Inactivation of *RAD52* and *HDF1* DNA repair genes leads to premature chronological aging and cellular instability

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The present study aims to investigate the role of radiation sensitive 52 (*RAD52*) and high-affinity DNA binding factor 1 (*HDF1*) DNA repair genes on the life span of budding yeasts during chronological aging. Wild type (wt) and *rad52*, *hdf1*, and *rad52 hdf1* mutant *Saccharomyces cerevisiae* strains were used. Chronological aging and survival assays were studied by clonogenic assay and drop test. DNA damage was analyzed by electrophoresis after phenol extraction. Mutant analysis, colony forming units and the index of respiratory competence were studied by growing on dextrose and glycerol plates as a carbon source. *Rad52* and *rad52 hdf1* mutants showed a gradual decrease in surviving fraction in relation to wt and *hdf1* mutant during aging. Genomic DNA was spontaneously more degraded during aging, mainly in *rad52* mutants. This strain showed an increased percentage of revertant colonies. Moreover, all mutants showed a decrease in the index of respiratory competence during aging. The inactivation of *RAD52* leads to premature chronological aging with an increase in DNA degradation and mutation frequency. In addition, *RAD52* and *HDF1* contribute to maintain the metabolic state, in a different way, during chronological aging. The results obtained could have important implications in the chronobiology of aging.

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1. Introduction

The process of aging and cellular senescence is a universal and multifactorial phenomenon, which takes place at all levels of biological organization (from genes to cells, tissues, systems and to the whole organism). Since the basic cellular mechanisms involved in the aging process are similar in all species, they can be extrapolated to humans. According to the genetic theory, genes are responsible for maximum lifespan, although only between 25% and 35% of longevity could be blamed on them. The rest would correspond to environmental factors, as demonstrated by McGue *et al.* (1993) in a study of Danish human twins, and Ljunqquist *et al.* (1998) in a study of Swedish identical twins.

In the 1940s scientists investigated the role of mutations in aging. The evidence that supported this idea came from the exposure to ionizing radiation. It was observed that radiation increases the frequency of genetic mutations and accelerates the aging process. However, later studies showed that radiation induces changes that mimic the changes of age. This finding diminished its effect when experiments with rats subjected to moderate amounts of radiation induced an increase in life expectancy of these animals (Warner *et al.* 1987).

Different theories of aging have been proposed. However, none of them explains the process in its entirety, since aging is considered to be a multifactorial and multicausal process (Warner *et al.* 1987). Aging is associated with two joint processes, progressive cell degeneration and the loss of regenerative capacity. Both processes occur in each stage of life and remain in a perfect balance under normal conditions. The process of aging happens to all the levels: from the molecular level, to the cellular and organ level (Mercado-Sáenz, *et al.* 2010).

Keywords. Aging; DNA breaks repair; *HDF1*; *RAD52*; senescence; yeast

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Among the model organisms currently in use for biogerontological research *S. cerevisiae* has a special importance. It is an eukaryotic organism (haploid or diploid) that conserves many basic molecular, genetic and metabolic pathways with high similarity to human cells, including tumors (Ruiz-Gómez *et al.* 2010a).

Two different forms of aging processes can be studied in yeast (chronological and replicative). A system to measure yeast longevity based on chronological longevity of a population of non-dividing yeast (chronological life span) have been developed previously by other authors and we have optimized it for our laboratory conditions (Maclean *et al.* 2003; Qin and Lu 2006). Yeasts have evolved to survive under adverse conditions such as starvation. Wild type strains are likely to exist in stationary phase only if all the nutrients required for growth become available. For this reason the aging studies are performed in either a medium containing no nutrients or limited concentration of them, such as water or synthetic dextrose complete (SDC) (Fabrizio *et al.* 2005a, b).

The alteration of cell physiology, metabolism and the accumulation of oxidative damage (detectable in both the genomic DNA and in the mitochondrial DNA), are produced spontaneously during life or by the action of chemical and physical agents (Ruiz-Gómez *et al.* 1999; Laqué-Rupérez *et al.* 2003; Ruiz-Gómez and Martínez-Morillo 2005). They could be considered as important factors in the development and progression of cancer and other age-related diseases. In this way, the inactivation of repair enzymes plays an important role (Maclean *et al.* 2003). Although some authors reported an increased mutation accumulation during aging, other authors found that DNA mutations do not cause aging (Kaya *et al.* 2015).

Basic carbon metabolism and intermediary metabolism play an important role in the lifespan of yeast. In this way, lowering glucose is well known to stimulate mitochondrial respiration. As a consequence, low glucose leads to increase both replicative and chronological life span (Breitenbach *et al.* 2012).

It has been reported that glycolytic/fermentative genes are upregulated in *Sch9*Δ, *Ras2*Δ and *Tor1*Δ mutants, while mitochondrial genes are down-regulated. In this way, it has been found that long-lived mutants show a general activation of glycerol biosynthetic genes (Longo and Fabrizio 2012). A connection between the molecular mechanisms that control replicative and chronological aging has been observed. Fabrizio *et al.*, (2004a, 2005a, b) found than Sir2 and Msn2/4 may play opposite roles in controlling both types of aging.

The intracellular signals in response to DNA damage and stress conditions lead to cell cycle arrest and enhance DNA repair processes and the activation of alternate biochemical pathways.

Cell cycle delay occurs during aging in response to the damage induced by reactive oxygen species (ROS) to allow the cells to repair. This mechanism is dependent of different proteins such as Rad9p, Swi6p, the expression of cyclins, the activation of cyclin–dependent kinases, etc., (Burhans and Heintz 2009; Aung-Htut *et al.* 2012).

One of the factors postulated to drive the aging process is the accumulation of DNA damage. The human disorder trichothiodystrophy (TTD) exhibit many symptoms of premature aging, including reduced life-span. TTD cells carrying additional mutations which enhance the DNA repair defect show a greatly accelerated aging phenotype, which correlates with an increased cellular sensitivity to oxidative DNA damage (Diderich *et al.* 2012).

