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Research paper

Characterization of long living yeast deletion mutants that lack mitochondrial metabolism genes *DSS1*, *PPA2* and *AFG3*

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ABSTRACT

Molecular mechanisms of aging and longevity are still mostly unknown. Mitochondria play central roles in cellular metabolism and aging. In this study, we identified three deletion mutants of mitochondrial metabolism genes (*ppa2*Δ, *dss1*Δ, and *afg3*Δ) that live longer than wild-type cells. These long-lived cells harbored significantly decreased amount of mitochondrial DNA (mtDNA) and reactive oxygen species (ROS). Compared to the serpentine nature of wild-type mitochondria, a different dynamics and distribution pattern of mitochondria were observed in the mutants. Both young and old long-lived cells produced relatively low but adequate levels of ATP for cellular activities. The status of the retrograde signaling was checked by expression of *CIT2* gene and found activated in long-lived mutants. The mutant cells were also profiled for their gene expression patterns, and genes that were differentially regulated were determined. All long-lived cells comprised similar pleiotropic phenotype regarding mitochondrial dynamics and functions. Thus, this study suggests that *DSS1*, *PPA2*, and *AFG3* genes modulate the lifespan by altering the mitochondrial morphology and functions.

1. Introduction

The term 'aging' refers to the complicated biological process of growing older; it may be defined as a multifactorial phenomenon that is characterized by a time-dependent reduction of physiological functions. In contrast, longevity is a parameter; the length of time that an individual remains alive and it is inversely proportional to the pace at which aging occurs (Brown, 2012). Mitochondria are of great concern in the biology of aging studies, as they are responsible for essential metabolic processes, generation of energy, cellular redox state and many other processes including cellular homeostasis and cell/organism physiology (Palková and Váchová, 2016).

According to the free radical theory of aging, organisms age because of the accumulation of free radical damages over time. The modified mitochondrial theory of aging is based on the fact that mitochondrial DNA (mtDNA) has a higher rate of mutation and less efficient repair machinery compared to nuclear DNA (López-Otín et al., 2013). Mutations in mtDNA that alter the expression of oxidative phosphorylation (OxPhos) complexes can lead to mitochondrial dysfunction and accelerate ROS generation (Wallace, 2010; Hacioglu et al., 2012).

The yeast *Saccharomyces cerevisiae* can grow by fermentation in the presence of glucose or by mitochondrial respiration in the presence of respiratory substrates such as glycerol. However, it primarily prefers fermentation and can inhibit aerobic respiration in the presence of glucose (Skinner and Lin, 2010). Therefore, since *S. cerevisiae* can grow either by fermentation or respiration, it is a suitable model to study the effects of the loss of genes needed for respiration, which can be lethal in other organisms. Yeast mutants defective in oxidative phosphorylation are unable to grow on media containing non-fermentable carbon sources. These respiratory-deficient mutants are often termed as petite that comprise either large deletions in the mitochondrial genome (rho-) or completely lack mitochondrial DNA (rho0). Mutants with lesions in the mitochondrial genome are referred to as cytoplasmic petite, whereas respiratory-deficient strains carrying mutations in the nuclear genome are referred to as nuclear petite or pet mutants (Merz and Westermann, 2009; Tzagoloff and Dieckmann, 1990). Alterations in mtDNA cause a progressive decline in mitochondrial function during aging (Attardi, 2002; Hekimi and Guarente, 2003; Woo and Poyton, 2009).

In fact, the role of mitochondria in the aging process is more

Abbreviations: mtDNA, mitochondrial DNA; ROS, reactive oxygen species; YPD, yeast extract, peptone, dextrose; YPG, yeast extract, peptone, glycerol; DCFH-DA, Dichloro-dihydro-fluorescein diacetate; FACS, Fluorescent activated cell sorting

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complicated than the proposed theories of aging. Multiple changes in mitochondrial function, structure, distribution, and dynamics contribute to aging or age-related features (Copeland et al., 2009). Studies in different model organisms have reported that a change in mitochondrial function can extend lifespan. Mutation or reduced function in nuclear genes encoding electron transport chain (ETC) components in yeast, *C. elegans*, *Drosophila*, and mice delay the aging process (Hacioglu et al., 2012; Hwang et al., 2012). How the mitochondrial signaling pathway modulates the aging process and the identity of the pathway constituents that transmit these longevity signals remain mostly unknown.

Multiple pathways are involved in the crosstalk from the mitochondrion to the nucleus and are conserved among eukaryotes (Woo and Poyton, 2009; Jazwinski, 2014; Butow and Avadhani, 2004). Cells can adapt to impaired mitochondrial functions by activating such an evolutionarily conserved communication pathway referred to as retrograde response (Jazwinski, 2014). As described in yeast and mammals, the response often results in changes in gene expression pattern and overall cell physiology leading to the prevention of cell death (Liu and Butow, 2006). In yeast, the major retrograde pathway (the RTG pathway) includes three activators Rtg1p, Rtg2p and Rtg3p in which Rtg2p transfers the signal from mitochondria to the Rtg1p/Rtg3p (heterodimeric) that translocates from the cytosol to nucleus and activates expression of numerous genes involved in yeast metabolic reprogramming (Palková and Váchová, 2016). This metabolic remodeling encompasses a shift in the utilization of lipid or acetate as the carbon source (Ždralević et al., 2012). The activation of anaplerotic reactions and peroxisomal functions, including the glyoxylate cycle, has long been considered to be a significant RTG pathway response in yeast and the *CIT2* gene, encoding the peroxisomal isoform of citrate synthase, as a typical target of the RTG pathway (Palková and Váchová, 2016). *CIT2* expression mainly depends on *RTG2* when cells lack mitochondrial DNA (Epstein et al., 2001).

With modern genetic techniques, it has been shown that single genes can have a dramatic effect on lifespan (McCormick et al., 2015). Many of these genes act together in known signaling pathways (Fontana et al., 2010; Johnson et al., 2013; Lapierre and Hansen, 2012). In yeast, chronological lifespan (CLS) and replicative lifespan (RLS) are two widely used methods for studying the Biology of aging (Kaeberlein, 2010a; Longo et al., 2012).

In the present investigation, we carried out yeast RLS assay that measures how many daughter cells a mother can produce before it ceases dividing, by using single deletion mutants of mitochondrial metabolism genes to find out whether manipulation to mitochondrial metabolism genes can extend replicative lifespan in the diploid yeast, *S. cerevisiae* (BY4743 background). Out of 144 mitochondrial targeted nuclear genes, we identified three mutants (*ppa2*Δ, *dss1*Δ, and *afg3*Δ) with extended lifespan. Biochemical and genetic characterization of these mutants suggested that altered mitochondrial morphology, functions and activated retrograde signaling path contributed to the lifespan extension in these mutants.

2. Materials and methods

2.1. Yeast strain and culture

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 mutants (*dss1*Δ, *ppa2*Δ and *afg3*Δ) were obtained from EUROSCARF and used in this study. Cells were grown at 30 °C both in solid and liquid YPD broth (1% yeast extract, 2% Peptone, 2% Dextrose and 2% Agar). The concentrations of the yeast cells according to the experimental conditions (optical density) at 600 nm wavelength (OD_{600}) were measured using a spectrophotometer.

2.2. Replicative aging assay

The conventional protocol was directly followed from Kaeberlein (2010b). Briefly, twenty-five cells were randomly chosen for each strain and placed onto a fresh region of YPD solid media. Growth was maintained under 30 °C incubation. A micromanipulator attached with Nikon-Eclipse50 microscope was used to dissect daughter cells from the mother cells after each division. The number of daughter cells produced by the mothers was counted and marked in the score sheet, and the statistical calculation was performed using the Wilcoxon Rank-Sum test. The strains that live longer were confirmed by replicate analysis.

2.3. Elutriation

Logarithmically growing cells were considered as young cells since there are only tiny fractions of old cells present in an exponentially growing population. Thus, young yeast cells were obtained by growing them in fresh 2% YPD growth medium for 6 h at 30 °C. Aged (20-generation old) cells were isolated using an elutriation system (Beckman Coulter Avanti J-26 XP). Cells grown in overnight cultures were loaded into the separation chamber of the elutriation system with a flow rate of 40 ml/min at 2500 rpm. Then the flow rate was fixed at 30 ml/min and the centrifugation speed to 1000 rpm to isolate cells larger than 15 μm (≤20 generation old cells). The resulting old cells were stored at −80 °C until they were analyzed. Isolated cells were checked by counting their bud rings after Fluorescent Brightener-28 staining (Sigma-Aldrich).

2.4. Glycerol spotting assay

Yeast strains were grown in YPG (3% glycerol) and YPD. Serial dilution was performed to obtain OD_{600} values of 0.2, 0.02, 0.002, and 0.0002. Five μl of each solution was dropped onto YPG and YPD-agar plates and incubated at 30 °C for 48 h.

