The Effect of RAD7 Gene's Null Mutation on Single-Cell Aging

Ismat Jahan Anee [a], *Reza Md Shahjahan* [a], *Shamimul Alam* [a], *Rowshan Ara Begum* [a], *Md. Asaduzzaman Shishir* [b], *Rakha Hari Sarker* [c] and *Ashfaqul Muid Khandaker* [a]*

Abstract

Background: Although different theories and hypotheses were postulated for aging, molecular mechanisms of its regulations are still vastly unknown. In time, a post-mitotic cell ages with the aggregation of mutations within its genome and reach senescence. The DNA repair system that protects its genome also malfunctions with time. This study aims to discover whether manipulating a DNA repair gene can regulate the cellular life span, especially the chronological life span using the single-cell model (*Saccharomyces cerevisiae*) in the field of aging. ***Methods:*** In this study, yeast mutant that lacks nucleotide excision repair (NER) gene *RAD7* along with the diploid wild type (DP-WT) yeast strain (BY4743) as control were used. *RAD7* encodes a protein that acts in the NER of UV-damaged DNA. To characterize the mutant cell, experiments i.e. chronological lifespan assay, growth-proliferation, respiration status, mtDNA distribution patterns, and interaction analysis using bioinformatics were conducted. MIPS functional classification using FunSpec software was used to determine *RAD7's* interaction with diverse genes. ***Results:*** In the chronological lifespan assay, it was found that the *RAD7* gene's null mutation (*Δrad7*) had a prolonged stationary phase compared to the wild-type strain. The mutant displayed respiration potency by growing well on a glycerol-based medium. Fluorescence microscopic observations disclosed that the mutants had a lower abundance of mtDNA compared to the control. Furthermore, *RAD7* gene interaction analysis demonstrated how it interacts with other genes in the organism. Mutation of this repair gene affects mtDNA distribution and mtDNA abundance, yet cells could survive in the chronological phase. mtDNA depletion is a sign of altered mitochondrial morphology and function that may activate a retrograde response from mitochondria to the nucleus.

Conclusion: From this study, it is suggested that the *RAD7* gene can regulate yeast cells' life span. However, the retrograde response was not investigated in this current study and thus further efforts are also required to draw a fine conclusion.

Keywords: DNA Repair Gene; mitochondria; cell aging; *RAD7*; *Saccharomyces cerevisiae*..

Abbreviations: NER, nucleotide excision repair; CLS, chronological lifespan; RLS, Replicative lifespan; NEF4, Nucleotide excision repair factor 4; NEF2, Nucleotide excision repair factor 2; OD: Optical density; Rad7p, *RAD7* protein; RAD16p, *RAD16* protein; DP-WT, Diploid Wild-Type; mtDNA, mitochondrial DNA; rpm, rotation per minute.

Significance | Deciphering aging on yeast model

*Correspondence: **Ashfaqul Muid Khandaker, PhD**, Assistant Professor, Branch of Genetics and Molecular Biology, Department of Zoology, University of Dhaka, Dhaka-1000, Bangladesh. **Contact no.**: +8801759044481; **Email**: muid.zoo@du.ac.bd

Edited by **Dr. Farzana Hossain, Shizuoka University, Japan**, and accepted by the Editorial Board May 30, 2022 (Received for review April 9, 2022)

Introduction

Every living form on earth experiences spontaneous biological modifications as it ages. Aging occurs due to an incremental

Author Affiliation:
[a] Department of Zoology, University of Dhaka, Dhaka-1000, Bangladesh.
[b] Department of Microbiology, Primeasia University, Dhaka-1213, Bangladesh.
[c] Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh.

Please cite this article:
Anee IJ, Shahjahan RM, Alam S, Begum RA, Shishir MA, Sarker RH and Khandaker AM (2022). The effect of RAD7 gene's null mutation on single-cell aging. *Microbial Bioactives*, 5(*1*), 171-180.

accumulation of cellular and molecular impairments over time. As organisms age, they face a constant deterioration in biological and cognitive capacity, decreased organismal function, different diseases, and eventually, death. On the contrary, longevity is the opposite of aging; it defines the time length an individual persists and it is inversely proportional to the speed of aging (Brown, 2012). In the last decade, research for the molecular determinants of aging had advanced rapidly and diverse molecular causes of aging were detected (Dodig et al., 2019; López-Otín et al., 2013). To decipher these pathways related to cellular aging, single eukaryotic cell, *Saccharomyces cerevisiae* (yeast cell) has long been widely approved as an effective model system (Denoth Lippuner et al., 2014; Khandaker & Koc, 2020; Valter D. Longo et al., 2012; Zimmermann et al., 2018). Budding yeast can reproduce perpetually in a culture medium whereas each yeast cell ages and ultimately dies. In yeast, many aging and disease-relevant pathways such as DNA repair mechanisms, protein folding and secretion, cell cycle regulation, stress response, mitochondrial homeostasis, nutrient signaling, hypostasis, proteostasis, and regulated cell death are well conserved with humans (Bilinski et al., 2017; Carmona-Gutierrez et al., 2018; Eisenberg & Büttner, 2014; Janssens & Veenhoff, 2016; Knorre et al., 2016; Lasserre et al., 2015; V.D. Longo & Fabrizio, 2002; Postnikoff et al., 2017; Tenreiro & Outeiro, 2010). Sequencing of nearly 90% of the ~6000 yeast genes has been done and labeled and based on the sequence resemblance, approximately 60% of the yeast genome is similar to humans (Stefanini et al. 2013). There are two fundamental approaches to determine the aging in yeast such as chronological lifespan and replicative lifespan (abbreviated as CLS and RLS respectively) (Kaeberlein, 2010; Valter D. Longo et al., 2012). The current study was conducted on CLS of yeast cells since it is a well-established yet simple model to understand the aging pattern. CLS is the duration of yeast cell remaining viable but at non-dividing phase (Khawaja et al. 2021).

Mitochondria play a pivotal role in aging and aging-associated diseases (Kudryavtseva et al., 2016; Tyrrell et al., 2020). The damage to mitochondrial genomes might be one of the primary reasons behind the regulation of aging (Wallace et al., 1998). The mitochondrial theory of aging is based upon the fact that mtDNA has a limited repair mechanism compared to the nuclear DNAthat leads to the rapid mutations in the mitochondrial genome (López-Otín et al., 2013). The mutation or deletion of mtDNA has an impact on oxidative phosphorylation complex expression which causes mitochondrial dysfunction (Hacioglu et al., 2012; Wallace, 2010).