According to the free radical theory of aging, this phenomenon is produced by the damage induced by ROS, generated mainly in mitochondria. In this way, the overexpression of antioxidant enzymes leads to a chronological life extension. In addition, an accumulation of oxidation-induced DNA damage and mutations has been observed in aged yeast (Longo and Fabrizio 2012).

Premature aging syndromes in humans, often produced by mutations, involve the incorrect maintenance of genomic integrity (Liu *et al.* 2005). Among the genes that affect aging are those that regulate DNA repair, telomere length, regulation of cellular stress response, etc. (Allen 1996).

The molecular mechanisms that regulate cellular aging are still poorly understood. Recent studies on premature aging syndromes suggest that the maintenance of genomic integrity is important to prevent the acceleration of the aging process (Busuttil *et al.* 2004). Progeroid syndromes (Werner's syndrome, Hutchinson-Gilford, Bloom, etc.) show clinical symptoms of premature aging, genetic instability, and an elevated cancer predisposition (Coppedè and Migliore 2010).

Whether the integrity of the genome is not restored, apoptotic cell death is activated to prevent the proliferation of severely damaged cells (Kitanovic and Wölfl 2006). In this way, chronologically aged yeast cultures die by apoptosis showing the accumulation of oxygen radicals, caspase activation (Herker *et al.* 2004) and the accumulation of oxidatively damaged proteins (McMurray and Gottschling 2004).

It has been reported in different model organisms and human cells an age-dependent accumulation of DNA mutations and DNA damage affecting DNA repair genes, caused mainly by oxidative DNA damage. In this way, mutations in p53 and in mitochondrial and cytosolic superoxide dismutase (SOD) have been found (Busuttil *et al.* 2005). These mutations are associated with a reduction in genomic instability. Moreover, *Sch9* mutants have been found to enhance genomic stability by down–regulating the expression of antioxidant genes, the activation of repair systems and the

regulation of the carbon metabolism (Longo and Fabrizio 2012). In addition, it is well stablished that the majority of mitochondrial proteins are encoded in the nucleus. A wide class of mutants with defects in mitochondrial function affects the transcriptional regulation of these nuclear genes. The level of the mitochondrial function is linked to the nuclear gene expression by retrograde regulation or signaling, involved by *RTG2*, *RTG1* and *RTG3* (Chen and Runge 2012).

Double-strand breaks (DSB) are repaired/rejoined in *S. cerevisiae* and mammalian cells in different pathways: homologous recombination (HR), single-strand annealing, and more or less direct end joining called illegitimate recombination or nonhomologous end joining (NHEJ). Among these three mechanisms observed in yeast and mammalian cells, the HR is the predominant mechanism in *S. cerevisiae* (Friedl *et al.* 1998). We have used as a model organism the haploid and wild type (wt) yeast *S. cerevisiae* and three derivated strains mutated for the genes of high affinity DNA binding factor 1 (*HDF1*)(=subunit of the ku complex [YKU70]), radiation sensitive 52 (*RAD52*) and their double combination (Ruiz-Gómez *et al.* 2010b). *Rad52* mutant cells cannot repair DSB via HR and show reduced ability for single-strand annealing (Friedl *et al.* 1998). *HDF1* is the yeast homolog of the Ku70 gene found in mammalian cells. Hdf1 protein forms a heterodimer with Ku80 homolog that binds with high affinity to DNA ends in DSB, resulting that NHEJ pathway is affected in *hdf1* mutant cells (Milne *et al.* 1996). In addition, in *rad52 hdf1* double mutants, all pathways of DSB repair or rejoining mechanisms are blocked (Friedl *et al.* 1998).

The inactivation of these genes contributes to an accelerated replicative aging but little is known about their influence on chronological aging as it is described in the Basic Biology of Aging database at the University of Washington: [www.uwaging.org]; where it can be downloaded the data sheet for *RAD52*: (*www.uwaging.org/genesdb/gene.php?id=122*), and for *HDF1 gene*: (*www.uwaging.org/genesdb/gene.php?id=122*). Little is known about the relationship between *rad52* and *hdf1* deletion and the metabolic status of yeast cells.

In addition, the *Saccharomyces* genome database [www.yeastgenome.org] shows that the disruption of *HDF1* decrease the replicative and chronological life span, and the inactivation of *RAD52* decrease the replicative life span but nothing is known about the chronological aging.

The called 'White petite' mutant is a class of mitochondrial mutant in which mitochondria are not functional. By having non-functional mitochondria, these cells can not get their energy through respiration (aerobic), so it must necessarily rely on the fermentation (anaerobic). It allows distinguishing them from wild strains, as they are unable to grow using glycerol as a carbon source. The index of respiratory competence (IRC) is the percentage of oxidative cells in relation to the total cells (oxidative + fermentative).

Little is known about the role of the DNA repair mechanisms, NHEJ and HR, in the chronological aging and genome stability, which could have important implications also in cancer induction and progression.

The present study aims to investigate the role of the NHEJ and HR DNA repair mechanisms on the life span of budding yeasts, the genome integrity, the spontaneous mutation frequency and the index of respiratory competence of cells, during the process of chronological aging of *S. cerevisiae*.

2. Material and methods

2.1 *Yeast strains and culture medium*

The experiments were carried out with the haploid yeast strain *S. cerevisiae* WS8105-1C and the *hdf1*, *rad52* and *rad52 hdf1* derivatives; listed in Table 1. These strains were supplied by Dr A. A. Friedl (Radiobiological Institute, Ludwig-Maximilians-Universität, Munich, Germany). Yeast cells were grown in Yeast extract-Peptone-Dextrose (YPD) broth (1% Bacto-yeast extract, 2% Bacto-peptone, 2% dextrose) with or without 2% Bacto-agar (Friedl *et al.* 1998).

To study chronological aging, yeast cells were grown in a SDC medium (2% dextrose, 0.17% yeast nitrogen base, 0.5% ammonium sulphate, 0.15% aminoacids mixture, 4X auxotrophic markers [ade, arg, trp, ura]) (Fabrizio and Longo 2003).