2.5. Confocal microscopic analysis of mitochondrial morphology and distribution

Mitochondria specific dye Mitotracker-Red–CMXRos (Thermo Fisher Scientific) was used to stain mitochondria in live cells. Mitochondria were visualized under a confocal fluorescence microscope (1000×) using 579/599 nm (Excitation/Emission) wavelength. Pictures were captured with a CCD camera (Andor Technology). Manufacturer's protocol was strictly followed in the staining procedure.

2.6. Mitochondrial density measurement

Mitochondria specific dye Mitotracker-Red –CMXRos (Thermo Fisher Scientific) was used to quantify mitochondrial density in young and old cell populations (Optical density, OD_{600} = 0.8 was adjusted for each sample). A flow cytometer (FACS-BD) was used to measure the fluorescent signals in the cells. Significant differences between wild type and mutant cells were determined by using the Wilcoxon rank sum test.

2.7. Measurement of mitochondrial DNA content

The qPCR based method for measuring mitochondrial DNA content was adopted from Masayesva et al. (Merz and Westermann, 2009; Masayesva et al., 2006). In brief, total DNA was isolated from each strain (OD_{600} = 0.8) by utilizing the traditional phenol-chloroform extraction method. The relative content of mitochondrial DNA was measured through quantitative real-time PCR (qPCR- BIO-RAD) by amplifying mitochondrial COX2 gene. COX2 forward primer 5′-CAGC AACACCAAATCAGAAGG-3′; reverse primer 5′-GTCCCACACAACTCAG AACATGCTC-3′ were used for COX2 amplification, and actin gene

(forward primer 5-ACGTTCCAGCCTTCTACGTTTCCA-3 and reverse primer 5-ACGTGAGTAACACCATCACCGGAA-3) was used for normalization. PCR was performed for 40 cycles with 100 ng template DNA in a 25 µl reaction mixture using Maxima SYBR green/ROX qPCR master mix (2×) kit (Thermo Scientific). PCR condition was set as 94 °C for 5 min, followed by 40 cycles of 94 °C - 30 s, 60 °C - 30 s, 72 °C - 30 s and 72 °C for 5 min. The $2^{-\Delta\Delta CT}$ method was applied to analyze the relative measurement of the mitochondrial DNA content among young and old of WT and long-lived cells. Triplicate measurements were done for each of the three biological replicas.

2.8. Measurement of cellular ATP level

The standard protocol for ATP extraction from total cell lysate was adopted from Ding et al. (Ding et al., 2013). Somatic cell ATP bioluminescent assay kit (Sigma Aldrich) was used for luminometric measurement of ATP levels through a Luminometer, Fluoroskan Ascent and FL (Thermo Scientific). Statistical analysis was done by the Student's *t*-test.

2.9. Flow cytometric measurement of endogenous ROS

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) is one of the most widely used techniques for directly measuring intracellular reactive oxygen species. OD_{600} values of cells were adjusted to 0.8 and transferred to a sterile centrifuge tube. A 500 µl aliquot of cells was stained with the 5 µm DCFH-DA. The cells were incubated at 30 °C for 30 min at 180 rpm and then washed three times with PBS buffer. Finally, cells were resuspended in 500 µl PBS and analyzed instantly with a flow cytometer (FACS-BD). Statistical analysis was performed by using two-tailed pair *t*-test.

2.10. TCA cycle activity test

Aconitase activity assay kit (Sigma Aldrich) was utilized to measure the relative performance of the TCA cycle among WT and mutant cells. A spectrophotometer, Multiskan Spectrum (Thermo Electron Corporation), was used for this colorimetric assay. The protocol and design provided by the kit manufacturer were directly followed. Two-tailed pair *t*-test was applied to compare the data.

2.11. In vivo measurement of cytoplasmic pH

Two µl of the pH-sensitive GFP tagged plasmid (pHluorin) p413-TEF (165 ng/µl) was transformed into each yeast strains by the LiAc method. The existence of plasmids containing GFP was confirmed by the fluorescence microscopy. The fluorescence spectrum of pHluorin was detected and confirmed at 390 nm and 470 nm excitations through Perkin Elmer Fluorescence Spectrometer LS 55. The protocol for pHluorin calibration and cytoplasmic pH measurement was adopted from (Orij et al., 2009). In brief, cells were treated with 100 µg digitonin per ml PBS and incubated for 15 min for mild permeabilization. Cells were then resuspended in citric acid/Na2HPO4 buffer of preset pH ranges from pH 5.0 to pH 9.0. The results were plotted against each pH range after background subtraction.

2.12. Real-time PCR (qPCR) analysis of retrograde signal gene

The expression of CIT2 gene was analyzed through a qPCR (Bio-Rad-iQ50) approach. The forward primer of CIT2 was 5′CGGTATTCG TTTCAGAGGTCG, and reverse was 5′GCTTCTGGTAGTGGTTGTGAG. The actin gene (forward primer 5-ACGTTCCAGCCTTCTACGTTTCCA-3 and reverse primer 5-ACGTGAGTAACACCATCACCGGAA-3) was used for normalization. Total RNA was isolated and purified from 30 million cells for each strain by using an RNA isolation kit (Ambion Technology). cDNA was made with the help of first strand cDNA synthesis kit (Thermo Scientific). 100 ng DNA in a 25 µl reaction was used with Maxima SYBR green/ROX qPCR master mix (2×) kit (Thermo Scientific). The $2^{-\Delta\Delta CT}$ method was applied to analyze the data.

2.13. Global gene expression analysis

Total RNAs were isolated from wild-type and deletion mutants with the help of RNA isolation kit (QIAGEN) and stored at −80 °C. Whole genome microarray analysis was performed by using Agilent's One ColorYeast ExpressionArray. Raw data were processed using the LIMMA package of Bio conductor using R programming. Linear model matrix was designed, and intensity values were applied to the design using “lmFit” function. The values were logarithmically transformed, a value of ± 1 (or higher/lower) showed two-fold or higher up/down expression. The molecular and biological functions of the up- and down-regulated genes were identified with MIPS (Munich Information Center for Protein Sequences) classification using the Fun-Spec analysis program.

2.14. Cloning and overexpression

Yeast genes *DSS1*, *PPA2* and *AFG3* were cloned from the yeast ORF collection (Thermo Scientific). Overexpression plasmids containing these genes were constructed by the Advanced Gateway cloning system. BP recombination reactions were performed between the ORFs containing plasmids and donor vector pDONR221 with the help of BP Gateway clonase enzyme mix (Invitrogen) to generate entry clones. Gene insertion into pDONR vector was confirmed by analyzing the *Bsr*GI restriction digestion pattern within the sequence. Then, LR recombination was performed between the entry clones and the destination vector pAG423-GPD (Addgene). After each reaction, the mixtures were transformed into OmniMax2T1 competent cells for the selection of antibiotic-resistant colonies that comprised the target genes in plasmids. Gene replacement into an expression vector was confirmed by analyzing the sequence-specific restriction of Eco-RV. Then the constructed target genes were retransformed into WT cells for overexpression. Traditional LiAc method was applied to this transformation.

3. Results and discussion

3.1. Deletion of AFG3, PPA2, and DSS1 genes extends replicative lifespan

Replicative life span refers to the number of daughter cells produced by a mother cell prior to senescence. We analyzed the replicative life span of wild-type (WT) diploid cells (BY4743), and it's isogenic 144 deletion mutants that lack mitochondrial metabolism genes (Table 1). Mitochondrial protein-encoding nuclear genes and their biochemical paths were derived by using the MITOP2-tool (Elstner et al., 2009). Due to a high number of samples, only ten cells from each strain were subjected in the initial screen. In subsequent analyses, 25 cells were followed in duplicate for each mutant, and an extended lifespan of three mutants was observed. The average replicative life span of WT (control) strain was measured as 25.05 while *afg3*Δ, *ppa2*Δ *and dss1*Δ were observed as 35.05, 32.1 and 30.1 respectively. Thus deletion of *AFG3*, *PPA2*, and *DSS1* genes extended lifespan by 40%, 28%, and 20%, respectively (Fig. 1). The replicative lifespan of these mutants was also analyzed in the BY4741 haploid background, and similar results were obtained (Fig. S1). Advanced Gateway cloning system was applied to clone *AFG3*, *PPA2*, and *DSS1* genes into a high copy number plasmid and therefore transformed into WT cell but overexpression of these genes did not alter the life span of the cells (Fig. S2).