DNA repair system plays a vital role in maintaining genome integrity. Any impairment of this DNA repair system can cause drastic changes in an organism like mutagenic diseases and cellular senescence (Gangloff & Arcangioli, 2017; Maynard et al., 2015). Multiple DNA repair systems are present to safeguard the genome from harmful DNA lesion effects. Nucleotide Excision Repair (NER) is one of the most vital DNA repair systems in both eukaryotes and prokaryotes. NER functions in the amendment of the DNA damage that occurred by ultraviolet (UV) light, inter-strand and intra-strand DNA crosslinks, and many other DNA lesions (Prakash and Prakash 2000). NER works generally in two sub-pathways, global genome repair (GGR) and transcription-coupled repair (TCR)(Svejstrup, 2007). GGR erases DNA impairments from non- transcribed chromatin along with the non-transcribed strand of genes transcribed by RNA-polymerase II (Lettieri et al., 2008). TCR excises lesions from RNA-polymerase II transcribed gene strands (Svejstrup, 2007).

The NER repair mechanism is well examined and characterized in *Saccharomyces cerevisiae* (Prakash & Prakash, 2000; Waters et al., 2012). NER genes in yeast can be divided into two classes; class 1 including *RAD1*, *RAD2*, *RAD3*, *RAD4*, *RAD10*, *RAD14,* and *RAD25* genes and class 2 includes *RAD7*, *MMS19*, *RAD16*, *RAD23* genes (Prakash and Prakash 2000). Several of these genes encode proteins that are crucial for the recognition of damage and incision of the DNA. Mutations in the NER genes result in extreme UV sensitivity and a deficiency in DNA repair can give rise to genome instability (Wu & Roks, 2014), and oxidative (both nuclear and mitochondrial) DNA damage (Melis et al. 2013), cancer and aging (Marteijn et al., 2014). The configuration and action of NER genes are highly conserved among eukaryotes from yeast to humans (Friedberg et al., 2005; Prakash et al., 1993).

RAD7, a NER gene is located on chromosome X in *Saccharomyces cerevisiae* and works in the NER system (Table 1) to correct the ultraviolet or in short, UV-damaged DNA (Lahari et al., 2018). The Rad7p and Rad16p generate a complex called Nucleotide Excision Repair Factor 4 (NEF4) (Guzder et al., 1997). NEF4 acts especially with DNA damaged by UV in an ATP-dependent method (Guzder et al., 1997, 1998). Rad4p and Rad23p form another complex, called Nuclear Excision Repair Factor 2 (NEF2), that binds cooperatively with NEF4 to the impaired DNA (Guzder et al., 1999). Unlike NEF2, NEF4 is not needed for incision, but the in vitro incision activity is improved when NEF2 and NEF4 are present (Guzder et al., 1997, 1999). When Rad16p is absent, Rad7p interacts physically with NEF2, and binds DNA but does not show specificity for DNA damage (Guzder et al., 1999). *RAD7* mutant, like *RAD16* mutant, is an expert in incision but flaws in repair synthesis and excision of the oligonucleotide, suggesting that Rad7p and Rad16p act in a NER post-incision phase (Reed et al. 1998). In the human body, NER deficiencies are correlated with Xeroderma pigmentosum (XP) and Cockayne syndrome (Foury, 1997). Homologs of Rad7p and Rad16p have been specified in *S. pombe* (Lombaerts et al., 1999). Here in this study, the effect of *RAD7* gene deletion on single-cell aging was investigated to determine whether it can regulate the lifespan of yeast (single-cell model). To ascertain the function of a particular gene it's important to knock out the gene to see the changes in the cellular

mechanism which provides an idea of the target gene's role in the organism. By employing the single deletion mutants of the *Δrad7* gene; life span assay, growth proliferation status, respiratory phenotypic characterization, mitochondrial DNA abundance, and gene-gene interaction were evaluated. Accordingly, the role of the DNA repair gene *RAD7* in cellular aging was analyzed in this contemporary study.

Methodology

Yeast strain and culture preparation

Wild-type strain of yeast BY4743 (mat a/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15 ura3Δ0/ura3Δ0) and the genetic background isogenic deletion mutant *Δrad7* were obtained from EUROSCARF and utilized for this research. Cells were grown at 30° Celsius temperature both in solid YPD (1% yeast extract, 2% Peptone, 2% Dextrose and 2% Agar) and liquid YPD (1% yeast extract, 2% Peptone, and 2% Dextrose) culture medium. The concentrations of the yeast cells were assessed by a "GENESYS 5 spectrophotometer" according to the experimental conditions (optical density) at 600 nm wavelength (OD600).

Preparation of main stock (control and mutant)

To conduct the experiments smoothly both control and mutant strain were cultured and stored as aliquots. Firstly, YPD liquid media (1% yeast extract, 2% Peptone, and 2% Dextrose) was prepared for DP-WT as control and mutant yeast strain *Δrad7*. DP-WT and mutant yeast strains were reserved in liquid YPD culture. For each type of sample, 7ml liquid YPD was poured into a 15 ml falcon tube. Later, 5 µL yeast cell from the mother stock was added to the 7 ml culture medium. The falcon tubes were then incubated in a shaking incubator at 30° Celsius temperature overnight for cell growth. The next day, 3ml glycerol was added to the grown culture medium and stored at -20° Celsius fridge.

Chronological lifespan assay

The chronological aging pattern of yeast cells is identified by performing the colony formation unit (CFU) assay (Demir & Koc, 2010; Parrella & Longo, 2008). At first, the solid YPD culture plates were prepared. Then, yeast strain (control and mutants) was streaked onto the solid YPD Plates using the frozen reserved stock. These plates were later incubated at 30° Celsius for 24 hours for cell growth. For survival experiments, Liquid YPD medium was prepared and overnight cultures grown in YPD medium were used to inoculate experimental cultures on day 0 at a density of $1.5*10^7$ cells /mL into 500 mL flasks with a flask volume : medium volume ratio of 5 : 1. Flasks were then incubated overnight in a shaking incubator at 180 rpm at 30° C for 15 days. Starting at day three, 1.0 ml sample culture was taken from each flask and Optical Density (OD600) was measured. OD 0.4 was adjusted by diluting with sterile water using the formula: $\frac{0.4}{OD\ Reading} \times 1000$.

Later serial dilutions were done at 1:100 ratio and viability was measured by plating diluted culture onto YPD plates. Culture plates were then incubated at 30° Celsius for 24 hours. The next day, as the cells were grown, single colonies were counted for each sample type. The same process was repeated every 72 hours for 15 days to measure the survival rate of the cells. On day three, the CFU number was considered to be the 100 percent survival of the strain. This same procedure was repeated 5 times during 15 days analysis. Data of two (Δ*rad7*, DP-WT) variants were arranged in an excel sheet. The survival graph was generated by conducting statistical analysis using "Online Application for Survival Analysis (OASIS)". Kaplam-meier test was performed to calculate survival percentage and log-rank test was done to determine significant difference.