To test the mutation frequency and the IRC, yeast cells were grown on a medium of Yeast extract-Peptone-Glycerol (YPGlyc) (containing a nonfermentable carbon source) (1% Bacto-yeast extract, 2% Bacto-peptone, 3% (*v*/v) Glycerol 99%) with 2% Bacto-agar (Wauters *et al.* 2001).

All reagents from Difco, Becton Dickinson and Co. Sparks, MD, USA).

Table 1. Yeast strains used in this study

Strain	Genotype
WS8105-1C	*MATα RAD HDF ade2 arg4-17 trp1-289 ura3-52*
WS8105-1C *hdf1Δ*	*MATα RAD hdf1Δ::URA3 ade2 arg4-17 trp1-289 ura3-52*
WS8105-1C *rad52Δ*	*MATα rad52::TRP1 HDF ade2 arg4-17 trp1-289 ura3-52*
WS8105-1C *rad52Δ hdf1Δ*	*MATα rad52::TRP1 hdf1Δ::URA3 ade2 arg4-17 trp1-289 ura3-52*

2.2 *Chronological aging*

Prior to start with the chronological aging, yeast cells were cultured to reach the stationary phase in SDC medium. From fresh YPD plates from each strain, one loop of cells was inoculated in 1 mL of SDC. After measuring the optical density at 600 nm (OD600), 20 mL SDC for each strain was inoculated with 1,500,000 cells mL^{-1} in four separate flasks. The medium was in a flask at a volume ratio of 1:5. Then, they were cultured during 4 days at 30°C, 300 rpm. During this time the number of cells was counted to be sure that the culture reaches the stationary phase. When early stationary phase was reached, it was the moment when the aging experiment began really, considering this day as day 0 in the experiment. During the aging process the strains were maintained at 30°C, 300 rpm. Viability was studied regularly by clonogenic assay and drop test, during 20 days (Fabrizio and Longo 2003).

2.3 *Cell density*

The number of cells was monitored daily by measuring the OD600 (Helios ε, Unicam, Cambridge, UK). The OD600 values less than 1.0 are widely known to be linear with the number of cells for *S. cerevisiae*.

2.4 *Cell cycle analysis*

The cell cycle distribution was measured by light microscopy (400 X) (Nikon Instech Co., Kanagawa, Japan). A morphological analysis of a portion of the samples prior to the chronological aging was performed classifying the cells as 'single cells (G0/G1) (cells with no buds), small buds (S) (cells in which the bud size was less than one-third the size of the mother cell), large buds (G2+M) (cells harbouring larger buds) and others (cells having multiple or protruded buds)' (Umezu *et al.* 1998).

2.5 *Survival assays*

Yeast survival was assayed by the drop test and clonogenic assay. For qualitatively assay (drop test), six 10-fold serial dilutions from each strain were prepared and five-microliter aliquots of each dilution were spotted onto YPD plates (Umezu *et al.* 1998). Then, the plates were incubated for three days at 30°C. For quantitatively evaluation, serial dilutions from each sample were prepared in sterile water and aliquots were spread on YPD plates. Then they were incubated for three days at 30°C before counting colonies. Then, the number of colonies grown was counted and the surviving fraction calculated. Samples seeded on YPGlyc plates were cultured for 10 days at 30°C. Dilutions with 10–30 colonies grown for drop test and with 30–300 for quantitatively evaluation were scored and compared as representatives to calculate the surviving fraction (Ruiz-Gómez *et al.* 2008).

2.6 *DNA damage analysis*

After chronological aging yeast genomic DNA was prepared by Phenol-Chloroform extraction and ethanol precipitation, according to the protocol describe by Hoffman and Winston (1987). Briefly, yeast cells were resuspended in lysis buffer containing 10 mM Tris/Cl Ethylenediaminetetraacetic acid (EDTA) pH 8.0, 2% Triton X-100, 1% lauryl sulphate (SDS) and 0.1 M NaCl. Then, 0.3 g of 0.45 mm glass beads and 0.2 mL of Phenol-Chloroform-isoamyl alcohol (25:24:1) were added. Samples were vortexed for 3 min and centrifuged 5 min at 15,300*g*. Aqueous phase was transferred to a clean eppendorf tube and DNA was precipitated with 100% ethanol. After centrifugation at 15,300 *g* for 2 min, the pellet was treated with RNase A 50 μg mL^{-1} (65°C, 10 min). Then, 0.1 mL 10 N ammonium acetate and 1 mL 100% ethanol were added. Samples were centrifuged again and the pellets washed briefly with 70% ethanol. DNA isolated was resuspended in 30 μL Tris/Cl-EDTA pH 8.0 buffer. DNA was electrophoresed in a 0.8% agarose gel (in 0.5 X Tris-Borate EDTA) at 80 V, 90 min. The gel was stained with ethidium bromide 10 mg mL^{-1} (López-Díaz *et al.* 2014). All reagents used in this section were from Sigma-Aldrich Co., St Louis, MO, USA). The DNA molecular weight marker used was Hyperladder I (Bioline Ltd, London, UK).

2.7 *Mutant analysis and colony forming units (CFU)*

The study of the spontaneous mutations during aging was possible due to the markers incorporated in the yeast genome (*ade2, arg4-17, trp1-289, ura3-52*), which makes the colonies red. When the genotype reverses to the wild type, the colonies appear in white and they are called 'revertants'. Another spontaneous mutation that can appears is the so-called 'white petite' that is characterized by the growth of small white colonies, due to the lower growth capacity caused by a mutation in mitochondria and the absence of growth on a nonfermentable carbon source. During the aging period, 300 cells from each sample were spread on YPD and YPGlyc plates and incubated at 30°C during 3 and 10 days respectively. Then, the number of colonies of each type was scored. Colony-forming units (CFU) percentages were calculated comparing the number of colonies grown from each type to the number of total colonies grown (Parrella and Longo 2008; Ruiz-Gómez *et al.* 2010a).

