast cells. The left panel (A) describes the budding lifespan denoting RLS expressed as a number of daughters produced. The right panel (B) describes the total lifespan (TLS) expressed in units of time. For all tested yeast mutants the difference in the number of generation is statistically significant. However, the significant differences observed in the number of buds (replicative lifespan) between the standard strain BY4741 and the tested mutants become much less significant when the length of life is expressed in units of time (Fig. 1, Table 2). The observed differences are not statistically significant. Only in the case of deletion of the *SFP1* gene can we observe a significant increase of lifespan expressed in units of time (Fig. 1B, Table 2). It is very interesting that one of the mutant strains known as "longevity" (e.g. *rpl20bΔ*), have shorter total lifespan than the wild type strain (Fig. 1B, Table 2). The total lifespan of the yeast cell consists of two phases: reproductive and postreproductive, the duration of which may show significant differences depending on the type of mutant. The obtained results show a statistically significant prolongation of

**Table 1**
Yeast *S. cerevisiae* strains used in this study.

| Strain | Genotype | Source |
|---|---|---|
| BY4741 | MATa *his3 leu2 met15 ura3* | *EUROSCARF* |
| *fob1Δ* | BY4741 *YDR110W::kanMX4* | *EUROSCARF* |
| *sch9Δ* | BY4741 *YHR205W::kanMX4* | *in this study* |
| *rpl20bΔ* | BY4741 *YOR312C::kanMX4* | *EUROSCARF* |
| *tor1Δ* | BY4741 *YJR066W::kanMX4* | *EUROSCARF* |
| *hxk2Δ* | BY4741 *YGL253W::kanMX4* | *EUROSCARF* |
| *sfp1Δ* | BY4741 *YLR403W::kanMX4* | *EUROSCARF* |