Proliferation status and Growth curve determination:

To understand the role of DNA repair genes on chronological lifespan, another experiment was conducted for growth and proliferation status determination. From the frozen working stock, yeast strains (DP-WT, *Δrad7*) were streaked onto solid YPD plates. These plates were later incubated at 30° Celsius for 24 hours for cell growth. Liquid YPD medium was prepared and two conical flasks were filled with 200ml liquid YPD, 100 ml in each. Single colonies from the culture plate (DP-WT, *Δrad7*) were inoculated into two conical flasks one after another and labeled properly. Flasks were then incubated overnight in a shaking incubator at 180 rpm at 30° Celsius. Starting on day three, 1ml sample culture was taken from each flask and Optical Density (OD_{600}) was measured. Serial dilution was done by adjusting OD 0.4 by diluting with sterile water. And on every third day, from the starting date, the optical density of each strain type was measured until all the cells of each variant reached senescence.

Determination of the respiratory status of the mutant:

Yeast (*Saccharomyces cerevisiae*) can only respire when grown on non-repressor carbon sources such as glycerol and ethanol. But respiratory-deficient mutants, also known as petites are unable to respire as they possess some mitochondrial dysfunction (Emerit et al., 2004; López-Otín et al., 2013).

So, conducted another experiment to determine whether these respiratory-deficient mutant cells could grow in the culture medium containing glucose or glycerol (Muid et al., 2019). Control and mutant strain were grown in YPG (3% glycerol) and YPD liquid medium overnight. The next day, the culture was renewed by adding 15 ml overnight grown yeast culture to 30 ml fresh YPD liquid medium. The new culture was put in a shaking incubator at 180 rpm for 4 hours at 30° C temperature. Then, serial dilution was performed to obtain OD600 values of 0.2, 0.02, 0.002, and 0.0002. 5 µL of each solution was then dropped onto YPG and YPD-agar plates and incubated at 30° C for 48 hours.

Fluorescence microscopic analysis of mtDNA abundance and distribution:

To find out the mtDNA distribution pattern in the mutant, wild-type, and mutant yeast strain were grown overnight in liquid YPD

media (Cone & Cone, 2018). The Optical Density (OD600) was adjusted between the range of 0.8-2.0 which was considered to be ideal for this experiment. Then, yeast cells and 100 percent ethanol at a ratio of 1:3 were taken in a 1.5 ml microfuge tube. The yeast-ethanol mixture was kept stagnant for 30-60 minutes at room temperature. After that, the microfuge tubes were centrifuged for 1 min at 2500 rpm. The supernatant was removed and cells were suspended in 1 ml 1x PBS buffer and centrifuged again at 2500 rpm for 1 min. The supernatant was removed and 200 µL 1xPBS/1:2000 DAPI stain solution (2.5 mg DAPI stain was added in 1 ml distilled water of which 0.5 µL is added in 1xPBS buffer to prepare the PBS-DAPI solution) was added in the microfuge tube. In the end, one drop of yeast (control and mutant) suspended in the PBS-DAPI stain mixture was poured onto a microscope slide, and a coverslip was put on top of the slide. The stained yeast cells were then observed using a "Nikon Eclipse 50i fluorescence microscope (manufactured by Japan)".

Interaction analysis

The proteins that interact with Rad7p were identified with the help of the yeast genome database (https://www.yeastgenome.org/) and molecular and biological functions were identified by MIPS (Munich Information Center for Protein Sequences) classification using the FunSpec (http://funspec.med.utoronto.ca/) software-based analysis program. Firstly, all physical and genetic interacting genes with the RAD7 gene were identified and analyzed in FunSpec software. All compiled knowledge sources and public data source information was used and the *p*-value cutoff was set at 0.01. This helped to find out the *RAD7* gene's interaction with other genes more precisely.

Results

The mutant cell, *Δrad7* demonstrated higher viability in the Chronological phase

The chronological lifespan assay was conducted for 15 days. In this experiment chronological lifespan of the mutant was analyzed to find out how the DNA repair gene acts to maintain cellular viability in the non-dividing phase. *Δrad7* mutant showed a higher rate of viability in comparison with the wild-type cells (Figure 1). The mutant demonstrated an adaptive regenerative pattern, and the mutant strain had a greater viability rate after three to six days of incubation period which was later dwindled. The mean lifespan of Diploid Wild-Type (DP-WT) and *RAD7* was 5.48 and 12.10 days respectively. *RAD7* had longer lifespan compared to wild-type ($P < 0.05$).

Growth and proliferation status of *Δrad7*

To understand the mutant strain's viability pattern in the non-dividing state, Optical Density (OD) was assessed at 600 nm (Figure 2). OD values were within the range of 0.8 – 2.00. In comparison with the wild-type strain, the mutant displayed higher viability in the stationary phase. However, in the prolonged stationary phase, the mutant *Δrad7* showed a consistent cell decrease pattern. On 34th, 37th, and the very last 58th day the OD value for *Δrad7* was 2.016, 1.720, and 1.700 respectively. This result shows that the growth of the *Δrad7* cells decreased gradually after the 34th day till the end of the experiment and in DP-WT, the cells stopped growing after the 40th day.

Respiratory status of *Δrad7*

The petite mutants show mitochondrial genome instability and mtDNA loss and some Petites are also long-lived (Schroeder & Shadel, 2014). Cells containing such defective mitochondria fail to use respiratory materials such as ethanol or glycerol. Mutants that are not respiratory efficient, can't grow on glycerol. On the other hand, improper growth on glycerol might have happened to mutations that do not entirely impede respiration. In the case of the *RAD7* gene being a nucleotide excision repair gene, ample information is available on its damage repair role but the effect of null mutation of DNA repair gene *RAD7* on mitochondrial function was not analyzed before. That's why this particular experiment was performed to find out whether the *RAD7* mutant was defective in respiration. The mutant was grown on a glycerol-rich culture medium. Both control and mutant *Δrad7* were grown normally in the glucose-containing medium. The mutant strain *Δrad7* could exploit glycerol as the carbon source in the medium, which proved these mutant cells' are not respiratory deficient. Though *Δrad7* was grown better in the glucose medium compared with the glycerol medium (Figure 3), deletion of the gene didn't impede the non-fermenting respiratory pathway of the mutant strain.

Abundance and distribution pattern of mtDNA in the mutant *Δrad7*

DAPI stained cells from the wild-type and mutant strain *Δrad7* were observed under a fluorescence microscope at 100× magnification to find out the mtDNA abundance and distribution pattern in these cells. In the cells of both wild-type and mutant yeast strain, the large round stained body was observed which appeared to be the nucleus (N) and the small dispersed ones were the mitochondrial DNA (mt). In wild-type cells, adequate mtDNA was finely distributed in a linear array. But in *Δrad7*, the mtDNA abundance was seen reduced (Figure 4). Fluorescence intensity of cells was later analysed by ImageJ software and significant difference in fluorescence intensity between DP-WT (higher intensity) and *Δrad7* (lower intensity) was found ($p < 0.05$).

Interaction Analysis

To understand the functional interaction of *RAD7*, bioinformatics analysis was performed using FunSpec software. MIPS functional classification was done ($p < 0.01$) (Figure 5; Table 2). Table 2 provides a precise idea of how this DNA repair gene works with other genes and their protein derivatives in the *Saccharomyces cerevisiae*.

