# MOLECULAR AND GENETIC INVESTIGATION OF AGING: THE ROLE OF MITOCHONDRIAL METABOLISM GENES ON LIFE SPAN DETERMINATION

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# ABSTRACT


## MOLECULAR AND GENETIC INVESTIGATION OF AGING: THE ROLE OF MITOCHONDRIAL METABOLISM GENES ON LIFE SPAN DETERMINATION

Molecular mechanism of aging and longevity is still a complex phenomenon. In the course of time, an organism or tissue or a post mitotic cell ages, becomes weak, starts losing energy and ultimately falls in death; that implies that mitochondria has a central role of in the aging process. So in this study it is subjected whether manipulations to mitochondrial metabolism genes can extend life span in yeast. 144 strains derived from the yeast (S.cerevisiae) open reading frame (ORF) deletion collection were screened to identify single deleted mitochondrial genes that increase life span. This has resulted in the isolation of three long-lived mutants' Δppa2 (28% extended), Δdss1(20% extended) and Δafg3 (40% extended) that are chosen for the current study. These long lived cells comprised relatively less amount of mtDNA at the young stage with effective proliferation rate while mtDNA content was highly decreased in old compared to wild type. Relatively less amount of ATP and absence of endogenous reactive oxygen species (ROS) level was observed both in long lived young and old cells. Long lived cell's mitochondria was viewed as aggregated. In addition, the elevation of the mitochondrial membrane potential (ΔΨmito ) was found to predominate the relative degree of longevity. All long lived cells comprised similar pleiotropic mitochondrial phenotype and whole genome microarray published the sets of genes that were commonly upregulated and downregulated. The induction of peroxisomal glyoxylate cycle along with TCA cycle is suggested upon CIT2 higher expression. Thus this investigation reveals the regulatory properties of these genes through the remodeling of mitochondrial morphology and function.

# ÖZET

## YAŞLANMANIN MOLEKÜLER VE GENETİK OLARAK ARAŞTIRILMASI: MİTOKONDRİYAL METABOLİZMA GENLERİNİN YAŞAM SÜRESİNİ TANIMLAMADAKİ ROLÜ

Yaşlanmanın ve uzun ömrün moleküler mekanizması halen karmaşık bir olgudur. Zamanla, bir organizma ya da doku veya mitoz sonrası hücre yaşlanır, zayıflar, enerji kaybetmeye başlar ve sonunda ölür, bu mitokondrinin yaşlanma sürecinde merkezi bir role sahip olduğunu göstermektedir. Sonuç olarak, bu çalışmada mitokondriyal metabolizma genlerinin manipülasyonlarının maya ömrünü uzatıp uzatamayacağı incelenmiştir. Maya (S. Cerevisiae) açık okuma çerçevesi (ORF) delesyon setinden elde edilmiş 144 suş, ömrü arttıran tek silinmiş mitokondriyal genleri tanımlamak için taranmıştır. Bu, mevcut çalışma için seçilen üç uzun ömürlü mutantın Δppa2 (%28 uzamış), Δdss1 (%20 uzamış) ve Δafg3 (%40 uzamış) izolasyonu ile sonuçlanmıştır. Yaşlılarda yabani tipe kıyasla mtDNA içeriği oldukça azalırken, uzun ömürlü hücreler etkili proliferasyon oranı ile genç aşamalarında yabani tipe kıyasla daha az mtDNA içerir, bu da mtDNA içeriğinin uzun ömürde etkili olduğunu göstermektedir. Hem uzun ömürlü genç hem de yaşlı hücrelerde endojen reaktif oksijen türleri (ROS) seviyesinin komple yokluğu ve ATP oranının görece azlığı gözlenmiştir. Uzun ömürlü hücre mitokondrilerinin kümeleştiği gözlenmiştir. Buna ek olarak, mitokondriyal membran potansiyelinin(ΔΨmito) yükselmesi uzun ömürde görece baskın bulunmuştur. Tüm uzun ömürlü hücreler, benzer pleitropik mitokondriyal fenotipe sahiptir ve tüm genom mikroarray genellikle upregüle ve downregüle edilen genlerin setlerini yayınladı. Retrograd tepki geni CIT2'nun uzun ömürlü genç hücrelerde nispeten daha yüksek ifadelenmesi, TCA döngüsü ile birlikte peroksizomal glioksilat döngüsünün indüksiyonu olduğunu düşündürmüştür. Sonuç olarak, bu araştırma mitokondriyal morfoloji ve fonksiyon biçimlenmesiyle bu genlerin düzenleyici özelliklerini ortaya koymaktadır.

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# CHAPTER 1

# INTRODUCTION

## 1.1. Why Do We Grow Old?

The term ‘Aging’ is one of the most complex biological processes of growing older, it may be defined as a multifactorial phenomenon that is characterized by a time dependent reduction of physiological function. In contrast, longevity is a parameter; the length of time that individuals will remain alive in the absence of death and inversely proportional to the pace at which ageing occurs (Katharine et al.2012). Genomic instability, epigenetic distortion, signaling dysregulation, Impaired of mitochondrial function, loss of proteostasis and senescence are the key hall marks of aging (Lopez et al. 2013).

## 1.2. The Central Role of Mitochondria in the Aging Process

The role of mitochondria in aging process is more complicated than proposed by the mitochondrial theory of aging. Multiple changes in mitochondrial function, structure, distribution, and dynamics contribute to aging or age-related features. Studies in different model organisms have reported that change in mitochondrial function can extend lifespan. Such as, Life span can be increased by reduced function of the mitochondria. Mutation or reduced function in nuclear genes encoding electron transport chain (ETC) components in yeast, C. elegans, Drosophila, and mice delay the aging process (Copeland et al., 2009); How the mitochondrial signaling pathway modulates the aging process and the identity of the pathway constituents that transmit these longevity signals remain unknown.

Mitochondria have a central role in the studies of ageing because they supply the majority of the organisms’ energy requirement from biological fuels. mitochondria as the chief target of radical damage, since there is a known chemical mechanism by which mitochondria can produce Reactive oxygen species (ROS) such as the superoxide radical, or via an indirect route the hydroxyl radical. These radicals then damage the

mitochondria's DNA and proteins (According to the free radical theory of aging, organisms age because of the accumulation of free radical damage over time. (Lopez et al.2013).