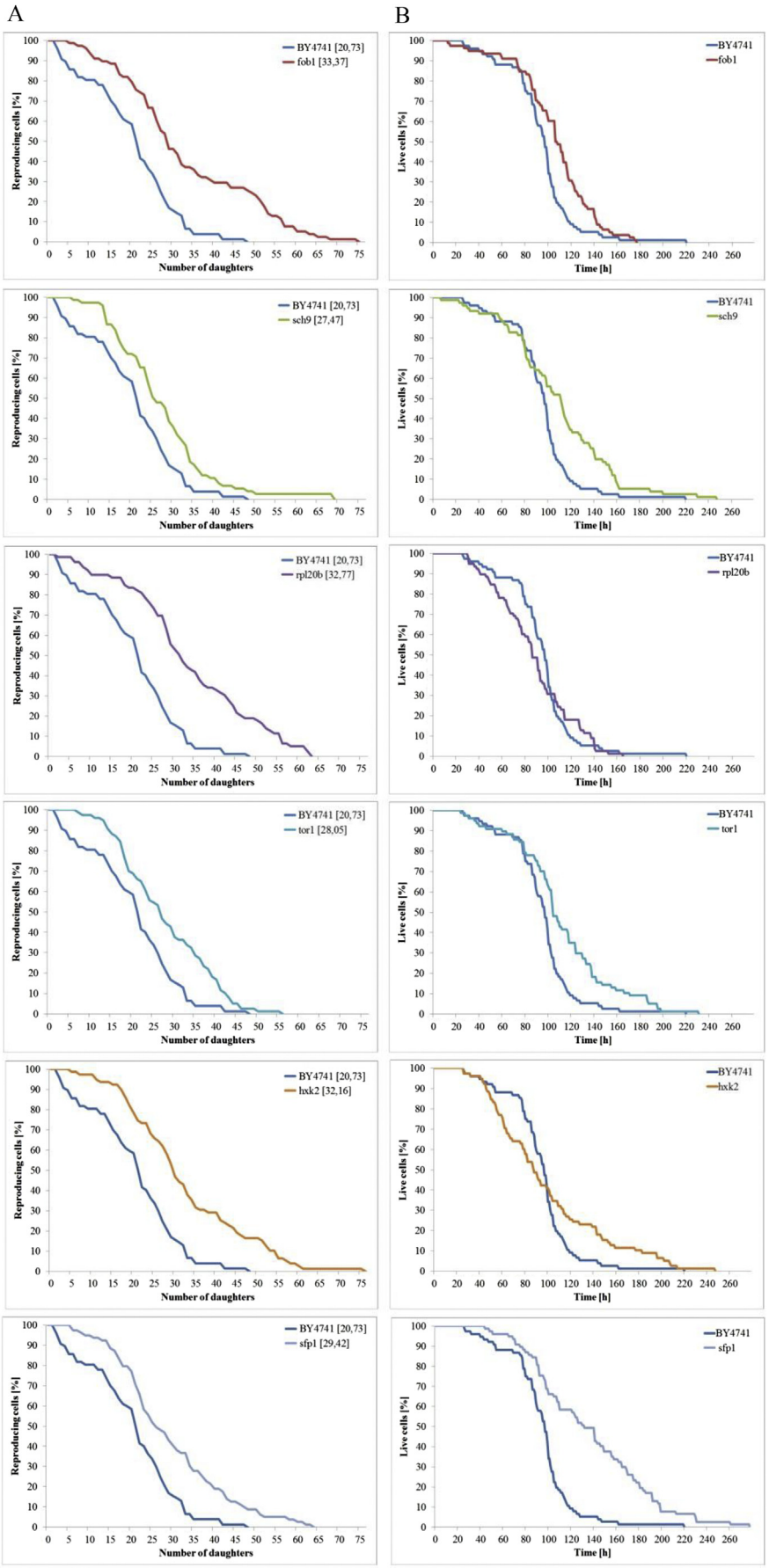


**Fig. 1.** Comparison of the reproductive potential (A) and the total lifespan (B) of the haploid wild type yeast strain BY4741 and isogenic mutant strains *fob1Δ*, *sch9Δ*, *rpl20bΔ*, *tor1Δ*, *hxk2Δ* and *sfp1Δ*. In parentheses are shown the mean value of the reproductive potential.

**Table 2**
Mean reproductive potential (number of generations), Reproductive lifespan, Postreroductive lifespan and Total lifespan of the Yeast Strains Studied (Mean ± SD, Combined Data from Duplicate Experiments). ***p < 0.01 compared to the wild type strain.

| Strain | Reproductive potential (number of generations) | Reproductive lifespan (h) | Postreroductive lifespan (h) | Total lifespan (h) |
|---|---|---|---|---|
| BY4741 | 20.73 ± 10.43 | 45.5 ± 28.9 | 49.4 ± 31.9 | 94.9 ± 28.03 |
| *fob1Δ* | 33.37 ± 16.4*** | 69.9 ± 37.6*** | 35.8 ± 29.9*** | 105.7 ± 32.8 |
| *sch9Δ* | 27.47 ± 11.58*** | 55.8 ± 29.2 | 52.8 ± 43.6 | 108.6 ± 45.1 |
| *rpl20bΔ* | 32.77 ± 14.99*** | 63.1 ± 28.6*** | 23.2 ± 31.2*** | 86.2 ± 33.7 |
| *tor1Δ* | 28.05 ± 10.88*** | 67.01 ± 34.34*** | 44.28 ± 43.90 | 111.30 ± 41.86 |
| *hxk2Δ* | 32.16 ± 14.25*** | 64.97 ± 32.97*** | 34.27 ± 37.60*** | 99.24 ± 50.27 |
| *sfp1Δ* | 29.42 ± 13.13*** | 98.4 ± 48.1*** | 37.3 ± 37.4 | 135.7 ± 51.5*** |

the "reproductive lifespan" in case of *fob1Δ, rpl20bΔ, tor1Δ*, *hxk2Δ* and *sfp1Δ* mutants, but not in case of *sch9Δ* mutant. The most increase in this parameter can be observe in case of *sfp1Δ* mutant. This difference in reproductive lifespan was approx. 60% in comparison to the wild-type strain. This may be due to the extension of the time duration of the single reproductive cycle. The post-reproductive lifespan as a second phase of the total lifespan mean the time from the last reproductive cycle to the cell death and is inversely proportional to the reproductive potential. Our results show that for almost all tested mutants, the exception of *sch9*Δ, the postreproductive lifespan is shortened in comparison to the wild-type strain. However, observed differences are statistically significant for *fob1Δ, rpl20bΔ, hxk2Δ*, whereas in case of *sch9Δ, tor1Δ*, *sfp1Δ* these differences are not statistically significant. The presented results cast doubt on the meaning of the term "longevity" used in the yeast gerontology studies. The most surprising thing is that among of analyzed "longevity mutants" do not appeared the longevity phenotype expressed in units of time used in animal gerontology.

In view of the above, we also collected all the data concerning the strains in question, including the data concerning the "short lived mutants" (Fig. 2A and B). Fig. 2A shows that the time of life (TLS) of strains is much more stable than the RLS value. The value of the trend line shows virtually no correlation as the correlation coefficient equals 0.01.