Since mitochondria are sensitive targets of reactive oxygen species (ROS), eukaryotic cells have developed several mitochondrial protein quality control systems that include mitochondrial proteases, which mediate protein homeostasis and prevent oxidant-induced

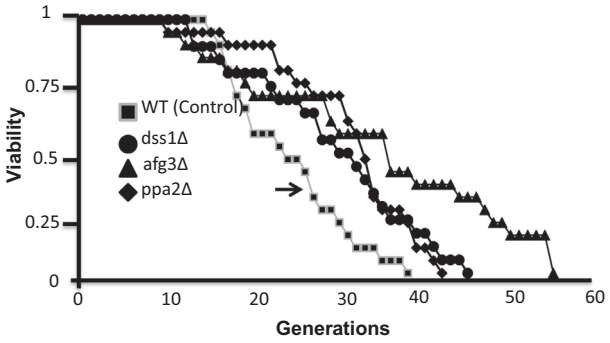

Fig. 1. Replicative Lifespan (RLS) analyses: Yeast RLS assay showing the lifespan curve of both control (WT) and the long living mutants. The average lifespan of WT and mutants' population were measured by counting the number of daughter cells produced by the mothers and obtained data were evaluated by using the Wilcoxon Rank-Sum test. Compared to the wild-type (WT) cells, *dss1*Δ, *ppa2*Δ and *afg3*Δ mutants showed 20%, 28% and 40% extended lifespan respectively. Arrow indicates the control curve of wild type (WT) cells.

mitochondrial damages (Volonte et al., 2016; Chen et al., 2011). In Yeast, Afg3 acts on the mitochondrial inner membrane as m-AAA protease (ATPase Associated Activity) that degrades misfolded or unassembled proteins (Arlt et al., 1998), thus involved in protein folding pathway (Table 1). Moreover, m-AAA has been proposed as a regulator of mitochondrial protein synthesis (Atorino et al., 2003). Yeast cells (BY4742 background) with the deleted *AFG3* gene are highly stress-resistant and live longer (Delaney et al., 2013), which is consistent with our observations regarding the lifespan extension of *afg3*Δ in the BY4743 background (Fig. 1). Recently, Volonte et al., 2016 found that AFG3L2, an m-AAA type of mitochondrial protease, is a novel caveolin-1-binding protein. Expression of a mutant form of AFG3L2 fails to localize to mitochondria and promotes degradation of ETC complex IV after oxidative stress.

Mitochondrial degradosome, encoded by *DSS1* (*MSU1*) and *SUV3*, plays a role in mitochondrial RNA metabolism by degrading aberrant RNAs. The RNase activity of this gene is necessary for mitochondrial biogenesis, and deletion of *DSS1* strongly inhibits mitochondrial translation (Dziembowski et al., 2003). Deletion of *DSS1* extends yeast replicative life span in the S288C background (Caballero et al., 2011), which is also consistent with our current investigation where we used BY4743 strain background and thus validated the data. However, our lab is the pioneer in identifying the long-lived cell, *ppa2*Δ in the same background. Ppa2 is a mitochondrial inorganic pyrophosphatase and required for mitochondrial DNA synthesis and mitochondrial function (Lundin et al., 1991). Thus, disruption of any of the genes (*DSS1/PPA2/AFG3*) was initially suspected to affect mitochondrial morphology and functions.

3.2. Mitochondrial morphology of ppa2Δ, dss1Δ and afg3Δ cells

To understand the mechanisms by which mutant cells live longer, we performed some mitochondria-related assays. Mitochondrial morphology and distribution were investigated through confocal fluorescence microscopy of cells stained with mitochondria-specific dye Mitotracker Red CmxRos. WT cell exhibited the serpentine nature of mitochondrial chain network as expected, while mutants' mitochondria were aggregated and formed aggregated spots in the cytoplasm (Fig. 2A).

In the absence of the beneficial mitochondrial m-AAA activity in cells leading to the accumulation of nonfunctional mitochondria (Volonte et al., 2016). However, the molecular mechanisms through which m-AAA protease is regulated remain poorly understood. Similarly, in the present study, mitochondria of the mutated strains were observed as aggregated spots instead of a network. The regulation of mitochondrial architecture is mediated by fusion/fission events, and mitochondria are selectively removed by mitophagy that degrades imperfectly functional mitochondria (Chistiakov et al., 2014). But our analyses showed that mitochondria were not destroyed; instead, mutants had sufficient levels of aggregated mitochondria (Fig. 2A, B), moreover, young *dss1*Δ and *ppa2*Δ cells had 1.7 fold and *afg3*Δ had 1.4 fold higher ($p < 0.05$) mitochondrial mass compared to wild type young cell (Fig. 2B); which suggested that they may functioned in a remodeled way in the mutants. The mitochondrial fission/fusion cycle plays a role in mitophagy, since deletion of *DNM1*, which is required for fission, attenuates mitophagy without eliminating it (Kanki et al., 2009). Interestingly, deletion of this gene extends yeast replicative lifespan (Scheckhuber et al., 2007). *DSS1*, *PPA2*, and *AFG3* genes interact with *DNM1* that has an important role in mitochondrial localization, fission, fusion and early mitophagy (Hoppins et al., 2011). *AFG3* genetically interacts with MIC family genes (*MIC12*, *MIC26*, *MIC19*, *MIC26*, *MIC27*, *and MIC60*) which act for setting focal adhesion of mitochondrial membrane and network (Costanzo et al., 2010). Taken together, it is likely that the deletion of *DSS1*, *PPA2*, and *AFG3* genes may lead to loss of the interactive network with the regulatory genes that may lead to mitochondrial aggregation.

3.3. mtDNA content and respiratory phenotypic characterization of long living cells

We, therefore, examined the mtDNA contents of the cells. In *Saccharomyces cerevisiae*, the mtDNA encodes components of the mitochondrial translational apparatus, as well as protein subunits of respiratory complexes III, IV and V (Contamine and Picard, 2000). The most common spontaneous events that result in altered mitochondrial function in budding yeast are either presence of mitochondrial DNA with reduced expression or partial loss of mitochondrial genome [ρ−] or total loss of the mtDNA [ρ0] which abolishes production of mitochondrial encoded proteins. Merz et al. 2009 (Merz and Westermann, 2009) classified all *pet* mutants (in the BY4742 background) in which

Table 1
List of mitochondrial genes that were analyzed in this study.

Pathway	ORF name
Carbohydrate metabolism	ACH1, IDP1, KGD2, AIP2, LIP5, IDH1, DLD1, KGD1, ACO1, MAE1, ICL2, ESP35, ALO1, PDB1, ACN9, LSC2, OSM1, MDH1, GPD3, IDH2, LAT1, CIT3, ALD5, GUT2, HPD1, PDA1, ACO2, FUM1, CIT1, ADH3, LSC1, PDX1, ALD7
Amino acid metabolism	GSD2, CHA1, PUT1, GCV3, GSD1, SHMT1, MET11, PUT2, ISO1, ARG7, LYS3, MET23, LYS10, AAT1, LEU4, ILV6, ARG5,6, ARG2, MIS1
Lipid metabolism	RPS25A, HMG1, CLS1, MRF1, OAR1, CEM1, PPT2, HMG2, MCR1, MCT1, PSD1
Nucleic acid metabolism	SED1, TST1, UNG1, RIM1, MGM9, MIP1, HMI1, FUN33, IPP2, OGG1, ADK2, MHR1, ABF2, DIN3, MGT1, MSH1, PHR1, NUC1, PPA2
Other (heme-ubiquinone) metabolism	SUV3, PET157, MRH4, REX2, CBP2, RNA12, CBP6, CYT2, DSS1, PET56, MRE2, SSB1, CYC3, MSS116, NAM1, MTF1, RPO41, HEM14, CBP1, MSS18, SLS1
Biogenesis of iron-sulfur cluster	ISA1, ISU2, SSC2, ISU1, ISA2, NFU1
Protein sorting	TOM6, TIM18, TIM13, TOM7, TOM37, MDJ2, IMP1, TOM72, FMP17, MSS2, IMP2, TOM5, FUN37, OXA1, GON1, HOT13, PNT1, CYC2, OCT1, MDJ1, TOM70
Protein folding	RCA1, PHB2, OMA1, PHB1, MCX1, HSP78, AFG3, SSA2, OSD1, CYP3, PRD1, MDM37, PIM1, SSC3, YIM1