The 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. Mutations in mtDNA that alter the expression of oxidative phosphorylation (OxPhos) complexes can lead to mitochondrial dysfunction and accelerated ROS generation. (Wallace et al.2010).

## 1.3. Mitochondria to Nuclear Signaling-"Retrograde Response"

The metabolic remodeling that the retrograde response encompasses a shift to the utilization of lipid/acetate as a carbon source. The glyoxylate cycle is an anaplerotic process that provides biosynthetic precursors and thus the retrograde response is a compensatory mechanism. Masa et al. 2011 described that cell can adapt to impaired mitochondrial functions by activaitng an evolutionary conserved communicaiton pathway from mitochondria to nucleus reffered as retrograde response. The prototypical target genes in this pathway are CIT2 (peroxisomal isoform of Citrate synthase that functions in glyoxylate cycle) and RTG2 which encodes regulatory proteins. CIT2 mainly depends on RTG2 when cells lacking mitochondrial DNA. RTG2 independent retrograde response was also shown by different study.

Another pathway, also known as mitochondrial unfolded protein response (HsP60), was demonstrated in various investigations including ETC deletion mediated longevity (Jenni durieux et al. 2011). Riekelt et al.2013 shoewed that mitochondrial ribosomal protein MRP knockdown triggers mitonuclear protein imbalance, reducing mitochondrial respiration and activating the mitochondrial unfolded protein response in C. elegans. UPRmt is induced by mitochondrial stress, subsequently activating a nuclear transcriptional response, inducing the chaperones HSP-6 (HSP-70 in mammals) and HSP-60 to restore mitochondrial proteostasis26, 27. UPRmt-mediated longevity is independent of ROS and induced by increased NAD+ /Sirtuin levels. Still scientists are hunting to uncover the detailed of the anti aging mechanism.

## 1.4. Yeast Model and Replicaitve Aging

The budding yeast Saccharomyces cerevisiae has been used as a model of organismal and cellular aging for more than 50 years. Yeast provides many advantages over other model systems because it includes short life span, well defined genetic and molecular methods, less expensive and easy to handle. High degree of evolutionary conservation of aging pathways has made yeast as an important model organism in aging-related research. Molecular and genetic studies of aging have gained pace over the last century and progress in aging research is now rapid by studying this single cell eucaryote (Matt Kaeberlein. 2010). Two different types of aging have been studied in yeast: replicative and chronological. The replicative life span of a yeast cell refers to the number of daughter cells produced by a mother cell prior to senescence. On the other hand, chronological life span measures the length of time a yeast cell can survive under non-proliferative conditions. To measure the yeast replicative lifespan, cells are initially spread at low density onto growth medium agar and incubated to allow bud emergence. Newly born daughter cells are then micromanipulated to fresh areas of the plate with the help of a micro manipulator. lifespans being determined by counting and removing the buds that they produced, until they size replicate (Kristan et al.2009).

## 1.5. Aim and Objectives of the Present Study

In this current study, 144 strains, derived from the yeast ORF deletion collection, were screened to identify the genes that play role in longevity. Three long lived mutats were isolated through the replicaitve aging assay and thus aimed to charaterize those long lived cells by means of mitochondrial morphology, biochemical functions and genetic analysis.

# CHAPTER 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 (dss1Δ, ppa2Δ, afg3Δ) were obtained from EUROSCARF and used in this study. Cells were grown in 30° C both in solid and liquid YPD broth (1% yeast extract, 2% Peptone, 2% and 2% Dextrose Agar). The concentrations of the yeast cells according to the experimental conditions (optical density) at 600 nm wave length (OD600) was measured using spectrophotometer.

## 2.2. Replicative Aging Assay

Generalized protocol was hired from Matt Kaeberlein et al. 2009. Briefly, in this experiment tweenty cells were randomly choosed from each strain and placed them into a fresh region of YPD solid media. Growth was maintained under 30˚C incubation. A micro manipulator attached with Nicon-Eclipse50 microscope was used to desect daughter cells from the mother cells in every generation. The number of daughter cells produced by the mothers were counted and marked in the score sheet and statistical calculation was performed using Wilcoxon Rank-Sum test. The strains that live longer were confirmed by repeating the assay twice.

## 2.3. Elutriation

Logarithmically growing cells is evaluated as a young population. So young yeast cells that were used during the experiments were obtained by growning them into fresh 2% YPD growth medium for 6 hours at 30 ° C. Aged cells were isolated from the culture with the help of a special rotor by centrifugal elutriation system (Beckman

Coulter Avanti J-26 XP). centrifugation and old cell isolation parametre was obtained according to the procedure recommended by the manufacturer. Cells grown at overnight were transferred to a separation chamber of the elutriation system with a pumping rate 40 ml/min at 2500 rpm. Then the pumping rate was fixed to 30 ml/min and centrifugation speed at 1000 rpm to get cells larger than 15 μm (senescent cells). The resulting primary (old) cells were stored at -80 C.

## 2.4. Glycerol Spotting Assay

Yeast strains with longer replicative lifespans were grown in YPG (3% glycerol) and in YPD. Spotting assay was performed with cells with adjusted OD600 values of 0.2. Serial dilution was performed to obtain OD600 values of 0.02, 0.002, and 0.0002. 5 μl of cell solutions was dropped onto YPGand YPD-agar plates and incubated at 30 °C for 48 h.

## 2.5. Oxygen Consumption Assay

WT and three long lived strains were grown in five ml YPD liquid culture media for an overnight at 30º c and then 100 μl were transferred into 200 ml fresh YPD liquid media to obtain a logarithomic phase of growth. The culture was incubated untill to reach OD600=0.8. The cells were washed with dH2O, suspended in glycerol media (YPG) and incubated at 30 °C for 30 min . To make a control sample, same amount of fresh YPG without cells was incubated at the same time. Hanna Instument H12400 logging DO meter was used to measure the dissolved oxygen value. The polarization and calibration proceure was performed using electrolite fluid followed by ınstrumental protocol. The percent of dissolved oxygen per minute, read by the probe (H176407/2) in each strain of culture containing 2×107 cells, was subtracted from the control (only liquid YPG) value. Triplicate measurement was perfomed to calculate the rate of oxygen consumption.