RAD7 interacts with *RAD16*, *RAD4*, *RAD6*, *RAD26,* and *SIR3* in the DNA repair pathway; also interacts with *RAD16*, *RAD4*, *and DEF1* in DNA damage response. Along with *BIM1* and *RAD4*; *RAD7* works in

Table 1| **A summary of DNA repair gene *RAD7* assayed in this study.**

Gene	Pathway involved	Homology	Function
RAD7	Nucleotide Excision Repair	*Homo sapiens* (*FBXL7*)	Binds UV damaged DNA using DNA –dependent ATPase activity and also repairs the non-transcribed chromatin.
		Mus musculus (*Lrr29*)	
		Mus musculus (*FBXl7*)	
		Rattus norvegicus (*FBXl7*)	
		Danio rerio (si:dkey-192l18.9; fbxl7)	
		Drosophila melanogaster (CG14891)	
		Caenorhabditis elegans(T01E8.1)	

Table 2| ***RAD7* interaction and their molecular and biological functions were identified and grouped with MIPS (Munich Information Center for Protein Sequences) classification utilizing the Fun-Spec web-based cluster interpreter for yeast. The *p*-values epitomized the probability of the intersection of a particular list with any specified functional category occurrence randomly.**

MIPS Functional Classification Chart					
Name of the Gene	**Category**	***p*-value**	**In category from cluster**	**k***	**f***
RAD7	DNA repair [10.01.05.01]	1.45498e-07	*RAD16 RAD4 RAD6 RAD26 RAD7 SIR3*	6	159
	DNA damage response [32.01.09]	7.88451e-06	*RAD16 RAD4 RAD7 DEF1*	4	77
	Transcription elongation [11.02.03.01.04]	0.00136737	*SPT4 ELC1*	2	31
	Proteasomal degradation (ubiquitin or proteasomal pathway) [14.13.01.01]	0.00137738	*RAD6 CUL3 DEF1*	3	128
	Cell cycle checkpoints (morphogenesis checkpoints, DNA- replication, DNA-damage, spindle and mitotic phase) [10.03.01.03]	0.00626952	*BIM1 RAD4*	2	67
	Amendment by ubiquitination and/or deubiquitination [14.07.05]	0.00863132	*RAD6 CUL3*	2	79

k*= Gene quantity from the input cluster in a particular category; f*= Total gene quantity in a particular category.

cell cycle checkpoints. *RAD7* also works in proteasomal degradation together with *RAD6*, and *CUL3*.

Discussion

The experiments conducted on *the Δrad7* mutant strain provided a notion that DNA repair gene *RAD7* can regulate cellular lifespan. The deletion of the gene caused greater viability, and so cells acquired a longer stationary phase (Figure 1). Later, Optical density measurement data for growth and proliferation analysis also validated the assay result (Figure 2). As the mutant grew well on fermentable carbon source (YPD) and demonstrated greater cellular viability, another experiment was conducted to find out whether this mutant could utilize its mitochondria's oxidative phosphorylation mechanism to grow on non-fermentable carbon sources like glycerol (YPG).

Mitochondrial mutations occur spontaneously in yeast and form small colonies named petite. The mitochondrial genome is damaged in these petite mutants and so they may cause large deletions of mtDNA or may even have a total loss of mtDNA (Huh et al., 2003; Walker et al., n.d.). Some petite mutants show mitochondrial genome instability. Previous studies demonstrated that cells can be petite and long-lived (Garcia et al., 2019; Ocampo et al., 2012; Ogata et al., 2016; Powell et al., 2000; Woo & Poyton, 2009) and also in some cases not petite and long-lived (Schroeder & Shadel, 2014). Mutants that are not respiratory efficient either can't grow on glycerol or grow poorly when the respiratory system is not entirely impaired. Yeast can grow by respiration when mitochondrial respiration is intact using non-fermentable carbon sources such as glycerol, lactate, or ethanol. This ability is a notably effective feature for determining mitochondrial

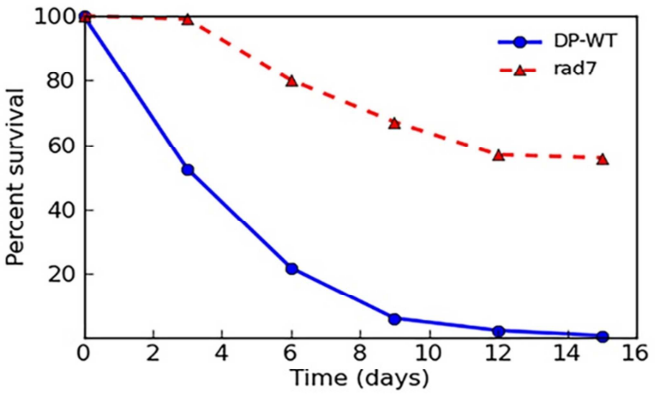

Figure 1| **Chronological lifespan analysis of the mutant *Δrad7* compared to control DP-WT.**

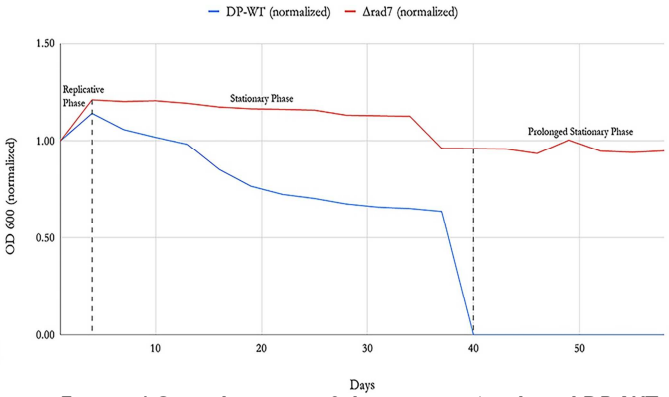

Figure 2| **Growth curves of the mutant *Δrad7* and DP-WT. DP-WT showed complete lysis on 40th day while *Δrad7* showed prolonged stationary phase.**

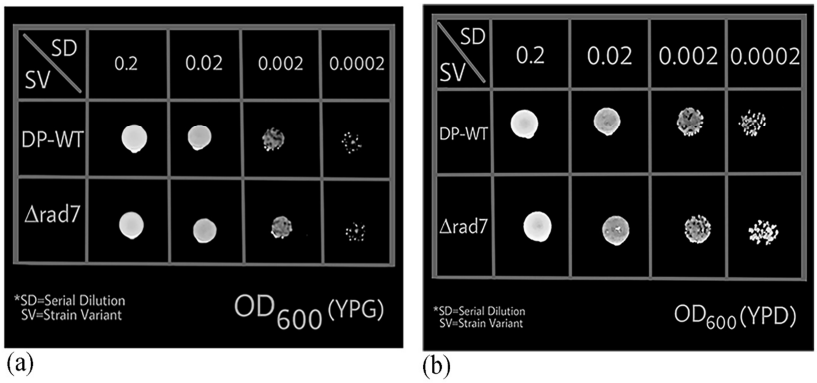

Figure 3| **Respiratory status of the mutant *Δrad7* and DP-WT; a) Cell grown on YPG (3% glycerol medium); b) Cell grown on YPD (2% dextrose medium).**