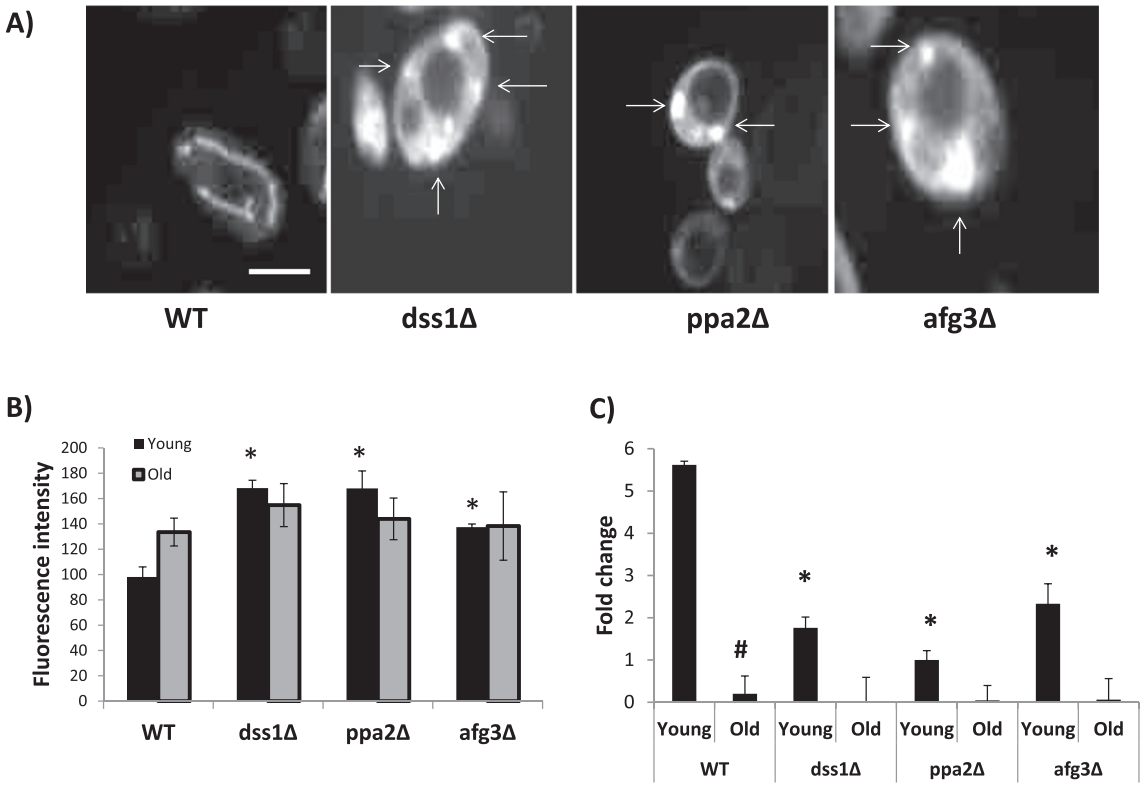

Fig. 2. Mitochondrial status of long living cells: A) Mitochondrial morphology. The confocal microscopic analysis was performed after Mitotracker Red CmxRos (Thermo Fisher Scientific) staining. WT cells comprised mitochondrial network where as long living mutants showed aggregated and colonized pattern of mitochondria in the cytoplasm. The arrows indicated the aggregation spots of the mitochondria. Scale bar = 5 μm. B) Mitochondrial mass. Mitochondrial mass was quantified by measuring the fluorescence intensity (579-excitation/599-emission) of the Mitotracker-Red–CMXRos stained cells through a flow cytometer (FACS-BD). Six samples from each strain were analyzed. Mutant young cells showed significantly higher ($*p < 0.05$) mitochondrial mass compared to WT counterpart. C) Mitochondrial DNA content. The relative content of mitochondrial DNA was measured through quantitative real-time PCR (qPCR- BIO-RAD) by amplifying the mitochondrial *COX2* gene. Wild-type cells showed decreased mtDNA levels as they aged ($\#p < 0.05$). Mutant young cells had significantly lower ($p < 0.05$) amount of mtDNA compared to WT young (significant differences are indicated with an asterisk). Triplicate measurements were done for each of the three biological replicas.

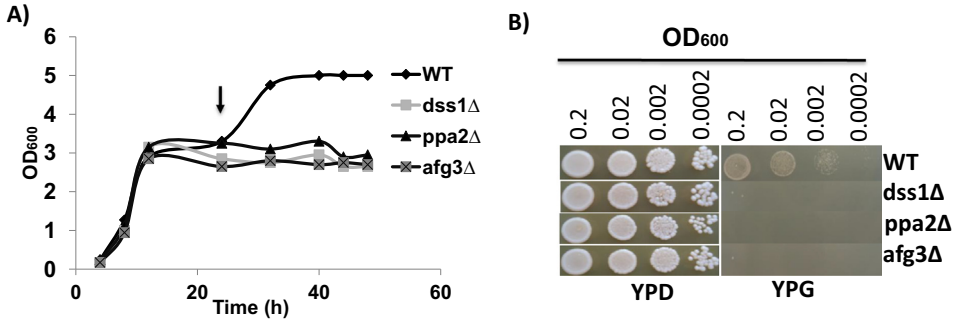

Fig. 3. Respiration and proliferation status of the long living cells: A) Growth curve analyses. Mutants showed almost similar proliferation rate compared to the WT until glucose was consumed (arrow). WT cells went through the diauxic shift, but the mutants did not have the second stage of the growth. B) Glycerol spotting assay. Long living mutants did not grow on glycerol (right panel) (YPG) as the carbon source, while they showed a similar colony morphology compare to WT in YPD plates (left panel).

DSS1 and *PPA2* genes were listed as essential for respiration but not for mitochondrial DNA maintenance and deletions of them confer gradual loss of mtDNA in the mutants which were visualized through DAP1 staining. In our present investigation, we did not test the functional state of mtDNA, but we measured the relative content of mtDNA through a quantitative real-time PCR analysis by amplifying the *COX2* gene that lies within the mitochondrial genome. We found that wild-type cells showed a decrease in their mtDNA levels as they age (5-fold less). Mutant cells had significantly lower (3–4 fold less) amount of mtDNA when compared to that of wild-type cells and their mtDNA was completely lost as they aged (Fig. 2B) which is consistent with the previous study done by Merz et al. 2009 (Merz and Westermann, 2009), regarding mtDNA depletion in the mutants. Thus, aging was associated with mtDNA loss in all cells, but the extent of lost was bigger in the long living mutants.

Loss of mtDNA leads to nuclear genome instability through a process of cell-cycle arrest, which indicates that the relative content of mtDNA is vital for cellular proliferation (Veatch et al., 2009). Our mutant cells comprised mtDNA but significantly less in amount compared to that of the wild type, but the presence of a certain amount of mtDNA was found enough for effective proliferation of the young cells under normal conditions. Reduction of mtDNA copy number may pass a signal to the nucleus to create a pleiotropic phenotype on mitochondrial morphology and function, thus, in turn, may facilitate longevity. mtDNA loss in the mutants could be linked to the respiration deficiency of the cells because respiratory deficient cells tend to lose their mtDNA (Veatch et al., 2009).

Cells with defective mitochondria cannot utilize respiratory substrates such as ethanol or glycerol. We tested whether long living mutants were defective in respiration by growing them on a glycerol-

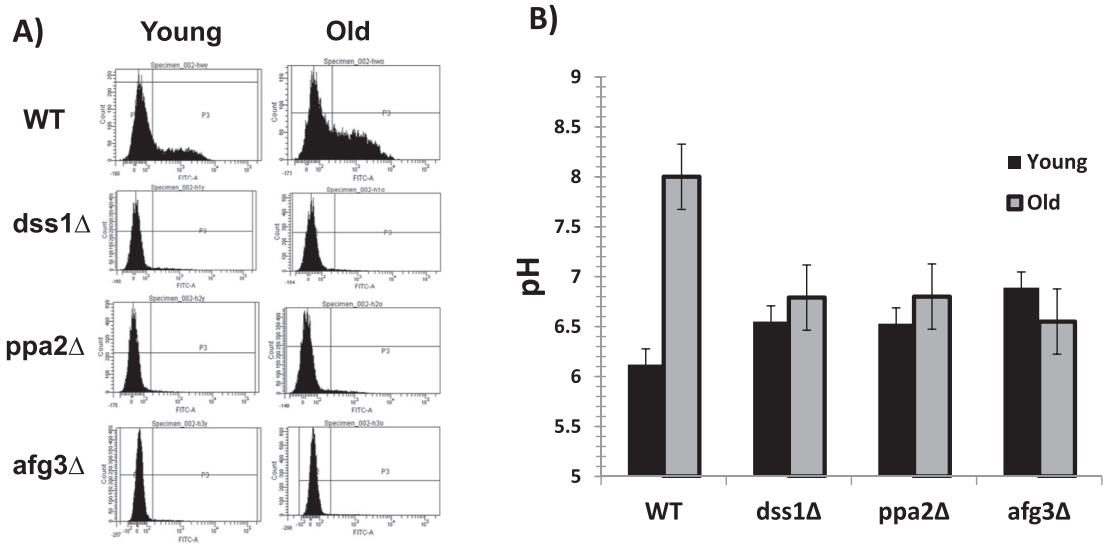

Fig. 4. Intracellular ROS and pH levels. A) Level of endogenous ROS. ROS specific stain DCF-DA was used to measure endogenous ROS level by a flow cytometer (FACS-BD). WT old cells showed a higher level of ROS compares to the young ones ($p \leq 0.05$). All long living mutants showed less ROS in both young and old stages. B) Intracellular pH determination. Ratiometric pHluorin p413 TEF (pH-sensitive GFP plasmid) was used in to measure cytoplasmic pH. The ratio of pHluorin emission by 512 and excitation ratio R390/470 was measured through Perkin Elmer Fluorescence Spectrometer LS 55. WT young cells exhibited an acidic pH, while replicatively old cells showed a higher (basic) pH value. Triplicate measurements were done for each of the two biological replicas.

containing media. Proliferation rates of cells in glucose were normal as they were assessed by a growth curve analysis in liquid YPD media (Fig. 3A). WT cells were able to go through the diauxic shift (able to utilize ethanol). However, the mutants were not able to grow after they consumed the glucose in the media (Fig. 3A). The mutants were not able to utilize glycerol as the carbon source (Fig. 3B) which showed that these cells had respiration deficiency.