## 2.6. Confocal Analysis of Mitochondrial Morphology and Distribution

Mitochondria specific dye Mitotracker-Red –CMXRos (Thermo Fisher Scientific) was used to stain mitochondria in live cells. Mitochondria was visualized under a confocal fluorescence microscope (1000×) using 579/599 nm (Exitation/Emision) wave length. Pictures were captured with a CCD camera (Andor technology). Manufacturer provided staining protocol was strictly followed in this study.

## 2.7. Mitochondrial Density Measurement

Then fluorescence intensity (579/599) of that dye was measured through a flow cytometer (FACS-BD) to quantify mitochondrial density within young and old cell population (Optical density, OD 600 =0.8 was adjusted for each sample).

## 2.8. Measurement of Mitochondrial DNA Content

Firstly, total DNA was isolated from 20 million cells of each strain by utilizing traditional phenol chloroform method and DNA concentration was adjusted through nano drop measurement. Relative content of mitochondrial DNA was measured through quantative real time PCR (qPCR- BIO-RAD) by amplifying cox2 that lies within mitochondrial genome. Cox2 forward primer 5'-CAGCAACACCAAATCAGAAGG-3' and reverse primer 5'-GTCCCACACAACTCAGAACATGCTC-3' were used for amplification and house keeping gene actin was used for normalization. PCR was performed for 40 cycles with 100 ng DNA in a 25-µL reaction mixture using Maxima SYBR green /ROX qPCR master mix(2X) kit (Thermo Scientific) and 50 nmol forward and reverse primers. PCR condition was set as 94˚C for 5 min, followed by 40 cycle of 94˚C -30 sec, 60˚C -30 sec, 72˚C- 30 sec and 72˚C for 5 min. The 2-ΔΔCT method was applied to analyse the relative measurement of the mitochondrial DNA content among young and old of WT and long lived cells. Triplicate measurement were done for each of the three biological replicas.

## 2.9. Measurement of Mitochondrial Membrane Potential

MitoProbe™ JC-1 Assay kit (Molecular Probes) was used for mitochondrial membrane potential measurements. Briefly, overnight YPD grown yeast strains were diluted and grown for an extra 3 h. OD600 values were adjusted to 0.7. Cells were pelleted and resuspended in 250 µl of YPD containing JC-1 dye. Control cells were resuspended in YPD without the use of dye. Cells were incubated at 30 °C for 30 min and were washed with PBS twice. After resuspension of cells in PBS, Red/green fluorescence intensity of cells were analyzed with 488 green and 550 red excitation by using standard filter Alexa fluor of Thermo scientific vario scan flash.

## 2.10. Measurement of Cellular ATP Level

Cellular ATP level of each older and younger yeast cell from Wild-type and isogenic deletion mutant (dss1δ, ppa2δ, afg3δ) were obtained. The standard protocol for ATP extraction from total cell lysate was adopted from Jin Ding et al., 2013. Instantly somatic cell ATP bioluminescent assay kit (Sigma Aldrich) was used for luminometric measurement of ATP level through a Luminometer, Fluoroskan Ascent and FL (Thermo Scientific).

## 2.11. Flow Cytometric Measurement of Endogenous ROS

Dichlorodihydrofluorescein diacetate (DCFH-DA) is one of the most widely used techniques for directly measuring intracellular reactive oxygen species. Wild-type yeast cells and yeast cells isogenic deletion of old and young mutant OD600 values were adjusted to 0.8 and transferred to a sterile centrifuge tube. Similar amount of cells were stained with the 5µm DCFH-DA. The cells were incubated at 30 ° C for 30 minutes at 180 rpm and then washed three times with PBS buffer. Finally cells were resuspended in 500µl PBS and analyzed instantly with a flowcytometer (FACS-BD).

## 2.12. TCA Cycle Activity Test

The mitochondrial enzyme Aconitase (In the conversion of Citrate to Iso-citrate step of the TCA cycle) activity is widely used by the biochemists to measure the TCA cycle activity hence the process is conserved among Eucaryotes. In this study, Aconitase activity assay kit (Sigma Adrich) was applied to measure the relative performance of TCA cycle among WT and long lived young and old cells. A spectrophotometer,Multiskan Spectrum (Thermo electron corporation), was used for this colorimetric assay. The protocol and design provided by the kit manufacurer was directly followed here.

## 2.13. In Vivo Measurement of Cytoplasmic pH

pH sensitive GFP tagged plasmid (pHluorin) p413-TEF was applied in this experiment. This plasmid was firstly amplified into bacterial competent cells and then isolated through geneJET plasmid miniprep kit (Thermo Scientific). 2µl of the plasmid (stock concentration was 165ng/µl) was transformed into each yeast strain by following traditional LiAc method with selectable marker His-. The existance of plasmid containign GFP was also confirmed by fluorescence microscope. The fluorescence spectrum of that pHluorin was detected and confirmed as 390 and 470 nm excitations through Perkin Elmer Fluorescecne Spectrometer LS 55. The protocol for pHluorin calibration and cytoplasmic pH measurement was adopted from Rick Orij et al. 2011. 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 ctric acid/Na2HPO4 buffer of known pH ranging from pH 5.0 to pH 9.0. The ratio of pHluorin emission by 512 and excitation ratio R390/470 were measured by the above mentıioned fluorescence spectrometer. The results were plotted against each pH range after background subtraction. These plots were used to calibrate in vivo cytoplasmic pH of young and old strains used in this study.