2.8 *Index of respiratory competence (IRC)*

The days 0, 7, 14 and 20 of aging, cells were seeded on YPD and YPGlyc plates. Then, they were cultured for 3–5 days at 30°C for YPD plates and 10 days at 30°C for YPGlyc plates. Colonies grown were scored and IRC calculated as (Parrella and Longo 2008):

$$\mathrm{IRC} = \frac{\text{Colonies grown on YPGlyc}}{\text{Total colonies on YPD}}$$

2.9 *Statistical analyses*

The Wilk-Shapiro rankit plot test was used to assess the normal distribution of the data. Additional statistical analyses were made with the test of two way Analysis of Variance (ANOVA) (treatment x time) and the Student's *t*-test. *P*-values reported are those of treatment effects. Differences were considered significant when $p<0.05$. Experiments were performed independently from 3–6 times, with 2–8 samples (n=4–25). The software used was IBM SPSS Statistics 22, licensed for Universidad de Málaga.

3. Results

In order to study the role of *RAD52* and *HDF1* genes on the chronological aging of yeast cells, and therefore the evaluation of the HR and NHEJ DNA DSB repair pathways during the aging phenomenon, the survival of the wt control strain and the derivatives *rad52*, *hdf1* and *rad52 hdf1* was evaluated. In addition, genome integrity, the frequency of spontaneous mutation and the metabolic status during chronological aging were also assayed.

3.1 *Chronological aging in rad52 and hdf1 mutants*

Prior to study the chronological aging of cells by itself, a 4 days of pre-culture was performed to permit the synchronization of cells reaching the plateau phase with cell densities of 7.5×10^{7} cells mL^{-1} (wt), 10.5×10^{7} cells mL^{-1} (*hdf1* mutant), 14.6×10^{7} cells mL^{-1} (*rad52* mutant) and 14.7×10^{7} cells mL^{-1} (*rad52 hdf1* double mutant).

Then, surviving fraction was evaluated periodically during the aging of cultures. As shown in figure 1A, the survival of wt strain and *hdf1* mutant did not decrease along the period of aging (20 days) ($p>0.05$, ANOVA). The lower tendency in surviving fraction and also the lower bend from day 7 to 17, observed for wt strain in relation to *hdf1*, could be due to the wide dispersion of data points by random, since no significant differences were observed and the tendency lines mathematically adjusted to the points showed a similar behavior. On the other hand, perhaps the high increment in respiration of wt during aging (figure 4) could produce the increase in surviving fraction of this strain from day 17 to 20. In contrast, *rad52* and *rad52 hdf1* mutants showed a gradual decrease in surviving fraction, reaching values of 10^{-3} at the end of the process. The differences observed for these mutant strains in relation to wt were statistically significant ($p<0.001$, ANOVA) (figure 1A).

The disruption of the HR pathway in *rad52* and *rad52 hdf1* mutants induced a decrease in survival along the aging process, but without significant differences between them ($p>0.05$, ANOVA). However, a great significant difference in survival was observed in relation to wt strain and *hdf1* mutant (blocked for NHEJ pathway) ($p<0.001$, ANOVA) (figure 1A). Cells defectives in *rad52* and *rad52 hdf1* genes showed a 10-fold decrease in surviving fraction after 10 days of aging in relation to wt and *hdf1* strain. At the end of the aging period (20 days), the survival obtained for strains mutated for *rad52* was 1000 times lower than that obtained for wt and *hdf1* (figure 1A).

Figure 1B shows the different growth of wt and mutant strains, evaluated by drop test, at days 0, 9, 15 and 20 of aging. At the end of the aging period, the growth of strains defectives in *rad52* gene (*rad52* and *rad52 hdf1*) was very low in relation to strains not mutated for *rad52* (wt and *hdf1*). The effect was really clear at day 20 of aging.

In addition, it was observed that aged cultures showed an increase in the number of lobed colonies. The lobed appearance of colonies is indicative of the difficulty of cells to continue dividing as cellular senescence process progresses (figure 1C).

These findings suggest that *rad52* and therefore the HR DNA strand break repair pathway, contribute to a premature chronological aging when this gene is disrupted and cells cannot repair this type of damage. DNA DSB could be produced spontaneously during aging.

These results suggest that inactivation of the HR DNA DSB repair pathway leads to a premature chronological aging. The HR repair pathway could be the most important for maintaining genome integrity and prevent premature chronological aging.

3.2 *Aging and genome integrity in rad52 and hdf1 mutants*

In order to study the integrity of the genome and the role of the DNA strand breaks repair pathways by HR and NHEJ; and therefore the spontaneous appearance of DSB in DNA during aging, a genomic DNA extraction and subsequent agarose gel electrophoresis was performed during cellular aging.

The intact genomic DNA content appears in the electrophoresis as a clear band located above the 10,000 bp, whereas the loss of DNA can result in a band of lower intensity or its disappearance.