3.4. Cellular ROS and pH levels

Mitochondria are both the place of ROS formation and the primary targets of ROS attack. Mitochondrial dysfunction is often associated with increased ROS production (Leadsham et al., 2013). Intracellular ROS is produced by mitochondrial respiration. Our mutants were respiration-deficient which prompted us to determine their ROS levels. A flow cytometric approach was performed to measure endogenous ROS produced by the cells by the usage of the ROS specific stain DCF-DA. During the course of the aging, wild-type cells experienced a higher level of ROS production ($p < 0.05$), although none of the mutants harbored intracellular ROS (Fig. 4A). Apparently, ROS is not the cause of aging, but it can modulate the lifespan (Koc et al., 2004; Salmon et al., 2010). We believe, our mutants benefited from the ROS less state, which in turn may facilitate longevity.

There is a relationship between intracellular pH and aging. Vacuolar pH increases as cells age replicatively and preventing this pH increase suppresses mitochondrial dysfunctions which extend the lifespan (Hughes and Gottschling, 2012). Old cells seem to accumulate proton pumper Pma1 in their cell membranes. By taking protons out of the cell, Pma1 significantly decreases the number of protons available to acidify the vacuole and thus disrupts the pH regulation. We wondered whether deletion of *PPA2*, *DSS1*, and *AFG3* genes are associated with pH change in the cell. Ratio metric pHluorin p413 TEF was used to measure cytoplasmic pH of long-living cells. WT young cells exhibited an acidic pH, while relatively old cells had a higher (basic) pH value. Consistent with the extended life spans, all of the mutants were able to maintain their intracellular acidity as they aged (Fig. 4B). Ability to keep their pH values within normal ranges (pH 6–7) could be one of the reasons why mutant cells live longer than wild-type cells.

3.5. ATP levels and TCA activity

Energy production abilities of the cells seem to be critical in lifespan determination (Hacioglu et al., 2012). Our mutants were unable to utilize mitochondrial paths to derive energy since they were deficient in respiration. In this case, we wanted to know whether they could synthesize comparable levels of ATP by glycolysis and how their ATP profiles change during the aging process. A significant difference was observed between wild-type young and old cells ($p < 0.05$). As seen in Fig. 5A, wild-type old cells had almost the half amount of the ATP that wild-type young cells harbored, thus aging reduced the amount of ATPs in normal cells. The mutants had different profiles for their young and old stages, but they all contained less amount of ATP ($p < 0.05$) when compared to those of the wild-type counterparts.

We also analyzed ATP levels in a time-dependent manner in cells growing in YPD media for 72 h (Fig. 5B). For the initial period, ATP contents were similar to those shown in Fig. 5A. After glucose was consumed (after 24 h of incubation), only wild-type cells were able to synthesize ATP. Although mitochondria are essential organelles, respiration or mtDNA is not required for the viability of budding yeast on fermentable medium, because glycolysis can make sufficient ATP needed for survival (Garipler et al., 2014). Yeast deficient of m-AAA have dramatic respiratory defects and also have reduced ATP synthesis due to the disrupted assembly of respiratory chain complexes leading to switch from oxidative phosphorylation to glycolysis (Tzagoloff et al., 1994). The mutant cells had impaired respiration, and thus they had to obtain their ATP by glycolysis which produces less ATP compared to the aerobic respiration reactions. Consistently, the mutants had a significantly lower level of ATP compared to the wild-type cells ($p < 0.05$); however, they were able to maintain a threshold level of ATP for their cellular processes. Thus a higher level of ATP did not provide longer lifespan to the wild-type cells.

TCA cycle is the master regulator of many biochemical reactions. The TCA cycle is the central metabolic pathway, and its activity is maintained during fermentation for biosynthetic reactions in yeast cells. The TCA activity assay was employed to further identify the deficiencies in the mitochondrial metabolism of the mutant cells. The activity of the TCA cycle was monitored by checking the activity of mitochondrial aconitase that converts citrate to isocitrate. Long living young cells (*ppa2*Δ and *afg3*Δ) exhibited slightly higher aconitase

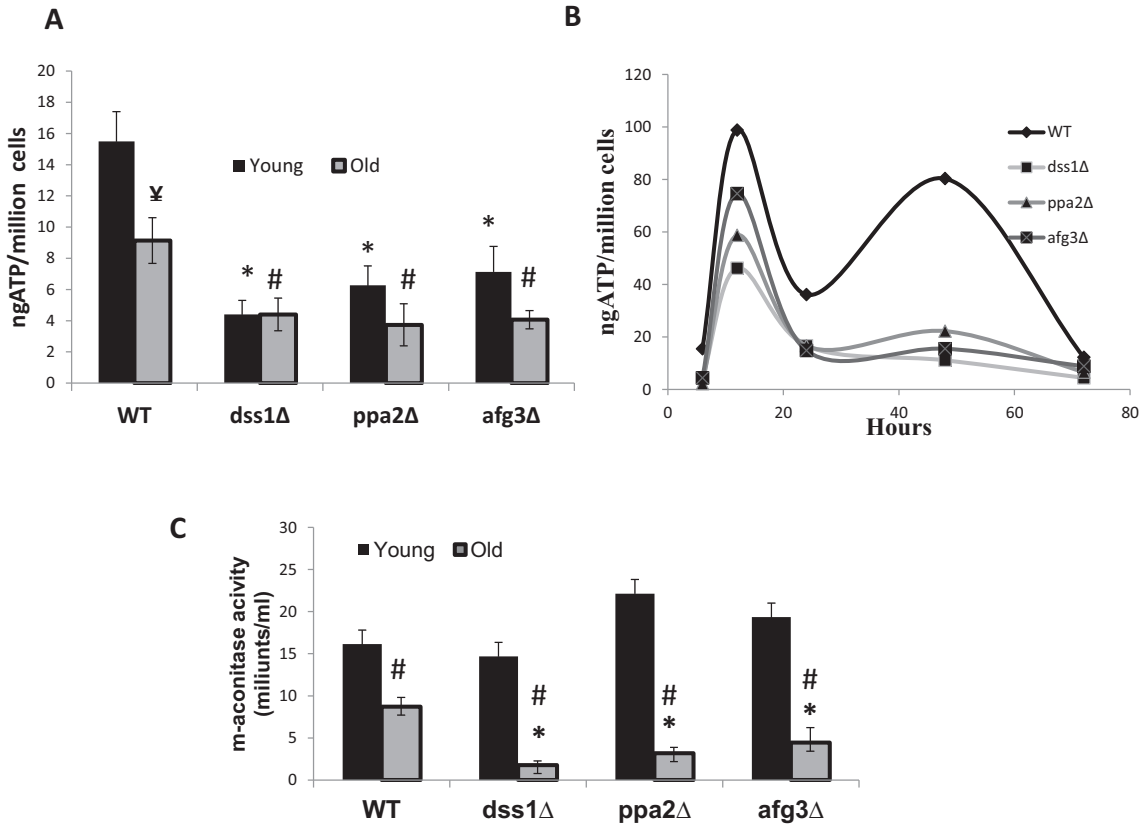

Fig. 5. Analysis of mitochondrial functions. A) Measurement of cellular ATP levels. ATP bioluminescent assay kit (Sigma Aldrich) was used for luminometric measurement ATP levels by a Luminometer, Fluoroskan Ascent and FL (Thermo Scientific). A significant difference was observed between wild-type young and old cells (¥$p < 0.05$). All long living mutants also showed less amount of ATP compares to the wild type counterparts (*$p < 0.05$ compared with wild type young and #$p < 0.05$ compared with wild type old cells). Triplicate measurements were done for each of the three biological replicas. B) ATP levels during exponential growth. Only wild-type cells were able to synthesize ATP after the diauxic shift. C) TCA cycle activity. Aconitase (TCA cycle enzyme) activity assay kit (Sigma Aldrich) was used to measure TCA cycle activity. Spectrophotometer, Multiskan Spectrum (Thermo Electron Corporation) was used for this colorimetric assay. Long living young cells (*ppa2*Δ and *afg3*Δ) exhibited slightly higher aconitase activity. All old cells showed significantly lower (#$p < 0.05$) aconitase activity compared to the corresponding young ones. Significantly less (*$p < 0.05$) aconitase activity was observed in the old mutant cells when compared with wild type old one. Triplicate measurements were done for each of the three biological replicas.

activity. As expected, compared to wild type (WT) young, WT-old cells rendered significantly less ($p < 0.05$) aconitase activity. Similarly in the mutants, old cells showed significantly lower ($p < 0.05$) aconitase activity when compared to its own young's. But the extent of decrease (between young and old) was greater in the mutants (about 2 fold in WT while 5–8 fold in mutants). When we compared the aconitase activity of mutant old cells with WT-old one, significant difference ($p < 0.05$) was observed (Fig. 5C). The long living mutants were able to utilize the TCA cycle in their young stage as determined by the activity of aconitase in cell extracts. The activation of the glyoxylate cycle in peroxisomes produces citrate to fuel the synthesis of α-ketoglutarate in the TCA cycle (Jazwinski, 2014). Thus the TCA cycle is compromised in cells experiencing mitochondrial defects, but flux through the pathway can be maintained by the action of the retrograde pathway (Lin et al., 2011).