## 2.14. Real Time PCR (qPCR) Analysis of Mito-nuclear Signal Genes

In this study the expression of three representative signal genes HSP60, RTG2 and CIT2 were analysed through qPCR (Bio Rad-iQ50). The primers for HSP60 were designed as forward TAGTGGTCCAAAGGAAGCTATTC and reverse CAAACGCTCTTGCAGTTTCTC. The Primer of RTG2 was designed for forward GTGTTCGAGAGGGTTCACTTTA and reverse ATAAGGACGGGACGCAATTAG. The forward primer of CIT2 was CGGTATTCGTTTCAGAGGTCG and reverse primer was GCTTCTGGTAGTGGTTGTGAG. B-actin was considered for normalization. Total RNA was isolated and purified from 30 million cells of each strain by using RNA kit (Ambion technology). Nano drop was used for quatification. DNAse 1 treatment was performed before the synthesis of cDNA. cDNA was made with the help of first strand cDNA sntehsis kit (Thermo scientific). 100 ng DNA in a 25-µL reaction was set using Maxima SYBR green /ROX qPCR master mix(2X) kit (Thermo Scientific) with 50 nmol forward and reverse primers. PCR was run as 95˚C for 10 min and 40 cycles of 60˚C for 15 sec, 60˚C for 30 sec and 72˚C for 30 sec. CT values were normalised and relative expression of the signal genes were measured through 2-ΔΔCT method.

## 2.15. Whole Genome Microarray and Transcriptional Analysis

At first, total RNA were isolated from wild-type and deletion mutants with the help of RNA isolation kit (QIAGEN) and stored at -80 C. Purity and quantitation were determined and measured using the NanoDrop spectrophotometer and banding pattern analysis in AGE. The resulting total RNA for whole genome microarray was performed with Agilent-one color-Yeast Expression array. RAW data were processed using the LIMMA package of Bioconductor using R programming. linear model matrix was designed and intensity values were applyed 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 (Munih Information Center for Protein Sequences) classification using FunSpec analysis program.

## 2.16. Cloning and Overexpression

Targetted genes DSS1, PPA2 and AFG3 were picked and amplified from E-coli ORFs collection set (AKOC lab, Izmir Institute of Technology). Overexpression construct of these genes were made with Gateway cloning system that comprised two main reactions, BP and LR. In this study, BP recombination reaction was performed between the ORFs containing plasmids and donor vector pDONR221 with the help of BP Gateway clonase enzyme mix (Invitrogen) in order to generate entry clones. Gene insertion into pDON vector was confirmed by analysing the BsrGI restriciton digestion pattern within the sequence. Then LR recombination was done between the generated entry clone and the destination vector pAG423 (Advanced Gateway 2μ high copy plasmid, HA tagged, GPD promoter-Addgene) with the help of LR Gateway clonase enzyme mix (Invitrogen). Homolog recombination between these BP and LR sites allowed insertion of desired genes into expression vector. After each reaction, the mixtures were transformed into OmniMax2T1 competent cell. For overexpressing that constructed target genes (replaced the expression vector) were retransformed in WT cells by maintaining traditional yeast transformation protocol (LiAc method).

# CHAPTER 3

# RESULTS

## 3.1. Identification of PPA2, DSS1, and AFG3 as Longevity Genes

Single deleted metabolism genes of S.cerevisiae (yeast) were used to investigate the regulatory role of mitochondrial metabolism genes on lifespan. Initially 144 mitochondrial targetted nuclear genes with reference to different pathways (table.1) were targetted and then total number of divisions of a mother cell measured by Replicaitve Life Span (RLS) until she ceases dividing. Thus the mutant PPA2Δ , AFG3Δ and DSS1Δ were identified as long lived cells and confirmed by measuring RLS of that mutants twice in this study.

Table 1. List of analyzed genes with reference to pathways.

| 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 |
| 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 |

Both Diploid (BY4743) and Haploid (BY4741) cells were also analyzed to ensure the result. Life span extended 40% due to AFG3 deletion (Fig.1). The gene AFG3 acts on mitochondrial inner membrane as m-AAA protease (ATPase Associated Activity) that degrades missfolded or unassembled protein (Heike Arlt et al.1998), thus involved in protein folding pathway (Table.2).