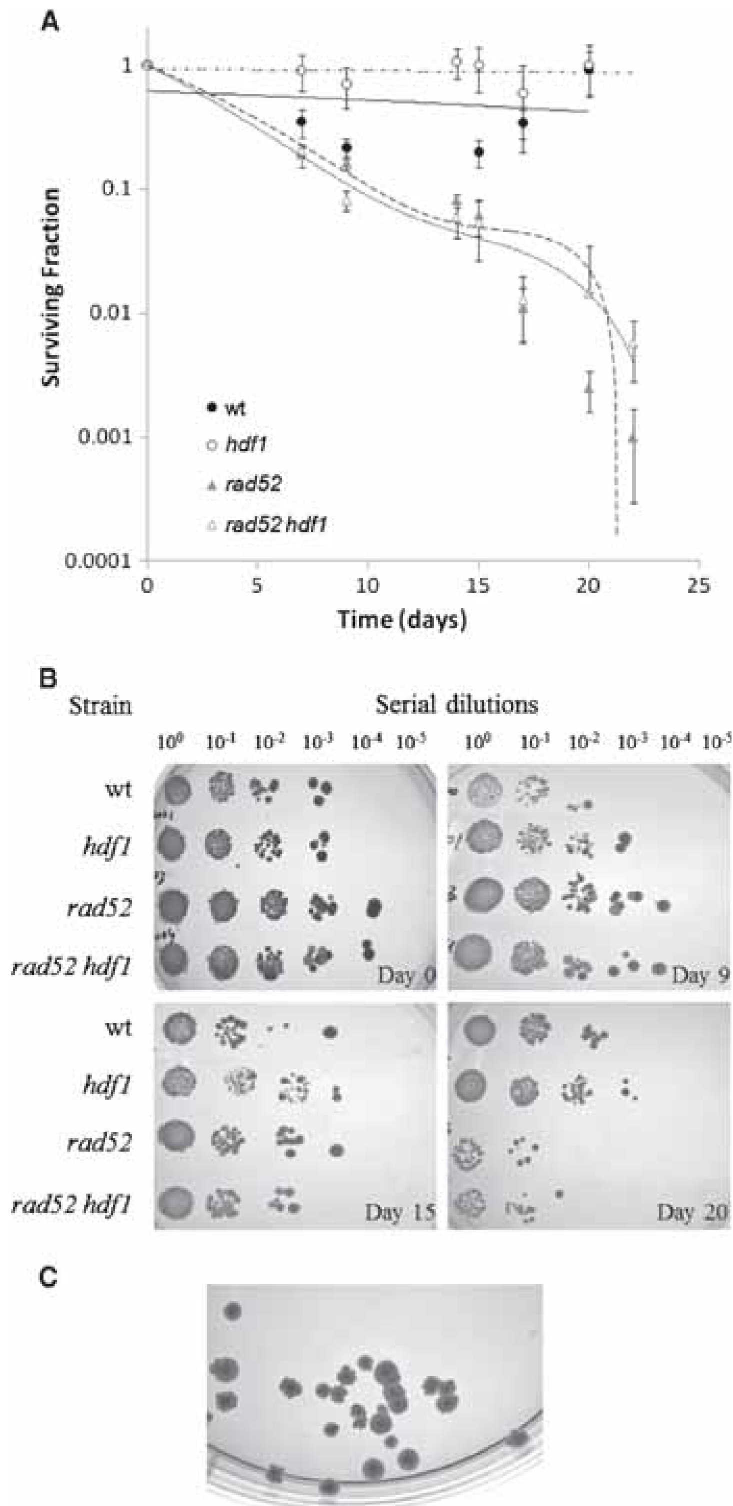

Figure 1. Chronological aging in wt, *hdf1*, *rad52* and *rad52 hdf1* yeast strains. (**A**) Surviving fraction obtained during 20 days of chronological aging. Mean±s.d. *hdf1 vs* wt: $p>0.05$; *rad52 vs* wt: $p<0.001$; *rad52 hdf1 vs* wt: $p<0.001$; *rad52 vs hdf1*: $p<0.001$; *rad52 hdf1 vs hdf1*: $p<0.001$; *rad52 vs rad52 hdf1*: $p>0.05$; ANOVA. (**B**) Chronological aging evaluated by drop test. (**C**) Aged colonies. The aging phenomenon resulted in the loss of the typical rounded shape of the colony, appearing lobed shapes indicative of chronological aging. wt: wild type (control); *hdf1*: mutant blocking the NHEJ pathway; *rad52*: mutant blocking the HR pathway; *rad52 hdf1*: double mutant blocked in both pathways. Experiments were performed 6 times; $n=25$.

As shown in figure 2, not aged controls wild type strain (wt), *hdf1*, *rad52* and *rad52 hdf1* mutants showed a clear band of genomic DNA (>10,000 bp) (day 0 of aging).

However, lower intensity bands were obtained during chronological aging for wt and *hdf1* strains (days 7, 14 and 20), in relation to their respective not aged controls (day 0). In addition, the genomic DNA bands corresponding to *rad52* mutants (*rad52* and *rad52 hdf1*) showed an important decrease in intensity at day 14 of aging and even disappeared at day 20 in *rad52* mutants. The decrease in the intensity of bands and their total disappearance are indicative of increased DNA degradation during chronological aging, in relation to the pattern obtained for wt control strain and for not aged cultures (day 0).

The results indicate that the process of chronological aging produces DNA DSB, which are generated spontaneously, and which cannot be repaired in *rad52* and *rad52 hdf1* mutants. DNA loss and even the disappearance of the whole DNA band is increased in aged *rad52* mutant. The sequential loss of DNA during aging suggests that chronological aging causes an alteration in the integrity of the genome due to defects in the repair of DNA strand breaks, mainly by HR (*rad52*) and in a lesser extent by NHEJ (*hdf1*). These results suggest that the HR DNA repair pathway could be the main repair mechanism operating in these strains during chronological aging. The typical pattern of DNA fragmentation by apoptosis was not observed.

3.3 *Aging and spontaneous mutation in rad52 and hdf1 mutants*

In order to assess the effect of aging on the frequency of spontaneous mutation in mutants unable to repair DNA DSB by HR and NHEJ, the frequency of occurrence of revertants and white petites were studied in the wild type (wt) strain and *hdf1*, *rad52* and *rad52 hdf1* mutants.

wt, *hdf1*, *rad52* and *rad52 hdf1* colonies are red/brown colored due to the incorporation of multiple auxotrophic markers into their genome. *ADE2* gene is the marker responsible for the red color. Wild type strains with *ADE2* active are white while *ade2* mutants accumulate a red pigment. Revertants lose the mutation and colonies reset to white. White petite mutants have a mutation in mitochondria, lose their function and colonies appear to be very small and white.