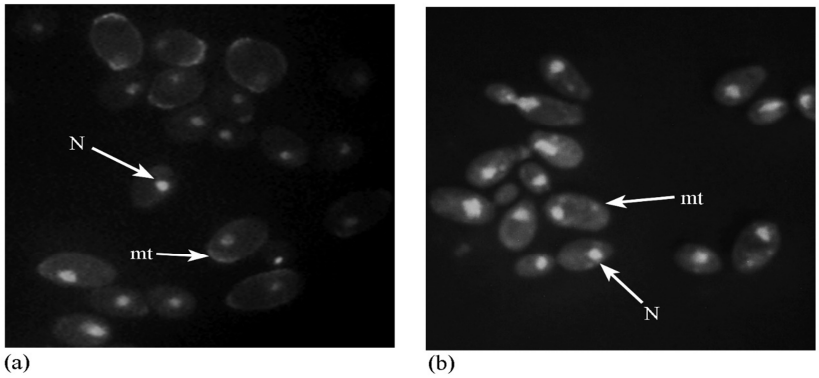

Figure 4| **a) DP-WT; b) *Δrad7*; Analysis of mtDNA distribution pattern of the mutant cells by staining the cell with DAPI and observed under fluorescence microscope at 100× magnification. (mt: mitochondrial DNA and N: Nucleus of the cells).**

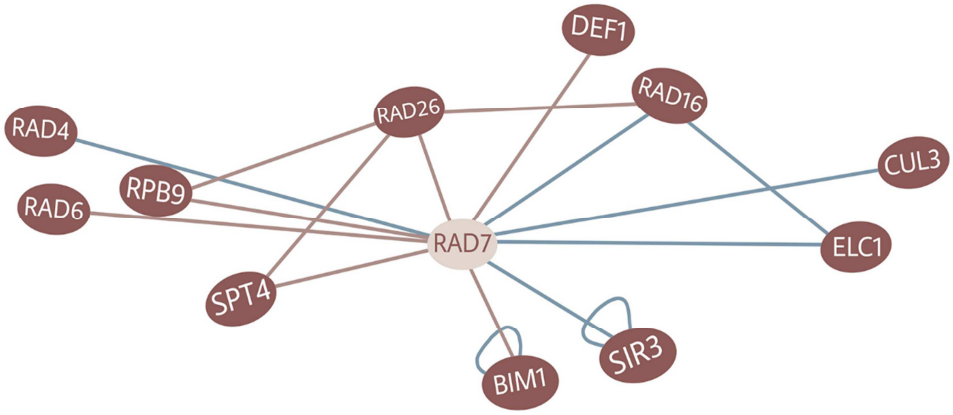

Figure 5| **RAD7 interaction network. The proteins that interact with RAD7 protein were analyzed with the help of *Saccharomyces* genome database. RAD26, DEF1, SPT4, BIM1, RPB9, RAD6, RAD16, CUL3, ELC1, SIR3, RAD4 were found to have a network with RAD7.**

damaged condition at a point when the cell is still viable (Parrella & Longo, 2008).

In the present investigation, we tested whether deletion of this gene has any impact on mitochondrial respiration. We found that *rad7* is not petite, can use the respiratory mechanism of mitochondria, and provide proof of its not having any major impact on the non-fermentable respiratory pathway (Figure 3). In addition, gene deletion had also an impact on mitochondrial allocation and function and so conducted additional experiments to analyze the distribution pattern of mitochondria in the mutant cells. In yeast, mtDNA remains into punctate (small and round) structures (Swayne et al. 2007). If a mutant strain or a subtype of a species lacks mitochondrial DNA, the absence of that mtDNA can be determined by utilizing the DNA staining procedure. So, a fluorescence microscopy experiment was performed by staining the cells with DAPI. In the WT cells, the mtDNA was typically distributed with a linear array at the edge of the cytoplasm. But in the mutant strain, *Δrad7*, mtDNA abundance was found to decrease compared to the wild and also exhibited a single spot in the cytoplasm (Figure 4). This provided an idea that the deletion of the RAD7 gene might provide an alternate function in the cell with a typical mitochondrial DNA distribution pattern and abundance. At the same time, call into the question, how do the cells obtain a prolonged stationary phase? This might be occurred by activating retrograde response, a signaling pathway enabled by the gradual decrease in mitochondrial membrane potential upon aging due to the mitochondrial dysfunction. Activation of retrograde response can slow down the inexorable death of yeast cells (Jazwinski, 2014).

Former experiments created an urge to know how this DNA repair gene, *RAD7* interlinks, and works with other genes. So, another experiment was carried out on the gene-gene interaction analysis to get an overall idea (Table 2; Figure 5). All the physically and genetically linked genes with *RAD7* were used to form the MIPS functional classification using FunSpec software. This network analysis showed that the *RAD7* gene works in cohort with *RAD16*, *RAD4*, *RAD6*, *RAD26*, and *SIR3* in the DNA repair pathway; works in a cluster with *RAD16*, *RAD4*, and *DEF1* in DNA damage response; works in modification by ubiquitination and deubiquitination in complex with *RAD6* and *CUL3*, *RAD7* works with SPT4 and *ELC1* in elongating transcription, works with *CUL3*, *RAD6* and *DEF1* gene in proteasomal degradation; and along with *BIM1* and *RAD4* gene works in cell cycle checkpoints (Table 2; Figure 5). Here, *RAD16* is NER protein and works as a subset of NEF4; and *RAD4* is a subunit of NEF2, identifies and binds damaged or altered DNA along with Rad23p throughout nucleotide excision repair (NER) (Lettieri et al., 2008); *RAD6* is a Ubiquitin-conjugating enzyme which is engaged in repair of post-replication errors as a heterodimer with Rad18p (Game & Chernikova, 2009); *RAD26* is protein engaged in transcription-linked nucleotide excision repair, rectifies UV-induced DNA lesions (Lee et al., 2002); *SIR3* is a silencing protein; cooperates with histone H3/H4 tails, Sir2p, and Sir4p, to determine transcriptionally silent chromatin (Moretti & Shore, 2001; Rine & Herskowitz, 1987); *CUL3* is the Ubiquitin-protein ligase gene (Michel et al. 2003), *ELC1* stands for Elongin C which forms a complex with Cul3p to provoke its proteolysis (Hyman et al., 2002); plays a role in global genomic repair (LeJeune et al., 2009), and *DEF1* is RNAPII degradation factor; generates a complex with Rad26p in chromatin (Woudstra et al., 2002). So, this interaction analysis proved that along with responding to DNA impairment and DNA repair, the *RAD7* gene also functioned in cell cycle checkpoints and proteasomal degradation. When this gene was absent, the activities might have been delayed and the cell might require a longer time to complete the cell cycling phase which in turn could prolong the chronological phase of the mutant strain. Further investigation is required to prove this conclusion generated based on the results driven from the specific experiments.