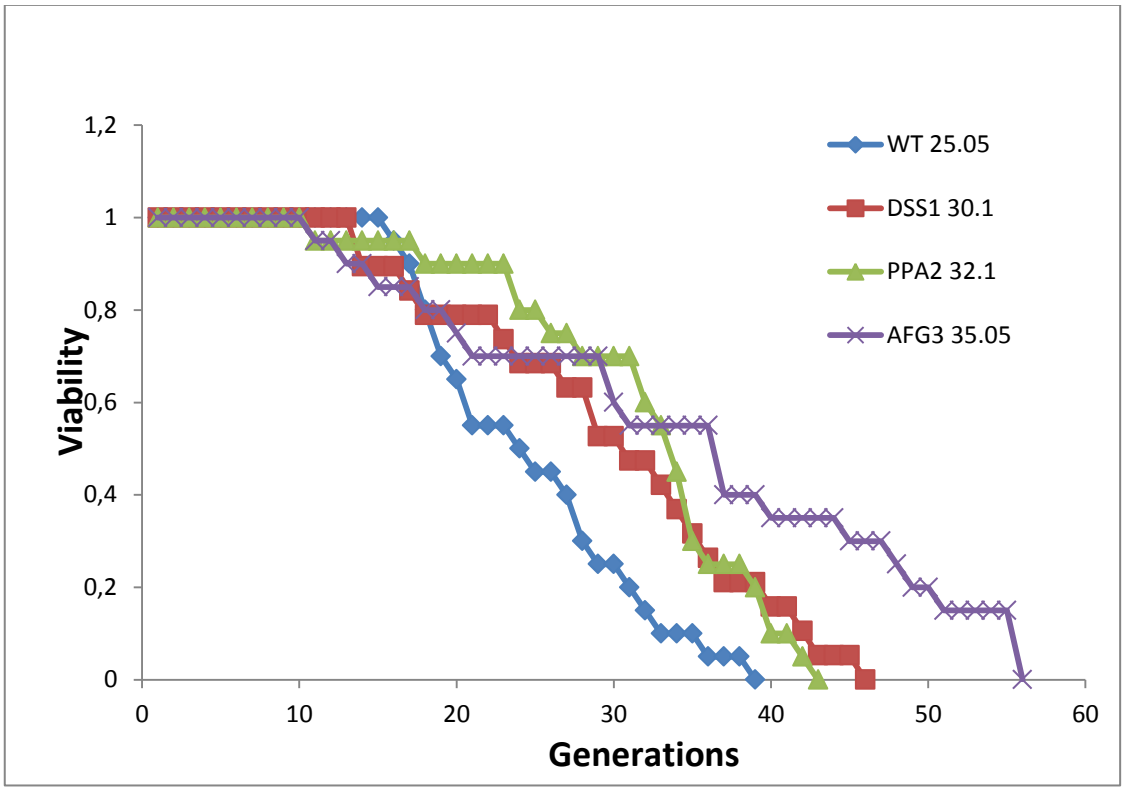


Figure 1. Replicative life span analysis of long lived mutants compared to wild type. Life span extended 20% in dss1Δ, 28% in ppa2Δ and afg3Δ has 40% extended from the wild type.

Mitochondrial inorganic pyrophosphatase PPA2 is required for mitochondrial DNA synthesis and mitochondrial function (Maria Lundin et.al 1990) but due to deletion of this gene, life span was observed as extended 28% (Fig.1). Andrzej et al.2003 identified DSS1 as mitochondrial degradosome that role in mitochondrial RNA metabolism by degrading aberrant RNAs (Table.2). In this study 20% life span extension was observed due to absence of the gene (Fig.1). Homology of these genes are also found in C. elegans, Drosophila and Human (Table.2, Source: Yeast genome data base).

Table 2. A brief introduction of the 3 longevity genes (GO source: yeast genome data base)

| Metabolism Pathway | ORF | Homology | Molecular function | Extended life span (In this study) |
|---|---|---|---|---|
| 1) Protein folding | AFG3 | AFG3 L2 (human) SPG-7(C-elegance) CG6512(Drosophila | The mitochondrial inner membrane m-AAA protease; mediates degradation of misfolded or unassembled proteins and is also required for correct assembly of mitochondrial enzyme complexes | 40% |
| 2) Nucleic acid | PPA2 | PPA2 (Human), Pyp1(C elegans); Nurf38 (Drosophila); | Mitochondrial inorganic pyrophosphatase, required for mitochondrial function, maintainance of mito genome and mitDNA synthesis | 28% |
| 3) Heme-ubiquinone | DSS1 | SHF1(human, Gallus) | mitochondrial degradosome, associates with the ribosome and mediates turnover of aberrant or unprocessed RNAs | 20% |

### 3.2. Long lived Cells' Mitochondrial Morphology, Density and Distribution

In the present study, mitochondrial morphology and distribution were investigated through confocal fluorescence microscope. Mitochondria staining dye Mitotracker Red CmxRos was used to visualize mitochondria in vivo. WT cell exhibited serpentine nature of mitochondrial chain network as expected while mitochondria aggregated and formed colonies within the all long lived cells (Fig.2). Both young and old long lived cells have similar fashion of aggregation. Dimitry et al. (2014) explained that the mitochondrial dynamics include the movement of mitochondria along the cytoskeleton, the regulation of mitochondrial morphology, and networking mediated by fusion (join)/fission (separation) events . Fission is selection of dysfunctional mitochondria and are selectively removed by mitophagy, an autophagy-lysosome system .

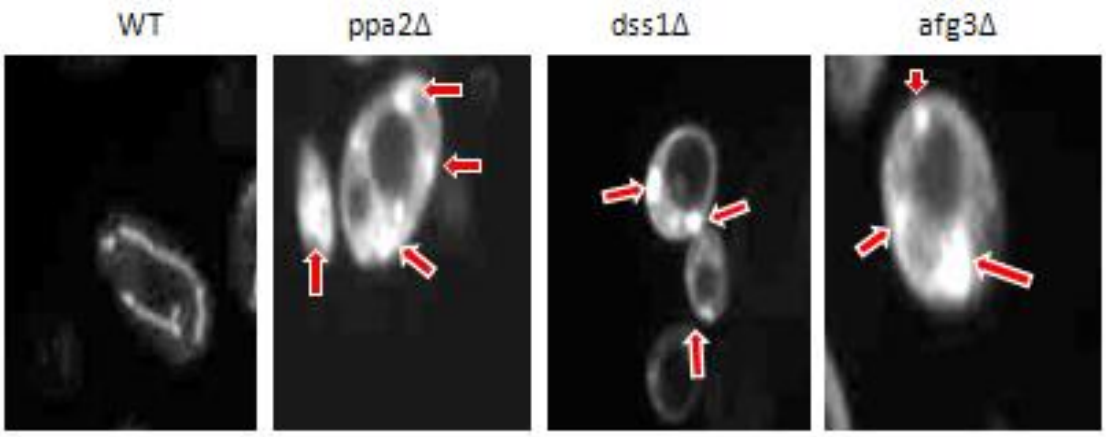


Figure 2. Confocal microscopic analysis of mitochondrial morphology and distribution pattern. WT mitochondria shows chain network. All long lived mutants exhibits mitochondrial aggregation and formed colonies in the cytosol (arrow indicated).

So mitochondrial density was measured by using the same dye that was used for visualization. After stained the mitochondria, Intensity was measured through a flow cytometer (FACS BD). Long lived cells in this study showed almost equal amount of mitochondrial mass compared to wild type (Fig.3) which suggested that mitochondria were not destroyed after breaking down of net work and in aggregated state.