As can be seen in figure 3, the inactivation of the HR (*rad52*) and NHEJ (*hdf1*) repair pathways produced changes in the frequency of spontaneous mutation (revertants and white petites) (at day 0) in relation to the wild type (wt) control strain (p<0.05, ANOVA). *rad52* and *hdf1* mutant strains showed an increased percentage of revertant colonies, which was higher in *rad52* mutant strains (21% for *rad52*, 14.67% for *rad52 hdf1*, 6.46% for *hdf1* and 2.21% for wt). A lower frequency of white petites in relation to wt strain, mainly for *hdf1* strain (p<0.05, ANOVA), was also observed at day 0 (5.5% for wt, 2.95% for *rad52*, 1.6% for *rad52 hdf1* and 0.74% for *hdf1*).

In relation to the aging process an increase in the frequency of appearance of revertants was obtained at the end of aging (days 13 to 20) in *rad52* mutant strain (p<0.05, ANOVA). *rad52* mutant showed a 21% of revertants at day 0, 18.88% at day 7 of aging, 20.58% at day 13 and 33.14% at the end of aging (day 20). No significant changes in the frequency of revertant colonies during the process of aging were observed for wt, *hdf1* and *rad52 hdf1* (figure 3A).

The number of white petites decreased during aging in all strains (p<0.05, ANOVA). The higher rate of decrease was obtained for wt strain. The frequency of occurrence of these mutants changed in wt strain from 5.5% on day 0 to 0.08% on day 20 (68.75-fold). In *hdf1* strain the percentage of white petite colonies decreased from 0.74 to 0.05% (14.8-fold). In *rad52* strain the value obtained ranged from 2.95 to 0.54% (5.46-fold). In *rad52 hdf1* double mutant a decreased from 1.6 to 0.92% (1.74-fold) on day 20 was found (figure 3B). No significant differences between mutant strains and wt control strain during aging were observed (p>0.05, ANOVA).

These results suggest that inactivation of the DNA DSB repair pathway by HR in combination with the chronological aging of yeast produces an increase in the frequency of spontaneous mutation (revertants) and may contribute to an increased genomic instability.

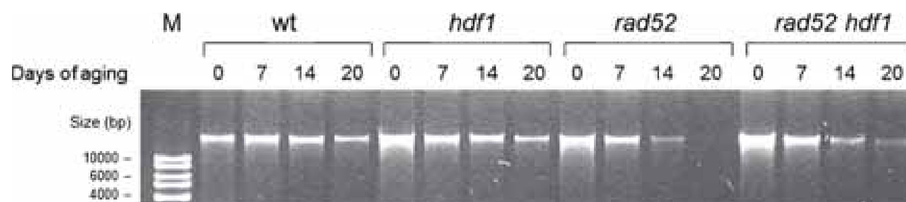

Figure 2. Degradation of genomic DNA during chronological aging in wt, *hdf1, rad52* and *rad52 hdf1* yeast strains. wt: wild type; *hdf1*: mutant blocking the NHEJ pathway; *rad52*: mutant blocking the HR pathway; *rad52 hdf1*: double mutant blocked in both pathways. M: Marker. Experiments were performed 4 times; n=4.

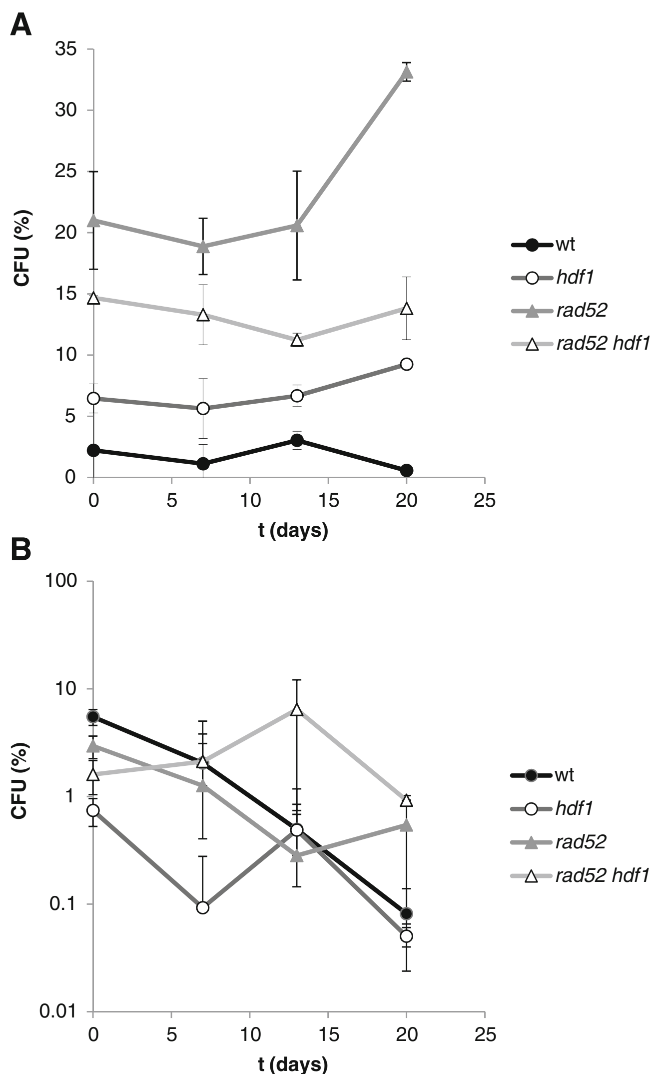

Figure 3. Spontaneous mutation during chronological aging. (**A**) Revertants. (**B**) White petites. Mean±s.d. *hdf1*, *rad52*, *rad52 hdf1* *vs* wt: $p<0.05$; ANOVA (revertants). *hdf1*, *rad52*, *rad52 hdf1 vs* wt: $p>0.05$; ANOVA (white petites). wt: wild type (control); *hdf1*: mutant blocking the NHEJ pathway; *rad52*: mutant blocking the HR pathway; *rad52 hdf1*: double mutant blocked in both pathways. Experiments were performed 3 times; $n=12$.