Conclusion

This study was intended to determine the effect of DNA repair gene *RAD7* manipulation, mostly on cellular lifespan regulation of the single-celled organism *Saccharomyces cerevisiae* (yeast). In our analysis, we found that the *RAD7* gene has a role in yeast cells' life span regulation. Deletion of the *RAD7* gene affected mtDNA distribution by reducing mtDNA abundance, yet cells could remain viable in the chronological phase with a prolonged lifespan. It is presumed that the mutant cells remain viable by activating the retrograde response, a back signal from mitochondria to the nucleus. From gene-gene interaction analysis findings, it can be predicted that *RAD7* may assist in the longer stationary phase by interfering with cell cycle checkpoints. The current study findings generated more stirring questions such as, is there any signaling pathway other than retrograde response working there? Is there any change in gene expressions in the mutant cells? Further comprehensive research regarding these issues is highly required to find out these mysteries.

Author Contribution

IJA performed the experiments, arranged the results, and wrote the manuscript. AMK guided and helped in research framework development. RMS, SA, MAS and RAB reviewed and revised the manuscript. MAS and RHS assisted in experimental facilities.

Acknowledgment

The work was partially funded by the Center for Advanced Studies in Research in Biological Sciences. IJA was supported by the National Science and Technology Fellowship granted through the Ministry of Science and Technology, Bangladesh. The authors are thankful to the

Chairman of the Department of Zoology, University of Dhaka, for his assistance. The authors are also grateful to Dr. Md. Arafat Al Mamun, Centre for Advanced Research in Sciences, University of Dhaka for their assistance in research facilities.

Competing financial interests

Authors have declared that no competing interest exists.

References

Bilinski, T., Bylak, A., & Zadrag-Tecza, R. (2017). The budding yeast Saccharomyces cerevisiae as a model organism: possible implications for gerontological studies. *Biogerontology*, *18*(4), 631–640. https://doi.org/10.1007/s10522-017-9712-x

Brown, K. (2012). Aging: The Mitochondrial Connection. *Journal of Clinical & Experimental Pathology*, *s4*(01). https://doi.org/10.4172/2161-0681.S4-003

Carmona-Gutierrez, D., Bauer, M. A., Zimmermann, A., Aguilera, A., Austriaco, N., Ayscough, K., Balzan, R., Bar-Nun, S., Barrientos, A., Belenky, P., Blondel, M., Braun, R. J., Breitenbach, M., Burhans, W. C., Büttner, S., Cavalieri, D., Chang, M., Cooper, K. F., Côrte-Real, M., … Madeo, F. (2018). Guidelines and recommendations on yeast cell death nomenclature. In *Microbial Cell*. https://doi.org/10.15698/mic2018.01.607

Cone, A., & Cone, A. (2018). *Yeast DAPI Staining*. 3–5.

Demir, A. B., & Koc, A. (2010). Assessment of chronological lifespan dependent molecular damages in yeast lacking mitochondrial antioxidant genes. *Biochemical and Biophysical Research Communications*, *400*(1), 106–110. https://doi.org/10.1016/j.bbrc.2010.08.019

Denoth Lippuner, A., Julou, T., & Barral, Y. (2014). Budding yeast as a model organism to study the effects of age. *FEMS Microbiology Reviews*, *38*(2), 300–325. https://doi.org/10.1111/1574-6976.12060

Dodig, S., Čepelak, I., & Pavić, I. (2019). Hallmarks of senescence and aging. *Biochemia Medica*, *29*(3), 483–497. https://doi.org/10.11613/BM.2019.030501

Eisenberg, T., & Büttner, S. (2014). Lipids and cell death in yeast. *FEMS Yeast Research*, *14*(1), 179–197. https://doi.org/10.1111/1567-1364.12105

Emerit, J., Edeas, M., & Bricaire, F. (2004). Neurodegenerative diseases and oxidative stress. *Biomedicine & Pharmacotherapy*, *58*(1), 39–46. https://doi.org/10.1016/j.biopha.2003.11.004

Foury, F. (1997). Human genetic diseases: a cross-talk between man and yeast. *Gene*, *195*(1), 1–10. https://doi.org/10.1016/S0378-1119(97)00140-6

Friedberg, E. C., Walker, G. C., Siede, W., Wood, R. D., Schultz, R. A., & Ellenberger, T. (2005). DNA Repair and Mutagenesis. In *DNA Repair and Mutagenesis*. ASM Press. https://doi.org/10.1128/9781555816704

Game, J. C., & Chernikova, S. B. (2009). The role of RAD6 in recombinational repair, checkpoints and meiosis via histone modification. *DNA Repair*, *8*(4), 470–482. https://doi.org/10.1016/j.dnarep.2009.01.007

Gangloff, S., & Arcangioli, B. (2017). DNA repair and mutations during quiescence in yeast. *FEMS Yeast Research*, *17*(1), 1–14. https://doi.org/10.1093/femsyr/fox002

Garcia, E. J., de Jonge, J. J., Liao, P.-C. C., Stivison, E., Sing, C. N., Higuchi-Sanabria, R., Boldogh, I. R., & Pon, L. A. (2019). Reciprocal interactions between mtDNA and lifespan control in budding yeast. *Molecular Biology of the Cell*, *30*(24), 2943–2952. https://doi.org/10.1091/mbc.E18-06-0356

Guzder, S. N., Sung, P., Prakash, L., & Prakash, S. (1997). Yeast Rad7-Rad16 Complex, Specific for the Nucleotide Excision Repair of the Nontranscribed DNA Strand, Is an ATP-dependent DNA Damage Sensor. *Journal of Biological Chemistry*, *272*(35), 21665–21668. https://doi.org/10.1074/jbc.272.35.21665

Guzder, S. N., Sung, P., Prakash, L., & Prakash, S. (1998). The DNA-dependent ATPase Activity of Yeast Nucleotide Excision Repair Factor 4 and Its Role in DNA Damage Recognition. *Journal of Biological Chemistry*, *273*(11), 6292–6296. https://doi.org/10.1074/jbc.273.11.6292

Guzder, S. N., Sung, P., Prakash, L., Prakash, S., & Sung, P. (1999). Synergistic Interaction between Yeast Nucleotide Excision Repair Factors NEF2 and NEF4 in the Binding of Ultraviolet-damaged DNA. *Journal of Biological Chemistry*, *274*(34), 24257–24262. https://doi.org/10.1074/jbc.274.34.24257

Hacioglu, E., Demir, A. B., & Koc, A. (2012). Identification of respiratory chain gene mutations that shorten replicative life span in yeast. *Experimental Gerontology*, *47*(2), 149–153. https://doi.org/10.1016/j.exger.2011.11.009