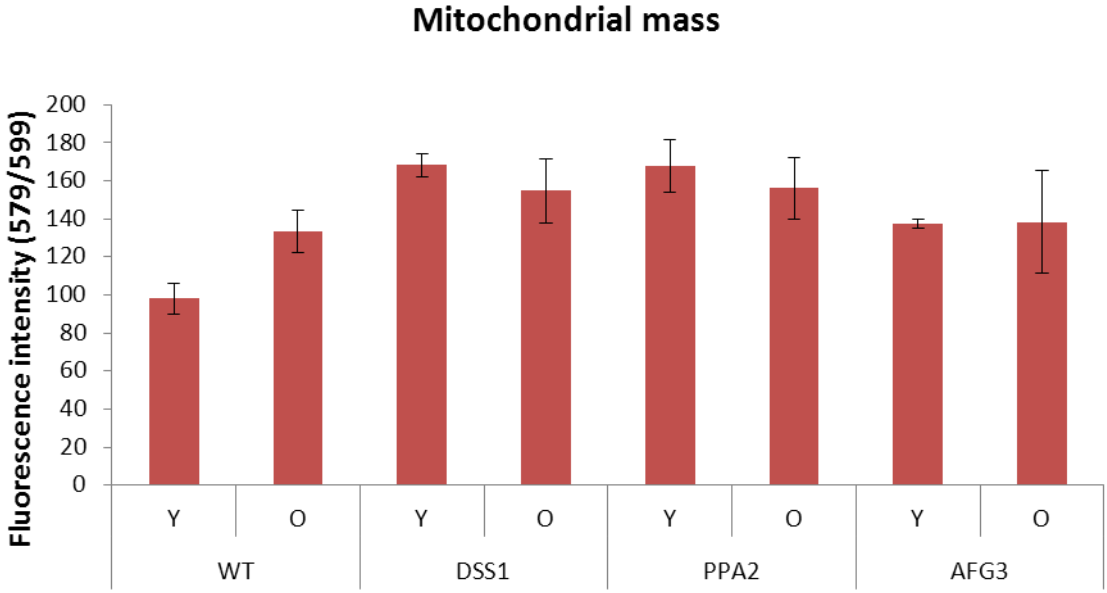


Fig 3. Fluorocytometric measurement of mitochondrial density by using mitotracker Red CMXRox dye.

## 3.3. Relative Content of Mitochondrial DNA

mtDNA encodes respiratory chain proteins as well as tRNAs and ribosomal RNAs that are required for synthesis of mtDNA-encoded proteins. 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 et al, 2000).

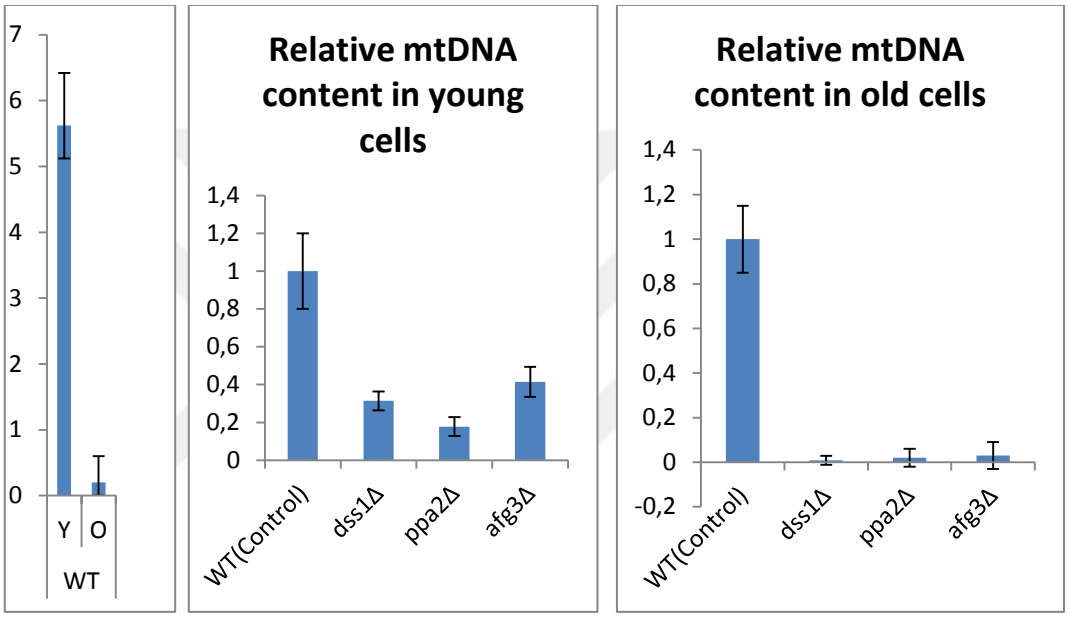


Figure 4. Quantitative analysis of mitochondrial DNA content by amplifying mitochondrial cox2 in a qReal time PCR

In this study, relative mitochondrial DNA content was quantified through quantitative real time PCR analysis by amplifying Cox2 gene that lies within mitochondrial genome. Presence of mtDNA in young long lived cells and absence of mtDNA in old cells were observed in this study. When relative amount of mtDNA was measured, relatively less amount of mtDNA was found in long lived young cells compared to WT young cell. While mtDNA was highly decrased in long lived old cells compared to wild type old cell (Fig.4).

## 3.4. The Respiration Status of Long Lived Cells

Cells with defective mitochondria cannot utilize respiratory substrates such as ethanol,glycerol or lactic acid. We tested whether long lived mutants are defective in respiration by growing them on a glycerol containing media. In this study, all long lived cells were not able to utilize glycerol as the carbon source to derive energy. Thus these long lived cells had respiration defficiency.

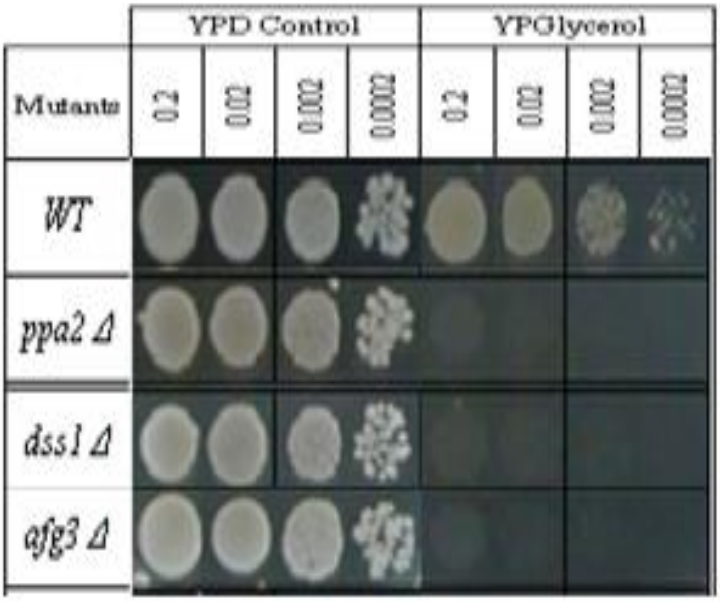


Figure 5. Glycerol spotting assay: respiratory phenotypic characterization of long lived cells.