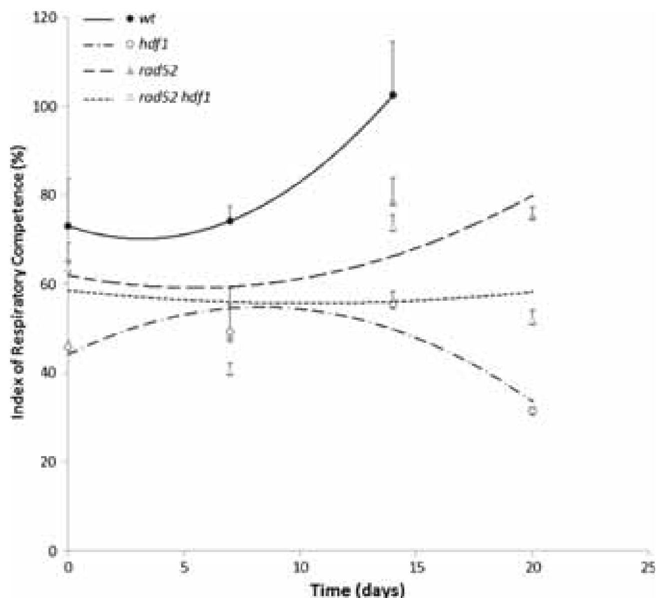

Figure 4. Index of respiratory competence (IRC) during aging. Mean±s.d. *hdf1 vs* wt: $p<0.01$; *rad52 vs* wt: $p<0.05$; *rad52 hdf1 vs* wt: $p<0.01$; *rad52 vs hdf1*: $p<0.01$; *rad52 hdf1 vs hdf1*: $p<0.05$; *rad52 vs rad52 hdf1*: $p<0.05$; ANOVA. Experiments were performed 3 times; $n=9$.

3.4 *Aging and index of respiratory competence in rad52 and hdf1 mutants*

In order to study the effect of aging on IRC in mutants unable to repair DNA DSB by HR and NHEJ, the percentage of oxidative colonies in relation to the total percentage of colonies (oxidative + fermentative) was studied, in wt strain and *hdf1*, *rad52* and *rad52 hdf1* mutants.

As shown in figure 4, an increase in the value of IRC was obtained during aging for the wt (control) strain. IRC value for this strain changed from 73.02% at day 0 until 102.47% (1.4-fold) at the end of aging. However, in *rad52*, *hdf1* and *rad52 hdf1* mutants a decrease in the IRC value was found after chronological aging in relation to the wt control strain.

The *hdf1* mutant strain suffered a gradual increase in IRC (45.73%, 49.18% and 55.49% on days 0, 7 and 14 of aging, respectively); and a decrease on day 20 of aging (IRC=31.43%) ($p<0.01$, ANOVA). The *rad52* mutant strain suffered a decrease on day 7 (ranging from 65.66% to 48.02%) and the rest of the stages of aging increased until the end, with values of 78.57% and 75.27%, on days 14 and 20 ($p<0.05$, ANOVA). The *hdf1 rad52* double mutant behaved similarly to the *rad52* strain except for the final stage where the value of IRC decreased. In the initial stages a value ranging from 63.61% to 40.37% was found. Then, the value increased to 72.90% and on day 20 decreased until 51.84% ($p<0.01$, ANOVA).

These results suggest that the inactivation of the DNA DSB repair pathways (HR and NHEJ) produce alterations in the cellular metabolic state resulting in a decrease in the index of respiratory competence in relation to the control strain. The inactivation of *hdf1* and *rad52* caused a decrease in the oxidative metabolism and thus an increase in the fermentative. The decrease was greater for *hdf1* strain, followed by the *rad52 hdf1* double mutant. The strain that showed a smaller decrease was the *rad52* mutant, as shown in figure 4 at day 0 of aging.

During the process of aging, a slight increase of IRC for *rad52* strain was found in relation to day 0 (1.15-fold). However, a decrease for *rad52 hdf1* double mutant (1.23-fold) and even an important decrease for *hdf1* strain (1.46-fold), in relation to day 0, were observed (figure 4).

Thus, inactivation of the DNA DSB repair pathways (HR and NHEJ) and chronological aging contributed in a different manner to the cellular metabolic state. On one hand, the inactivation of both pathways induced a decrease in the oxidative metabolism, involving mainly the *HDF1* gene; while the aging process produced an increase in the oxidative metabolism in the wt strain and *rad52* mutants, and in contrast an increase in the fermentative metabolism in the *hdf1* mutant strain.

These results suggest that the DNA break repair pathways by HR (*RAD52*) and NHEJ (*HDF1*) contribute to maintain the metabolic state in a different way, during aging.

4. Discussion

The radiosensitivity of the wild type strain used (WS8105-1C) and its *hdf1*, *rad52* and *rad52 hdf1* derivatives have been described previously by Friedl *et al.* (1998). As it is showed in the paper reported by these authors, the inactivation of DNA DSB repair mechanisms (HR and/or NHEJ) lead yeast strains population to a decreased surviving fraction in the radiation dose-response curves; being *rad52 hdf1* the most radiosensitive strain followed by *rad52*, *hdf1* and wild type (the most radioresistant) (Friedl *et al.* 1998).

RAD52 gene is not only involved in the repair of DNA DSB, but also in protecting telomeric chromosomal area, in the protection and change of telomere structure and even in the maintenance of telomere length. According to the theory of shortening of telomeres in each cell division, the process of shortening of telomeres leads to cellular damage and ultimately to cellular death associated with aging (Bryan *et al.* 1998). Di Primio *et al.* (2005) reported that HR and NHEJ pathways compete with one another while sharing some molecular components.

Apoptosis is a physiological process that occurs in all body tissues. It is a way to keep the form and function of the organs and remove defective or malignant cells. Apoptosis plays an important role in the aging of various organs among which include, brain, bone and immune system. Excessive apoptosis also leads to pathological situations. New evidence has shown that apoptosis plays a role in the pathophysiology of Parkinson's disease, Alzheimer's disease and arteriosclerosis; all age-related diseases (Madeo *et al.* 1999). The results obtained do not show the typical apoptosis pattern in DNA degradation during aging. In contrast to Fabrizio and Longo (2008), the chronological aging observed in the strains used in this study did not induce apoptosis.