3.6. Activation of the retrograde signaling path in ppa2Δ, dss1Δ, and afg3Δ

The retrograde signaling path allows the communication between mitochondria and the nucleus to coordinate mitochondrial protein synthesis during biogenesis and also to communicate mitochondrial malfunctions, initiating compensatory responses in the nucleus. One of the hallmarks of the retrograde response is its capacity to extend the replicative lifespan of the cells. Since our long-lived mutants (BY4743 background) had decreased mtDNA along with impaired mitochondrial functions and aggregated morphology, we reasoned that retrograde signaling path might play roles in these cells to compensate mitochondrial functions and extend the lifespan. The activity of the retrograde pathway can be assessed by examining the expression of *CIT2* gene that encodes peroxisomal citrate (González et al., 2017). Another function of Cit2p is to stimulate peroxisome biogenesis in periods of mitochondrial stress (Epstein et al., 2001) and glyoxylate cycle regulation (Jazwinski, 2014). So to check the status of the retrograde signaling, we analyzed the transcript level of the *CIT2* gene, which is a known transcriptional target of the retrograde regulation. A real-time PCR approach was utilized for quantification of *CIT2* gene expression (Fig. 6). It was significantly up-regulated (2–5 fold higher) in all three mutants. Thus, we found that retrograde signaling was activated in our mutants. Thus activation of the retrograde signaling could be another factor that extended the lifespan of these cells. It is also reported that the complete loss of mitochondrial genome in JM43 background yeast (JM43ρ0) cell extends life span independently of retrograde regulation (Woo and Poyton, 2009). Extension of RLS in several long-lived, large subunit ribosomal protein deletion mutants entirely depend on Gcn4 (Jazwinski, 2014). In contrast, Gcn4 dependent pathway was thought to activate in *afg3*Δ (BY4742 background), though polysome profile of *afg3*Δ cells resembles the general decrease in mRNA translation without an imbalance of free 40S and 60S subunits (Delaney et al., 2013). However, crosstalk between the retrograde response and other signaling pathways also exists in cells (Jazwinski, 2014).

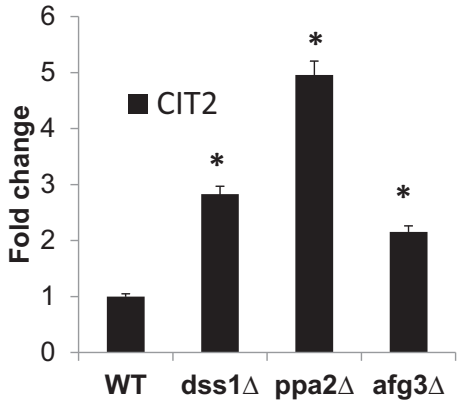

Fig. 6. Quantitative Real-time PCR analysis of CIT2 mRNA levels. Retrograde response pathway gene *CIT2* had a significantly higher expression (*$p < 0.05$) among all long-lived mutants. Triplicate measurements were done for each of the three biological replicas. Bars represent the mean values ± SD.

3.7. Global gene expression analyses of the mutants

Regarding mitochondrial function and morphology, similar pleiotropic phenotypes were observed among all mutants, indicating that they may share a common gene expression pattern that confers longevity. So, to further characterize the long living mutants, we analyzed their expression patterns and compared them to that of wild-type cells to see how they differ from the wild-type cells regarding up- or down-regulated genes (Supplementary Tables 1 and 2). Fun Spec, a web-based cluster Interpreter for Yeast, the analysis was done with MIPS (Munich Information Center for Protein Sequences) to identify the molecular and biological function of up and down-regulated genes (Supplementary Tables 3 and 4).

Data obtained from MIPS functional clustering reveals that all the repressed genes that are commonly found in long-lived cells are involved in the metabolic pathways of sulfur-containing amino acid methionine and cysteine metabolisms along with sulfate assimilation and free radical detoxification paths. Cysteine metabolic pathway involves in sulfate to sulfide conversion and glutathione synthesis that modulate free radical detoxification (Gorrini et al., 2013; Wu et al., 2004). We speculate that free radical detoxification is not needed for our long living cells hence they exhibited almost no ROS.

Our data suggested that the mutant cells favor the glyoxylate cycle since *CIT2* gene and genes that are important for fatty acid metabolism in glyoxylate cycle such as *POT1* and *POX1* (Hiltunen et al., 2003) and *FET3*, an iron transporter, which accumulates iron into mitochondria, had a higher level of expression in young cells. In yeast chronological aging, activated glyoxylate cycle couples with lower Krebs cycle activity and this metabolic shift leads to an increase in both production and utilization of succinate, which encounters with better cell viability (Samokhvalov et al., 2004). It is also possible that our long-lived mutants benefitted from such a metabolic shift.

More surprisingly, *STL1* (Glycerol/H+ symporter) gene showed a very high-level expression (32-fold). It is reported that osmotolerant yeast cells exhibit *STL1* transporter activity to maintain intracellular glycerol content (Tulha et al., 2010). Glycerol biosynthesis also has essential roles in budding yeast for maintaining cytosolic redox balance, especially under anaerobic conditions (Wang et al., 2001). The retrograde signaling stimulates the glycolytic breakdown of glucose to glycerol (Ruiz-Roig et al., 2012). Additionally, glycerol/H+ proton symporter overexpression may lead to accumulation of protons and confers an acidic cytoplasm. All the long-lived mutants we analyzed obtained a slightly acidic cytoplasm (pH 6.3–6.7), shown in Fig. 4B, however, we did not address whether overexpression of *STL1* had a role in pH regulation in these mutants.

The microarray data also showed that purine biosynthesis genes had a higher level of expression, especially for the synthesis of IMP which is the precursor of nucleic acids, AMP, GMP, cAMP (Stenesen et al., 2014). Apart from purines, genes (*CLB2*, *SWI5*, *CIK1*, *SFG1*, *HOF1*, *BUD4*, and *CWP1*) that play roles in cytoskeleton and budding were also up-regulated (Supplementary Tables 1 and 3). Activation of these genes may increase the number of buddings and thus extend the replicative lifespan.

Apart from our microarray analyses, we analyzed previously published data (Koc et al., 2004) to assess the expression patterns of *DSS1*, *AFG3* and *PPA2* genes in the old cells. None of these genes had a significant change in their transcript levels in 20-generation old cells. Furthermore, the Yeast Interactome Database yielded no functional interaction among these genes.

4. Conclusion

Overall, we characterized three long living mutants biochemically and genetically. We suggested that altered mitochondrial morphology (mitochondrial aggregation, a significant reduction of mtDNA content) and functions (respiratory deficiency, the absence of endogenous ROS and decreased ATP level) triggered mitochondrial stress to activate retrograde signal path that harbor lifespan prolongation of these mutants. Commonly found overexpressed and repressed genes further supported the conserveness of the pathway in these mutants. However, we did not determine why the lifespan extension of these mutants varied from each other or, why the extended RLS in *afg3*Δ (40%) was at a higher degree than two others (20% increased in *dss1*Δ, and 28% increased in *ppa2*Δ). These issues should be further investigated.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2019.05.001.

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References

Arlt, H., Steglich, G., Perryman, R., Guiard, B., Neupert, W., Langer, T., 1998. The formation of respiratory chain complexes in mitochondria is under the proteolytic control of the m-AAA protease. EMBO J. 17, 4837–4847. https://doi.org/10.1093/emboj/17.16.4837.

Atorino, L., Silvestri, L., Koppen, M., Cassina, L., Ballabio, A., Marconi, R., Langer, T., Casari, G., 2003. Loss of m-AAA protease in mitochondria causes complex I deficiency and increased sensitivity to oxidative stress in hereditary spastic paraplegia. J. Cell Biol. 163, 777–787. https://doi.org/10.1083/jcb.200304112.

Attardi, G., 2002. Role of mitochondrial DNA in human aging. Mitochondrion 2, 27–37.