On the other had, long lived mutants have similar growth patern and rendered same colony shape compared to wild type when they are grown in YPD (Fig.5). The proliferation rate was viewd throgh analysing the growth curve in YPD demonstrated that long lived strains do not have any growth defect and rate of proliferation was parallel to WT untill the glucose was present. As expected from the standard pattern, WT cells also started a second phase of growth so called diauxic shieft (able to utilize ethanol), (Fig.6).

These long lived respiratory defficient Cells were expected to have decreased rate of oxygen intake. As expected, the long lived mutants consumed relatively less amount of oxygen and had lower rates of oxygen uptake compared to that of wild type cells (Fig.7).

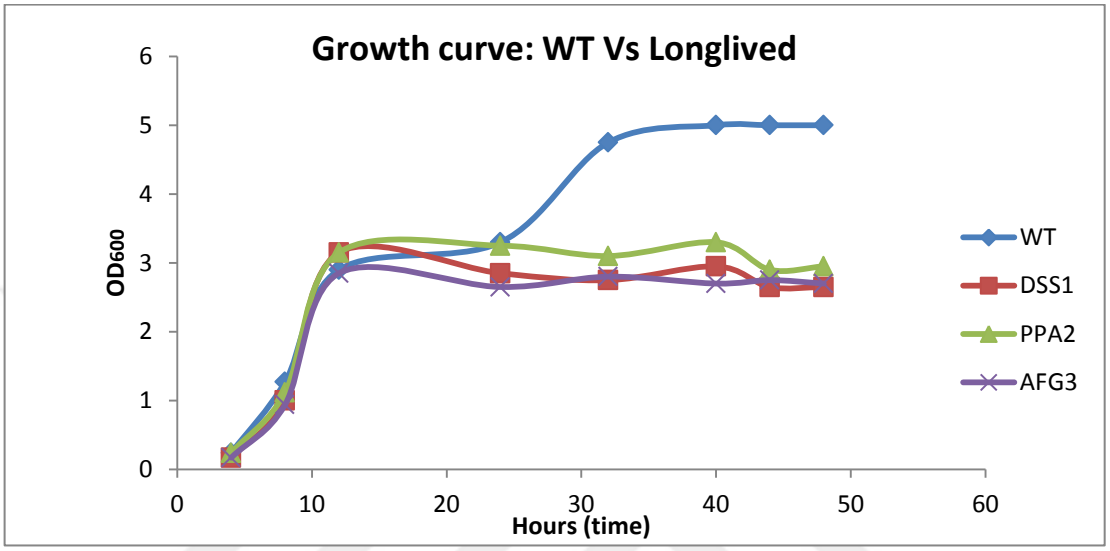


Figure 6. Comparing Growth curve of long lived cells and WT in YPD media

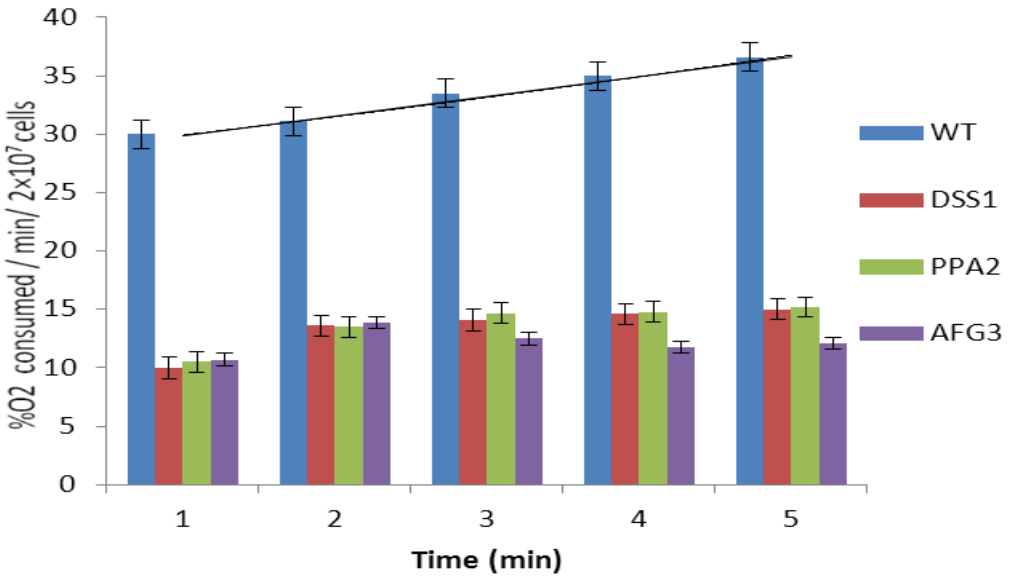


Figure 7. Rate of respiration: oxygen consumption assay showing the decrease of consumed oxygen by long lived cells.

## 3.5. Endogenous ROS

Mitochondrial ROS produced continuously throughout life at a species-specific rate which is independent from mitochondrial oxygen consumption but related to the rate of aging and longevity of each species (Reinald et al.2011). In this present investigation, flow cytometric analysis was performed to measure the level of endogenous ROS produced by the live cells with the help of the commonly used ROS specific stain DCF-DA (Fig.8). High endogenous ROS were observed in WT senescent cells compared to young WT cells as expected from the previous study. Interestingly, complete absence or extremely low level of endogenous ROS was observed in both young and old of all these long lived mutants (Fig.8).