Fig. 2B presents the data on post-reproductive lifespan (PRLS) of all strains tested. It appears that the negative correlation between PRLS and RLS is evident, although the tested strains strongly differ in genetic background. The trend line suggests a strong negative correlation between these parameters. The value of the correlation coefficient is −0.84.

## 4. Discussion

The opinions on the origin of the reproduction limit of the budding yeast and consequently the classification of the phenomenon have changed since its discovery in 1959 [1]. The authors of the discovery considered the phenomenon to be the consequence of accumulation of chitin-saturated structures, or bud scars, formed in yeast within the mother cell wall during each mitotic cycle [23–25], as the rigidity of the structures prevents formation of subsequent buds in the same place [1]. It was assumed that the surface of cell membranes covered with the bud scars is less active metabolically and when a substantial part of the cell surface is covered with the scars, the cell ceases to reproduce. However, the authors also noticed that the volume of such cell increases, which can additionally disturb proliferation. A similar conclusion was drawn from studies conducted by another group of scientists twenty years later, where it was suggested that the limit of reproduction resulted from the budding process, i.e. the cytokinesis mechanism specific to that group of unicellular fungi [26]. Such group-specific features are now considered "private", in contrast to the "public" universal features, somewhat precluding the use of yeast as a model organism in gerontology [27].

Despite such discouraging views, yeast became such a model organism in gerontological studies, since it was taken for granted that the cell reproduction limit is a consequence of accumulation of a hypothetical universal "senescence factor" within the mother cell [2]. However, despite many studies, the nature of the factor is still illusive [28,29]. In the studies of the yeast replicative lifespan (RLS), age and longevity are expressed primarily as the number of daughter cells produced by the mother cell. The use of such unit of age and longevity of yeast which is unique for gerontology has been challenged since the very beginning of the studies [16]. Expressing the age and longevity of yeast in a number of daughters suggested

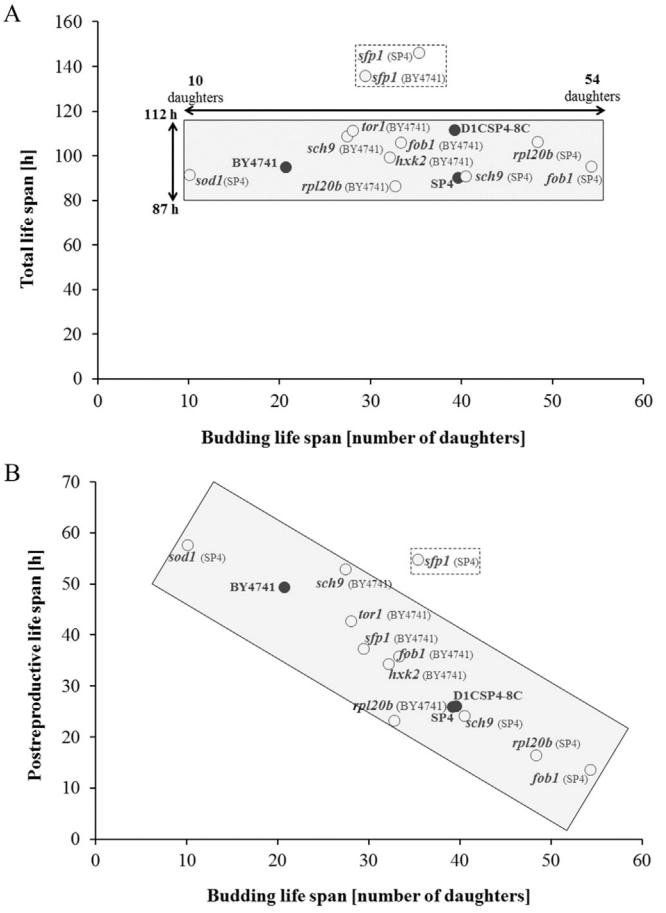


**Fig. 2.** Relationship between the mean number of daughters (RLS) and the mean lengths of the total lifespan (A) and post-reproductive lifespan (B). Yeast mutants are represented by open circles and their wild-type counterparts by solid circles.

that the focus was on fecundity rather than longevity; for that reason, it was later proposed that the length of a cell's reproductive phase should be published, since such information was available in relevant protocols [17]. Unfortunately, the suggestion was not followed. Results presented in numerous papers show that a number of genes or factors tested (e.g. chemical or environmental factors) can modulate reproductive potential of yeast cells (e.g. increase the number of daughters produced), yet their effect on the length of life is unknown since the number of daughters is not a unit of time. Therefore, an analysis of the time of life is required in order to verify whether genes or other tested factors contribute to the increased time of life or whether they only increase the reproductive potential of cells. For that reason we applied the method of establishing when a yeast cell dies [17,21], in order to evaluate longevity of yeast cells in units of time, thus allowing for the comparison with animal longevity.