Brown, K., 2012. Aging: the mitochondrial connection. J. Clin. Exp. Pathol. s4. https://doi.org/10.4172/2161-0681.S4-003.

Butow, R.A., Avadhani, N.G., 2004. Mitochondrial signaling: the retrograde response. Mol. Cell 14, 1–15. https://doi.org/10.1016/S1097-2765(04)00179-0.

Caballero, A., Ugidos, A., Liu, B., Öling, D., Kvint, K., Hao, X., Mignat, C., Nachin, L., Molin, M., Nyström, T., 2011. Absence of mitochondrial translation control proteins extends life span by activating Sirtuin-dependent silencing. Mol. Cell 42, 390–400. https://doi.org/10.1016/j.molcel.2011.03.021.

Chen, B., Retzlaff, M., Roos, T., Frydman, J., 2011. Cellular strategies of protein quality control. Cold Spring Harb. Perspect. Biol. 3, a004374. https://doi.org/10.1101/cshperspect.a004374.

Chistiakov, D.A., Sobenin, I.A., Revin, V.V., Orekhov, A.N., Bobryshev, Y.V., 2014. Mitochondrial aging and age-related dysfunction of mitochondria. Biomed. Res. Int. 2014. https://doi.org/10.1155/2014/238463.

Contamine, V., Picard, M., 2000. Maintenance and integrity of the mitochondrial genome: a plethora of nuclear genes in the budding yeast. Microbiol. Mol. Biol. Rev. 64, 281–315. https://doi.org/10.1128/MMBR.64.2.281-315.2000.

Copeland, J.M., Cho, J., Lo, T., Hur, J.H., Bahadorani, S., Arabyan, T., Rabie, J., Soh, J., Walker, D.W., 2009. Extension of Drosophila life span by RNAi of the mitochondrial respiratory chain. Curr. Biol. 19, 1591–1598. https://doi.org/10.1016/j.cub.2009.08.016.

Costanzo, M.; Baryshnikova, A.; Bellay, J.; Kim, Y.; Spear, E. D.; Sevier, C. S.; Ding, H.; Koh, J. L. Y.; Toufighi, K.; Mostafavi, S.; Prinz, J.; St. Onge, R.P. VanderSluis B. Makhnevych, T. Vizeacoumar, F. J. Alizadeh, S. Bahr, S. Brost, R. L. Chen, Y. Cokol, M. Deshpande, R. Li, Z. Lin, Z.-Y. Liang, W. Marback, M. Paw, J. San Luis, B.-J. Shuteriqi, E. Hin Yan Tong, A. van Dyk, N. Wallace, I. M. Whitney, J. A. Weirauch, M.

T. Zhong, G. Zhu, H. Houry, W. A. Brudno, M. Ragibizadeh, S. Papp, B. Pál, C. Roth, F. P. Giaever, G. Nislow, C. Troyanskaya, O. G. Bussey, H. Bader, G. D. Gingras, A.-C. Morris, Q. D. Kim, P. M. Kaiser, C. A. Myers, C. L. Andrews, B. J. Boone, C. The genetic landscape of a cell. Science 2010, 327, 425–431.

Delaney, J.R., Ahmed, U., Chou, A., Sim, S., Carr, D., Murakami, C.J., Schleit, J., Sutphin, G.L., An, E.H., Castanza, A., Fletcher, M., Higgins, S., Jelic, M., Klum, S., Muller, B., Peng, Z.J., Rai, D., Ros, V., Singh, M., Wende, H. Vander, Kennedy, B.K., Kaeberlein, M., 2013. Stress profiling of longevity mutants identifies Afg3 as a mitochondrial determinant of cytoplasmic mRNA translation and aging. Aging Cell 12, 156–166. https://doi.org/10.1111/acel.12032.

Ding, J., Bierma, J., Smith, M.R., Poliner, E., Wolfe, C., Hadduck, A.N., Zara, S., Jirikovic, M., Van Zee, K., Penner, M.H., Patton-Vogt, J., Bakalinsky, A.T., 2013. Acetic acid inhibits nutrient uptake in Saccharomyces cerevisiae: Auxotrophy confounds the use of yeast deletion libraries for strain improvement. Appl. Microbiol. Biotechnol. 97, 7405–7416. https://doi.org/10.1007/s00253-013-5071-y.

Dziembowski, A., Piwowarski, J., Hoser, R., Minczuk, M., Dmochowska, A., Siep, M., Van der Spek, H., Grivell, L., Stepien, P.P., 2003. The yeast mitochondrial degradosome: its composition, interplay between RNA helicase and RNase activities and the role in mitochondrial RNA metabolism. J. Biol. Chem. 278, 1603–1611. https://doi.org/10.1074/jbc.M208287200.

Elstner, M., Andreoli, C., Klopstock, T., Meitinger, T., Prokisch, H., 2009. Chapter 1 the mitochondrial proteome database. MitoP2. Methods Enzymol. 457, 3–20.

Epstein, C.B., Waddle, J.A., Hale, W., Dave, V., Thornton, J., Macatee, T.L., Garner, H.R., Butow, R.A., 2001. Genome-wide responses to mitochondrial dysfunction. Mol. Biol. Cell 12, 297–308. https://doi.org/10.1091/mbc.12.2.297.

Fontana, L., Partridge, L., Longo, V.D., 2010. Dietary restriction, growth factors and aging: from yeast to humans. Science 328, 321–326. https://doi.org/10.1126/science.1172539.

Garipler, G., Mutlu, N., Lack, N.A., Dunn, C.D., 2014. Deletion of conserved protein phosphatases reverses defects associated with mitochondrial DNA damage in Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. U. S. A. 111, 1473–1478. https://doi.org/10.1073/pnas.1312399111.

González, B., Mas, A., Beltran, G., Cullen, P.J., Torija, M.J., 2017. Role of mitochondrial retrograde pathway in regulating ethanol-inducible filamentous growth in yeast. Front. Physiol. 8, 148. https://doi.org/10.3389/fphys.2017.00148.

Gorrini, C., Harris, I.S., Mak, T.W., 2013. Modulation of oxidative stress as an anticancer strategy. Nat. Rev. Drug Discov. 12, 931–947. https://doi.org/10.1038/nrd4002.

Hacioglu, E., Demir, A.B., Koc, A., 2012. Identification of respiratory chain gene mutations that shorten replicative life span in yeast. Exp. Gerontol. 47, 149–153. https://doi.org/10.1016/j.exger.2011.11.009.

Hekimi, S., Guarente, L., 2003. Genetics and the specificity of the aging process. Science 299, 1351–1354 (80-.).

Hiltunen, J.K., Mursula, A.M., Rottensteiner, H., Wierenga, R.K., Kastaniotis, A.J., Gurvitz, A., 2003. The biochemistry of peroxisomal β-oxidation in the yeast Saccharomyces cerevisiae. FEMS Microbiol. Rev. 27, 35–64.

Hoppins, S., Collins, S.R., Cassidy-Stone, A., Hummel, E., DeVay, R.M., Lackner, L.L., Westermann, B., Schuldiner, M., Weissman, J.S., Nunnari, J., 2011. A mitochondrial-focused genetic interaction map reveals a scaffold-like complex required for inner membrane organization in mitochondria. J. Cell Biol. 195, 323–340. https://doi.org/10.1083/jcb.201107053.

Hughes, A.L., Gottschling, D.E., 2012. An early age increase in vacuolar pH limits mitochondrial function and lifespan in yeast. Nature 492, 261–265. https://doi.org/10.1038/nature11654.

Hwang, A.B., Jeong, D.-E., Lee, S.-J., 2012. Mitochondria and organismal longevity. Curr. Genomics 13, 519–532. https://doi.org/10.2174/138920212803251427.

Jazwinski, S.M., 2014. The retrograde response – a conserved compensatory reaction to damage from within and from without. Prog. Mol. Biol. Transl. Sci. 127, 133–154. https://doi.org/10.1016/B978-0-12-394625-6.00005-2.

Johnson, S.C., Rabinovitch, P.S., Kaeberlein, M., 2013. mTOR is a key modulator of ageing and age-related disease. Nature 493, 338–345. https://doi.org/10.1038/nature11861.

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

Kaeberlein, M., 2010b. Erratum: lessons on longevity from budding yeast (Nature (2010) 464 (513–519)). Nature 464, 1390.

Kanki, T., Wang, K., Baba, M., Bartholomew, C.R., Lynch-Day, M.A., Du, Z., Geng, J., Mao, K., Yang, Z., Yen, W.-L., Klionsky, D.J., 2009. A genomic screen for yeast mutants defective in selective mitochondria autophagy. Mol. Biol. Cell 20, 4730–4738. https://doi.org/10.1091/mbc.E09-03-0225.