Huh, W.-K., Falvo, J. V., Gerke, L. C., Carroll, A. S., Howson, R. W., Weissman, J. S., & O'Shea, E. K. (2003). Global analysis of protein localization in budding yeast. *Nature*, *425*(6959), 686–691. https://doi.org/10.1038/nature02026

Hyman, L. E., Kwon, E., Ghosh, S., McGee, J., Chachulska, A. M. B., Jackson, T., Baricos, W. H., Boguszewska Chachulska, A. M., Jackson, T., & Baricos, W. H. (2002). Binding to Elongin C Inhibits Degradation of Interacting Proteins in Yeast. *Journal of Biological Chemistry*, *277*(18), 15586–15591. https://doi.org/10.1074/jbc.M200800200

Janssens, G. E., & Veenhoff, L. M. (2016). Evidence for the hallmarks of human aging in replicatively aging yeast. *Microbial Cell*, *3*(7), 263–274. https://doi.org/10.15698/mic2016.07.510

Jazwinski, S. M. (2014). The retrograde response: A conserved compensatory reaction to damage from within and from without. *Progress in Molecular Biology and Translational Science*, *127*, 133–154. https://doi.org/10.1016/B978-0-12-394625-6.00005-2

Kaeberlein, M. (2010). Lessons on longevity from budding yeast. *Nature*, *464*(7288), 513–519. https://doi.org/10.1038/nature08981

Khandaker, A. M., & Koc, A. (2020). Mitochondrial Targeted AFG3 Abolishment Triggers Higher Mitochondrial Membrane Potential (ΔΨm) in Young Yeast. *Microbial Bioactives*, *3*(1), 119–124. https://doi.org/10.25163/microbbioacts.31003A0713270620

Khawaja, A.-A.-W., Belak, Z. R., Eskiw, C. H., & Harkness, T. A. A. (2021). High-Throughput Rapid Yeast Chronological Lifespan Assay. In *Methods in Molecular Biology* (pp. 229–233). https://doi.org/10.1007/978-1-0716-0868-5_18

Knorre, D., Sokolov, S., Zyrina, A., & Severin, F. (2016). How do yeast sense mitochondrial dysfunction? *Microbial Cell*, *3*(11), 532–539. https://doi.org/10.15698/mic2016.11.537

Kudryavtseva, A. V., Krasnov, G. S., Dmitriev, A. A., Alekseev, B. Y., Kardymon, O. L., Sadritdinova, A. F., Fedorova, M. S., Pokrovsky, A. V., Melnikova, N. V., Kaprin, A. D., Moskalev, A. A., & Snezhkina, A. V. (2016). Mitochondrial dysfunction and oxidative stress in aging and cancer. *Oncotarget*, *7*(29), 44879–44905. https://doi.org/10.18632/oncotarget.9821

Lahari, T., Lazaro, J., Marcus, J. M., & Schroeder, D. F. (2018). RAD7 homologues contribute to Arabidopsis UV tolerance. *Plant Science*, *277*(August), 267–277. https://doi.org/10.1016/j.plantsci.2018.09.017

Lasserre, J.-P., Dautant, A., Aiyar, R. S., Kucharczyk, R., Glatigny, A., Tribouillard-Tanvier, D., Rytka, J., Blondel, M., Skoczen, N., Reynier, P., Pitayu, L., Rötig, A., Delahodde, A., Steinmetz, L. M., Dujardin, G., Procaccio, V., & di Rago, J.-P. (2015). Yeast as a system for modeling mitochondrial disease mechanisms and discovering therapies. *Disease Models & Mechanisms*, *8*(6), 509–526. https://doi.org/10.1242/dmm.020438

Lee, S.-K., Yu, S.-L., Prakash, L., & Prakash, S. (2002). Yeast RAD26 , a Homolog of the Human CSB Gene, Functions Independently of Nucleotide Excision Repair and Base Excision Repair in Promoting Transcription through Damaged Bases. *Molecular and Cellular Biology*, *22*(12), 4383–4389. https://doi.org/10.1128/MCB.22.12.4383-4389.2002

LeJeune, D., Chen, X., Ruggiero, C., Berryhill, S., Ding, B., & Li, S. (2009). Yeast Elc1 plays an important role in global genomic repair but not in transcription coupled repair. *DNA Repair*, *8*(1), 40–50. https://doi.org/10.1016/j.dnarep.2008.08.010

Lettieri, T., Kraehenbuehl, R., Capiaghi, C., Livingstone-Zatchej, M., & Thoma, F. (2008). Functionally distinct nucleosome-free regions in yeast require Rad7 and Rad16 for nucleotide excision repair. *DNA Repair*, *7*(5), 734–743. https://doi.org/10.1016/j.dnarep.2008.01.016

Lombaerts, M., Peltola, P. H., Visse, R., den Dulk, H., Brandsma, J. A., & Brouwer, J. (1999). Characterization of the rhp7+ and rhp16+ genes in Schizosaccharomyces pombe. *Nucleic Acids Research*, *27*(17), 3410–3416. https://doi.org/10.1093/nar/27.17.3410

Longo, V.D., & Fabrizio, P. (2002). Visions & Reflections¶Regulation of longevity and stress resistance: a molecular strategy conserved from yeast to humans? *Cellular and Molecular Life Sciences (CMLS)*, *59*(6), 903–908. https://doi.org/10.1007/s00018-002-8477-8

Longo, Valter D., Shadel, G. S., Kaeberlein, M., & Kennedy, B. (2012). Replicative and Chronological Aging in Saccharomyces cerevisiae. *Cell Metabolism*, *16*(1), 18–31. https://doi.org/10.1016/j.cmet.2012.06.002

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., & Kroemer, G. (2013). The Hallmarks of Aging. *Cell*, *153*(6), 1194–1217. https://doi.org/10.1016/j.cell.2013.05.039

Marteijn, J. A., Lans, H., Vermeulen, W., & Hoeijmakers, J. H. J. J. (2014). Understanding nucleotide excision repair and its roles in cancer and ageing. *Nature Reviews Molecular Cell Biology*, *15*(7), 465–481. https://doi.org/10.1038/nrm3822

Maynard, S., Fang, E. F., Scheibye-Knudsen, M., Croteau, D. L., & Bohr, V. A. (2015). DNA Damage, DNA Repair, Aging, and Neurodegeneration. *Cold Spring Harbor Perspectives in Medicine*, *5*(10), a025130. https://doi.org/10.1101/cshperspect.a025130

Melis, J. P. M., van Steeg, H., & Luijten, M. (2013). Oxidative DNA Damage and Nucleotide Excision Repair. *Antioxidants & Redox Signaling*, *18*(18), 2409–2419. https://doi.org/10.1089/ars.2012.5036

Michel, J. J., McCarville, J. F., & Xiong, Y. (2003). A Role for Saccharomyces cerevisiae Cul8 Ubiquitin Ligase in Proper Anaphase Progression. *Journal of Biological Chemistry*, *278*(25), 22828–22837. https://doi.org/10.1074/jbc.M210358200