Looking at the presented results, the following conclusions can be drawn. None of the analyzed "longevity mutants" lived substantially longer than the standard strain. On the basis our studies, only one of the deletion mutants *sfp1Δ* lived for a longer time, but that particular mutant had been earlier considered "short lived" [30]. Its long generation time suggests that longevity could be a simple consequence of a higher number of daughters produced, along with the extended cell cycle. This result may be so controversial, especially in comparison with result Herren et al. [30], but our unpublished data show, that longevity effect of this gene is strongly depend on the genetic background. In BMA64-1A genetic background deletion of *SFP1* gene leads to a significant reduce of the reproductive potential, whereas in SP-4 genetic background [31] has no effect on the number of daughter cells produced (*data unpublished*).

The conclusion that mutations resulting in "longevity" of yeast do not actually cause longevity *sensu stricto* casts doubt on the usefulness of this particular organism as a model in gerontology. Such a controversial conclusion calls for an analysis of the dependence of the length of life of yeast on the RLS value. We therefore analyzed the available data and presented them in Fig. 2A and B. The data presented in Fig. 2 confirmed the previous finding that the post-reproductive lifespan (the time that passes from the last generation until cell death). of yeast negatively correlates with RLS. This simply means that the time of life of the mother after it produces its last daughter strongly depends on fecundity of the former. High fecundity mutants die soon after forming their last daughters. In the case of other organisms, there is an inverse relationship between the number of offspring and the maximum lifespan [32]. For yeast cells, the inverse relationship is observed only between the number of daughters and the post-reproductive lifespan. The number of daughters produced by the mother cell has little influence on the total lifespan. However, dependence of the length of life (TLS) on the RLS value (Fig. 2A) allows for the following conclusions. The differences in TLS are much lower than the differences in RLS. Most of the strains studied so far lived between 87 and 112 h, whereas the differences in the RLS values of those strains were up to five times greater. These differences do not depend of genetic background of the standard strains. The only exception is the *sfp1Δ* strain living much longer. The conclusion is that the time of life of yeast cells is relatively constant, irrespective of the presence of various deletion mutations strongly influencing the value of RLS. Consequently, the value of RLS can no longer be treated as a measure of longevity of yeast cells.

The main argument supporting usefulness of yeast as a model organism in gerontology was based on the finding that deletion of homologous genes results in longevity *sensu stricto* of yeast and *C. elegans* [33]. However, the presented data show that even the flagship "longevity mutants" do not live longer than the "short lived mutants" [18,21].

One can conclude, therefore, that the postulate [34,35] envisaging the use of yeast to reveal universal mechanisms of aging and longevity finds no experimental support. Mechanisms of aging can be in general divided into two main groups, namely "public" and "private" [27]. Public mechanisms of aging refer to highly conserved genes and metabolic pathways that are similar in all organisms. One of key examples may be the *TOR* kinase-encoding gene that regulates many physiological and cellular processes, including metabolism, growth, and aging [36]; another example is *S6K1* which regulates mRNA translation via phosphorylation of several substrates in ribosomal protein subunit 6 [37]. Many studies indicate that inhibition of S6 kinase, TOR or ribosomal protein subunits contributes to increased lifespan in many species [13,38,39].

Furthermore, minor differences in the total lifespan of various yeast mutants strongly suggest that death of yeast cells has a quasi-programmed character and is independent on the processes associated with aging. The earlier postulates [1,26] as well as the hypertrophy hypothesis [40,41] suggest the causative role of the choice of budding as the mechanism of cytokinesis in determining the reproduction limit of yeast cells. This opinion is consistent with the postulated hyperfunction theory proposed by Mikhail Blagosklonny [42] and later modified by Gems and de la Guardia [43]. It suggests that aging is not the result of accumulation of molecular damages but is caused by hyperfunction, especially excess biosynthesis, which leads to hypertrophy, whereas hypertrophy leads to dysfunction and consequently to increased mortality.

The presented opinions further support the paradigm shift of cellular senescence of human somatic cells [44]. Hence the explanation of the molecular mechanisms leading to cessation of cell reproduction and eventually to cell death would require a completely new experimental approach.

## Conflict of interest

The authors declare that they have no conflict of interest.

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## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.03.085.

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