Elevated levels of reactive oxygen species (ROS) in apoptosis cells triggered by chronological aging have been found by some authors (Herker *et al.* 2004; Aung-Htut *et al.* 2012). Stationary phase cultures are characterized by an accumulation of endogenously produced intracellular ROS in response to aging. Kitanovic and Wölfl (2006) report that aged wild-type cells display metabolic activity but their ability to replicate and form colonies is impaired. Yeast cells growing by respiration on non-fermentable carbon source such as glycerol express a high antioxidant activity related to a respiratory adaptation process (Kitanovic and Wölfl 2006).

An increase in ROS level has been found in both chronologically and replicatively aged cells not exposed to external stressors (Fabrizio *et al.* 2004b). Chronological aging in non-dividing yeast is associated with the inactivation of the superoxide-sensitive mitochondrial aconitase. In addition, replicative life span extension by calorie restriction requires an increase in mitochondrial respiration. On the other hand, the inefficient respiration or defective mitochondrial activity observed in aged cells could be caused by a ROS increase. However, the role of mitochondria in aging is still not clear (Fabrizio *et al.* 2004b).

In contrast, although our results showed a drastic decrease in respiration for *rad52*, *hdf1* and *rad52 hdf1* mutants, probably caused by an increase in ROS as suggested by Fabrizio *et al.* (2004b) for this type of phenotype; the results observed suggest that the mitochondrial genome stability was not affected in these mutants because the mutation rate for white petites decreased during aging, in general terms, being the frequency of white petites not statistically significant in relation to wt strain, during the aging period, as shown in figure 3B. In this way, the main contribution to the pattern of strand breaks observed in figure 2 could be probably due to the nuclear genome instability rather than to the mitochondrial genome.

Yeast cells accumulate DNA damage during the aging process. In this way, the antioxidant defense plays an important role in preserving the genomic integrity (Muid *et al.* 2014). Mitochondrial dysfunction is associated with increased levels of nuclear DNA damage and thus it could be the cause of aging and aging related diseases. However, this connection is poorly understood (Beach *et al.* 2015).

Mitochondria are the major source of ROS and their DNA is vulnerable to oxidative damage. Mitochondrial genome has a higher mutation rate than the nuclear genome (Lynch *et al.* 2008). Aging is often viewed in terms of gradual degeneration and accumulation of damage. In this way, point mutations represent an important form of cellular damage. Kaya *et al.* (2015) reported that DNA mutations do not cause aging in yeast. However they state that aging may result from accumulative damage.

There are scientific data suggesting that the repair capacity is related to the length of life (Park *et al.* 1999). Other

data suggest that the repair mechanisms become less effective with aging, which aggravates the process of aging. In addition, the polymorphism of genes that promote the repair of damaged DNA could influence the repair effectiveness (De Boer *et al.* 2002).

The analysis of mutants affected in both replicative and chronological aging has led to reinforce the importance of maintaining the respiratory phenotype. Among the genes identified for enhance the longevity stand out *SIR4* that mediates transcriptional repression at telomeres, *SIR2* that plays an important role in aging and *UTH1* required for induction of apoptosis (Aung-Htut *et al.* 2012).

Genomic instability is considered an important factor in aging, associated with a decline in DNA repair pathways and an increase in DNA damage, as a causal factor (Burhans and Weinberger 2012). As mentioned previously, oxidative stress plays an important role in chronological aging. In this way, the inactivation of Rad27p has been shown to reduce the lifespan associated with increased levels of DNA damage in stationary phase cells (Laschober *et al.* 2010). In addition, it has been reported a strong evidence between chronological lifespan extension and the failure of stationary phase cells to efficiently be arrested in G0/G1, mainly associated to replication stress (Burhans and Weinberger 2012). The integrity of mitochondrial DNA plays an important role in the correct function of this organelle. In this way, Mbantenkhu *et al.* (2011) reported that the yeast mitochondrial nucleoid protein, Mgm101, is related to the Rad52–type recombination proteins; being required for repeat–mediated recombination and to avoid mitochondrial DNA fragmentation.

The analysis of genomic DNA from *S. cerevisiae* by gel electrophoresis to study the damage induced in DNA molecules permit the detection of low level DNA damage and the quantification of the DNA content. The conditions of DNA electrophoresis combined with the DNA marker used in this work are sensible enough to detect DNA concentrations as low as 15 ng per band. Other authors have studied the DNA fragmentation by agarose gel electrophoresis to detect DNA damage (Potenza *et al.* 2004). Therefore, the DNA electrophoresis is sensible enough to detect a low degree of DNA damage.

Our study demonstrates that the inactivation of the HR DNA DSB repair pathway leads to a premature chronological aging. This repair mechanism has an important function in the maintenance of the genome integrity and stability, and thus it prevents the premature chronological aging. In addition, we found that the inactivation of the HR and NHEJ pathways contributes to maintain the metabolic state in a different way, during aging.

A biomarker of aging is a biological parameter which alone or in combination with others and in the absence of disease can predict the functional capacity at a certain age which may not coincide with the chronological age (Baker and Sprott 1988). It is therefore not surprising that many researchers are critical of the ability to find or devise biomarkers of aging, since this is a process that is not homogeneous and it does not occur at a single speed in different individuals or even between different organs and tissues in the same organism. However, the results might suggest that the *RAD52* gene could be considered as a candidate biomarker of the real physiological age of the individuals or cells; at least for further new research projects.

In conclusion, the inactivation of the DNA DSB repair pathway by HR leads to premature chronological aging. This repair mechanism is the most important for maintaining genome integrity and prevents the premature aging of the strains used in this study. The chronological aging leads to an increase in DNA degradation, which is higher when the HR repair pathway is inactivated. In addition, this repair mechanism contributes to an increased frequency of spontaneous mutations during chronological aging. The loss of the ability to repair DNA DSB alters the cellular metabolic state resulting in a decrease of the oxidative metabolism. Both mechanisms (HR and NHEJ) contribute to maintain the metabolic state, in a different way, during chronological aging.

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