Moretti, P., & Shore, D. (2001). Multiple Interactions in Sir Protein Recruitment by Rap1p at Silencers and Telomeres in Yeast. *Molecular and Cellular Biology*, *21*(23), 8082–8094. https://doi.org/10.1128/MCB.21.23.8082-8094.2001

Muid, K. A., Kimyon, Ö., Reza, S. H., Karakaya, H. C., & Koc, A. (2019). Characterization of long living yeast deletion mutants that lack mitochondrial metabolism genes DSS1, PPA2 and AFG3. *Gene*, *706*(April), 172–180. https://doi.org/10.1016/j.gene.2019.05.001

Ocampo, A., Liu, J., Schroeder, E. A., Shadel, G. S., & Barrientos, A. (2012). Mitochondrial Respiratory Thresholds Regulate Yeast Chronological Life Span and its Extension by Caloric Restriction. *Cell Metabolism*, *16*(1), 55–67. https://doi.org/10.1016/j.cmet.2012.05.013

Ogata, T., Senoo, T., Kawano, S., & Ikeda, S. (2016). Mitochondrial superoxide dismutase deficiency accelerates chronological aging in the fission yeast Schizosaccharomyces pombe. *Cell Biology International*, *40*(1), 100–106. https://doi.org/10.1002/cbin.10556

Parrella, E., & Longo, V. D. (2008). The chronological life span of Saccharomyces cerevisiae to study mitochondrial dysfunction and disease. *Methods*, *46*(4), 256–262. https://doi.org/10.1016/j.ymeth.2008.10.004

Postnikoff, S. D. L., Johnson, J. E., & Tyler, J. K. (2017). The integrated stress response in budding yeast lifespan extension. *Microbial Cell*, *4*(11), 368–375. https://doi.org/10.15698/mic2017.11.597

Powell, C. D., Quain, D. E., & Smart, K. A. (2000). The impact of media composition and petite mutation on the longevity of a polyploid brewing yeast strain. *Letters in Applied Microbiology*, *31*(1), 46–51. https://doi.org/10.1046/j.1472-765x.2000.00766.x

Prakash, S., & Prakash, L. (2000). Nucleotide excision repair in yeast. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *451*(1–2), 13–24. https://doi.org/10.1016/S0027-5107(00)00037-3

Prakash, S., Sung, P., & Prakash, L. (1993). DNA REPAIR GENES AND PROTEINS OF SACCHAROMYCES CEREVISIAE. *Annual Review of Genetics*, *27*(1), 33–70. https://doi.org/10.1146/annurev.ge.27.120193.000341

Reed, S. H., You, Z., & Friedberg, E. C. (1998). The yeast RAD7 and RAD16 genes are required for postincision events during nucleotide excision repair: In vitro and in vivo studies with rad7 and rad16 mutants and purification of a Rad7/Rad16-containing protein complex. *Journal of Biological Chemistry*, *273*(45), 29481–29488. https://doi.org/10.1074/jbc.273.45.29481

Rine, J., & Herskowitz, I. (1987). Four Genes Responsible for a Position Effect on Expression From HML and HMR in Saccharomyces cerevisiae. *Genetics*, *116*(1), 9–22. https://doi.org/10.1093/genetics/116.1.9

Schroeder, E. A., & Shadel, G. S. (2014). Crosstalk between mitochondrial stress signals regulates yeast chronological lifespan. *Mechanisms of Ageing and Development*, *135*, 41–49. https://doi.org/10.1016/j.mad.2013.12.002

Stefanini, I., De Filippo, C., & Cavalieri, D. (2013). Yeast as a Model in High-Throughput Screening of Small-Molecule Libraries. In *Diversity-Oriented Synthesis* (pp. 455–482). John Wiley & Sons, Inc. https://doi.org/10.1002/9781118618110.ch14

Svejstrup, J. Q. (2007). Contending with transcriptional arrest during RNAPII transcript elongation. In *Trends in Biochemical Sciences*. https://doi.org/10.1016/j.tibs.2007.02.005

Swayne, T. C., Gay, A. C., & Pon, L. A. (2007). Fluorescence Imaging of Mitochondria in Yeast. In *Methods in molecular biology (Clifton, N.J.)* (Vol. 372, pp. 433–459). https://doi.org/10.1007/978-1-59745-365-3_31

Tenreiro, S., & Outeiro, T. F. (2010). Simple is good: yeast models of neurodegeneration. *FEMS Yeast Research*, *10*(8), 970–979. https://doi.org/10.1111/j.1567-1364.2010.00649.x

Tyrrell, D. J., Blin, M. G., Song, J., Wood, S. C., Zhang, M., Beard, D. A., & Goldstein, D. R. (2020). Age-Associated Mitochondrial Dysfunction Accelerates Atherogenesis. *Circulation Research*, *126*(3), 298–314. https://doi.org/10.1161/CIRCRESAHA.119.315644

Walker, J. M., Ditor, S. E. E., & Metabolites, P. S. (n.d.). *METHODS IN MOLECULAR BIOLOGY™*.

Wallace, D. C. (2010). Mitochondrial DNA mutations in disease and aging. *Environmental and Molecular Mutagenesis*, n/a-n/a. https://doi.org/10.1002/em.20586

Wallace, D. C., Brown, M. D., Melov, S., Graham, B., & Lott, M. (1998). Mitochondrial biology, degenerative diseases and aging. *BioFactors*, *7*(3), 187–190. https://doi.org/10.1002/biof.5520070303

Waters, R., Evans, K., Bennett, M., Yu, S., & Reed, S. (2012). Nucleotide Excision Repair in Cellular Chromatin: Studies with Yeast from Nucleotide to Gene to Genome. *International Journal of Molecular Sciences*, *13*(9), 11141–11164. https://doi.org/10.3390/ijms130911141

Woo, D. K., & Poyton, R. O. (2009). The absence of a mitochondrial genome in rho0 yeast cells

extends lifespan independently of retrograde regulation. *Experimental Gerontology*, *44*(6–7), 390–397. https://doi.org/10.1016/j.exger.2009.03.001

Woudstra, E. C., Gilbert, C., Fellows, J., Jansen, L., Brouwer, J., Erdjument-Bromage, H., Tempst, P., & Svejstrup, J. Q. (2002). A Rad26–Def1 complex coordinates repair and RNA pol II proteolysis in response to DNA damage. *Nature*, *415*(6874), 929–933. https://doi.org/10.1038/415929a

Wu, H., & Roks, A. J. M. (2014). Genomic instability and vascular aging: A focus on nucleotide excision repair. *Trends in Cardiovascular Medicine*, *24*(2), 61–68. https://doi.org/10.1016/j.tcm.2013.06.005

Zimmermann, A., Hofer, S., Pendl, T., Kainz, K., Madeo, F., & Carmona-Gutierrez, D. (2018). Yeast as a tool to identify anti-aging compounds. *FEMS Yeast Research*, *18*(6), 1–16. https://doi.org/10.1093/femsyr/foy020