Koc, A., Gasch, A.P., Rutherford, J.C., Kim, H.Y., Gladyshev, V.N., 2004. Methionine sulfoxide reductase regulation of yeast lifespan reveals reactive oxygen species-dependent and -independent components of aging. Proc. Natl. Acad. Sci. U. S. A. 101, 7999–8004. https://doi.org/10.1073/pnas.0307929101.

Lapierre, L.R., Hansen, M., 2012. Lessons from C. elegans: signaling pathways for longevity. Trends Endocrinol. Metab. 23, 637–644. https://doi.org/10.1016/j.tem.2012.07.007.

Leadsham, J.E., Sanders, G., Giannaki, S., Bastow, E.L., Hutton, R., Naeimi, W.R., Breitenbach, M., Gourlay, C.W., 2013. Loss of cytochrome c oxidase promotes ras-dependent ros production from the er resident nadph oxidase, yno1p, in yeast. Cell Metab. 18, 279–286. https://doi.org/10.1016/j.cmet.2013.07.005.

Lin, A.-P., Anderson, S.L., Minard, K.I., McAlister-Henn, L., 2011. Effects of excess succinate and retrograde control of metabolite accumulation in yeast tricarboxylic cycle mutants. J. Biol. Chem. 286, 33737–33746. https://doi.org/10.1074/jbc.M111.266890.

Liu, Z., Butow, R.A., 2006. Mitochondrial retrograde signaling. Annu. Rev. Genet. 40, 159–185. https://doi.org/10.1146/annurev.genet.40.110405.090613.

Longo, V.D., Shadel, G.S., Kaeberlein, M., Kennedy, B., 2012. Replicative and chronological aging in Saccharomyces cerevisiae. Cell Metab. 16, 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.

Lundin, M., Baltscheffsky, H., Ronne, H., 1991. Yeast PPA2 gene encodes a mitochondrial inorganic pyrophosphatase that is essential for mitochondrial function. J. Biol. Chem. 266, 12168–12172. https://doi.org/10.1177/019263650008462009.

Masayesva, B.G., Mambo, E., Taylor, R.J., Goloubeva, O.G., Zhou, S., Cohen, Y., Minhas, K., Koch, W., Sciubba, J., Alberg, A.J., Sidransky, D., Califano, J., 2006. Mitochondrial DNA content increase in response to cigarette smoking. Cancer Epidemiol. Biomark. Prev. 15, 19–24. https://doi.org/10.1158/1055-9965.EPI-05-0210.

McCormick, M.A., Delaney, J.R., Tsuchiya, M., Tsuchiyama, S., Shemorry, A., Sim, S., Chou, A.C.-Z., Ahmed, U., Carr, D., Murakami, C.J., Schleit, J., Sutphin, G.L., Wasko, B.M., Bennett, C.F., Wang, A.M., Olsen, B., Beyer, R.P., Bammler, T.K., Prunkard, D., Johnson, S.C., Pennypacker, J.K., An, E., Anies, A., Castanza, A.S., Choi, E., Dang, N., Enerio, S., Fletcher, M., Fox, L., Goswami, S., Higgins, S.A., Holmberg, M.A., Hu, D., Hui, J., Jelic, M., Jeong, K.-S., Johnston, E., Kerr, E.O., Kim, J., Kim, D., Kirkland, K., Klum, S., Kotireddy, S., Liao, E., Lim, M., Lin, M.S., Lo, W.C., Lockshon, D., Miller, H.A., Moller, R.M., Muller, B., Oakes, J., Pak, D.N., Peng, Z.J., Pham, K.M., Pollard, T.G., Pradeep, P., Pruett, D., Rai, D., Robison, B., Rodriguez, A.A., Ros, B., Sage, M., Singh, M.K., Smith, E.D., Snead, K., Solanky, A., Spector, B.L., Steffen, K.K., Tchao, B.N., Ting, M.K., Wende, H. Vander, Wang, D., Welton, K.L., Westman, E.A., Brem, R.B., Liu, X., Suh, Y., Zhou, Z., Kaeberlein, M., Kennedy, B.K., 2015. A comprehensive analysis of replicative lifespan in 4,698 single-gene deletion strains uncovers conserved mechanisms of aging. Cell Metab. 22, 895–906. https://doi.org/10.1016/j.cmet.2015.09.008.

Merz, S., Westermann, B., 2009. Genome-wide deletion mutant analysis reveals genes required for respiratory growth, mitochondrial genome maintenance and mitochondrial protein synthesis in *Saccharomyces cerevisiae*. Genome Biol. 10, R95. https://doi.org/10.1186/gb-2009-10-9-r95.

Orij, R., Postmus, J., Beek, A. Ter, Brul, S., Smits, G.J., 2009. In vivo measurement of cytosolic and mitochondrial pH using a pH-sensitive GFP derivative in Saccharomyces cerevisiae reveals a relation between intracellular pH and growth. Microbiology 155, 268–278. https://doi.org/10.1099/mic.0.022038-0.

Palková, Z., Váchová, L., 2016. Aging 1287–1288.

Ruiz-Roig, C., Noriega, N., Duch, A., Posas, F., de Nadal, E., 2012. The Hog1 SAPK controls the Rtg1/Rtg3 transcriptional complex activity by multiple regulatory mechanisms. Mol. Biol. Cell 23, 4286–4296. https://doi.org/10.1091/mbc.E12-04-0289.

Salmon, A.B., Richardson, A., Pérez, V.I., 2010. Update on the oxidative stress theory of aging: does oxidative stress play a role in aging or healthy aging? Free Radic. Biol. Med. 48, 642–655.

Samokhvalov, V., Ignatov, V., Kondrashova, M., 2004. Inhibition of Krebs cycle and activation of glyoxylate cycle in the course of chronological aging of Saccharomyces cerevisiae. Compensatory role of succinate oxidation. Biochimie 86, 39–46. https://doi.org/10.1016/j.biochi.2003.10.019.

Scheckhuber, C.Q., Erjavec, N., Tinazli, A., Hamann, A., Nyström, T., Osiewacz, H.D., 2007. Reducing mitochondrial fission results in increased life span and fitness of two fungal ageing models. Nat. Cell Biol. 9, 99–105. https://doi.org/10.1038/ncb1524.

Skinner, C., Lin, S.J., 2010. Effects of calorie restriction on life span of microorganisms. Appl. Microbiol. Biotechnol. 88, 817–828.

Stenesen, D., Suh, J.M., Seo, J., Yu, K., Lee, K., Min, K., Graff, J.M., 2014. Caloric Restriction. vol. 17. pp. 101–112. doi: https://doi.org/10.1016/j.cmet.2012.12.006. Dietary.

Tulha, J., Lima, A., Lucas, C., Ferreira, C., 2010. Saccharomyces cerevisiae glycerol/H +symporter Stl1p is essential for cold/near-freeze and freeze stress adaptation. A simple recipe with high biotechnological potential is given. Microb. Cell Factories 9. https://doi.org/10.1186/1475-2859-9-82.

Tzagoloff, A., Dieckmann, C.L., 1990. PET genes of Saccharomyces cerevisiae. Microbiol. Rev. 54, 211–225.

Tzagoloff, A., Yue, J., Jang, J., Paul, M.F., 1994. A new member of a family of ATPases is essential for assembly of mitochondrial respiratory chain and ATP synthetase complexes in Saccharomyces cerevisiae. J. Biol. Chem. 269, 26144–26151.

Veatch, J.R., McMurray, M.A., Nelson, Z.W., Gottschling, D.E., 2009. Mitochondrial dysfunction leads to nuclear genome instability via an Iron-sulfur cluster defect. Cell 137, 1247–1258. https://doi.org/10.1016/j.cell.2009.04.014.

Volonte, D., Liu, Z., Shiva, S., Galbiati, F., 2016. Caveolin-1 controls mitochondrial function through regulation of m-AAA mitochondrial protease. Aging (Albany NY) 8, 2355–2369. https://doi.org/10.18632/aging.101051.

Wallace, D.C., 2010. Mitochondrial DNA mutations in disease and aging. Environ. Mol. Mutagen. 51, 440–450.

Wang, Z., Zhuge, J., Fang, H., Prior, B.A., 2001. Glycerol production by microbial fermentation: a review. Biotechnol. Adv. 19, 201–223.

Woo, D.K., Poyton, R.O., 2009. The absence of a mitochondrial genome in rho(0) yeast cells extends lifespan independently of retrograde regulation. Exp. Gerontol. 44, 390–397. https://doi.org/10.1016/j.exger.2009.03.001.

Wu, G., Fang, Y.-Z., Yang, S., Lupton, J.R., Turner, N.D., 2004. Glutathione metabolism and its implications for health. J. Nutr. 134, 489–492 (doi:14988435).

Ždralević, M., Guaragnella, N., Antonacci, L., Marra, E., Giannattasio, S., 2012. Yeast as a tool to study signaling pathways in mitochondrial stress response and cytoprotection. Sci. World J. 2012, 1–10. https://doi.org/10.1100/2012/912147.