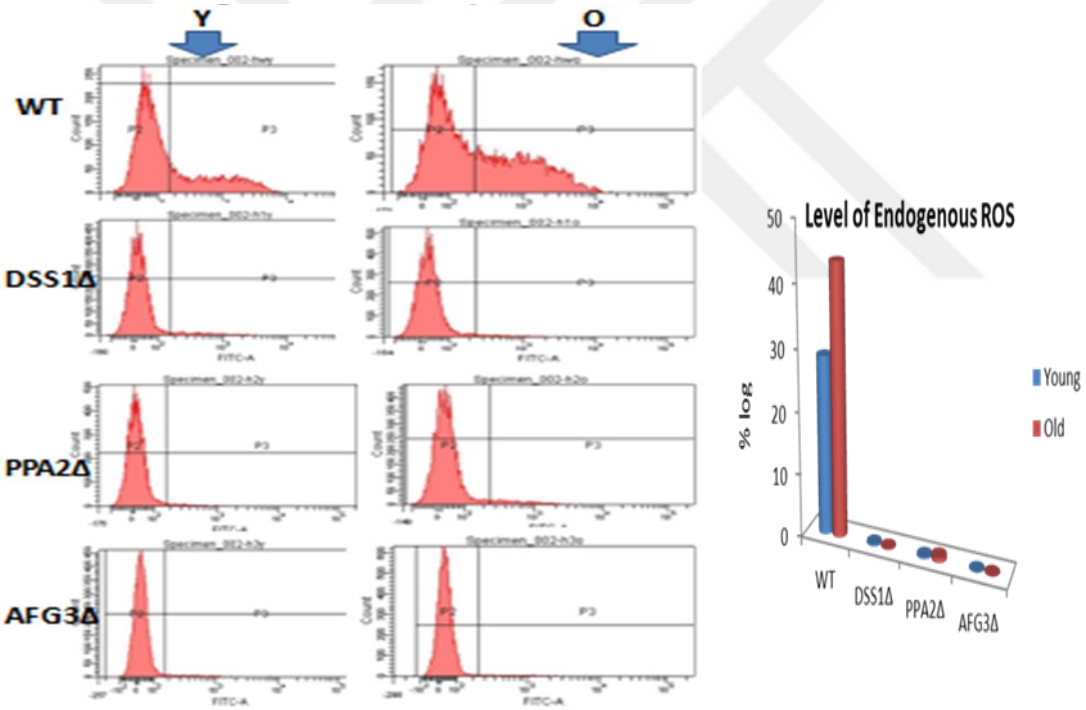


Figure 8. Flowcytometric measurement of endogenous ROS. Long lived cells showing absence of endogenous ROS while WT old cells accumulated higher level of endogenous ROS compare to young.

## 3.6. Mitochondrial Membrane Potential

The mitochondrial membrane potential affects mitochondrial integrity during the life span, and it also is important in driving the transport of biochemical precursors out of the mitochondria and into the cytoplasm where they are available for the synthesis of new daughter cells and maintain mitochondrial membrane potential, which drives the transport of biosynthetic precursors derived from the Krebs cycle (Michal Jazwinski 2004). When yeasts are fermenting glucose, the bulk of the ATP is generated at the substrate level in glycolysis. The ATP is used among others in the maintenance of mitochondrial membrane potential. In the present investigation, it is found that both young and old long lived cells maintained their mitochondrial membrane potential. In addition, PPA2Δ young cell showed about 1.5 fold higher and afg3Δ young showed about 2.5 fold higher membrane potential compared to all. No significant difference was observed between WT young and old cells (Fig.9).

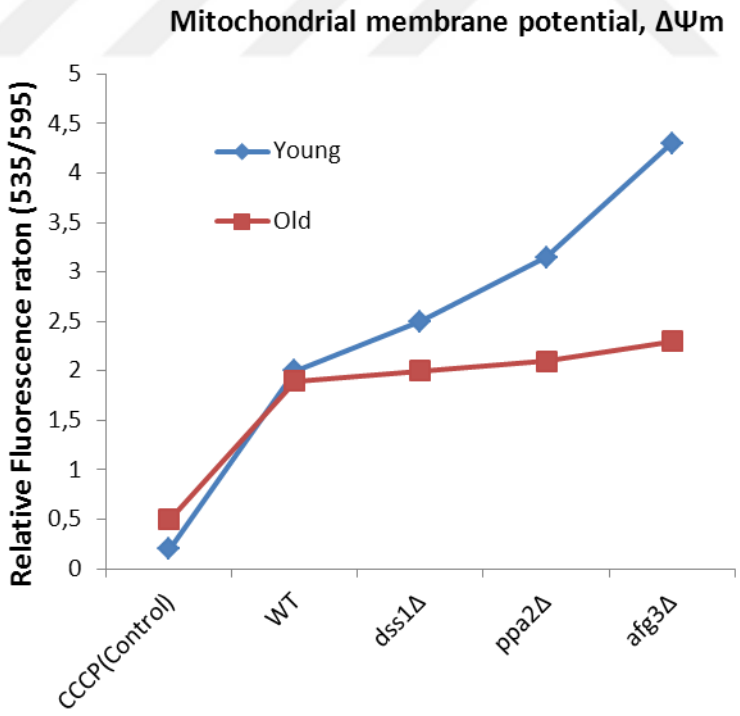


Figure 9. Mitochondrial membrane potential: Young long lived cells showed higher membrane potential.

## 3.7. Cellular ATP Level

In this study, luminometric detection was performed to measure the relative level of cellular ATP. As mid log phase was considered as young stage, the ATP level of young cells were measured directly from the mid log phase of growing culture. The replicatively old cells that were produced at the early stationary phase of growth after tweenty four hour, were isolated and subjected instantly for ATP extraction. It is observed that ATP level become doubled in Wild type young cells compared to old (Fig.10. A). Long lived young cells produced less amount of ATP compared to WT young cell whereas no significant difference was found between all old cells ($P \geq .05$). ATP level during growth was aslo measured in a time dependent manner. Similar type of fluctuation of ATP level was observed until glucose is present (Fig.10. B).

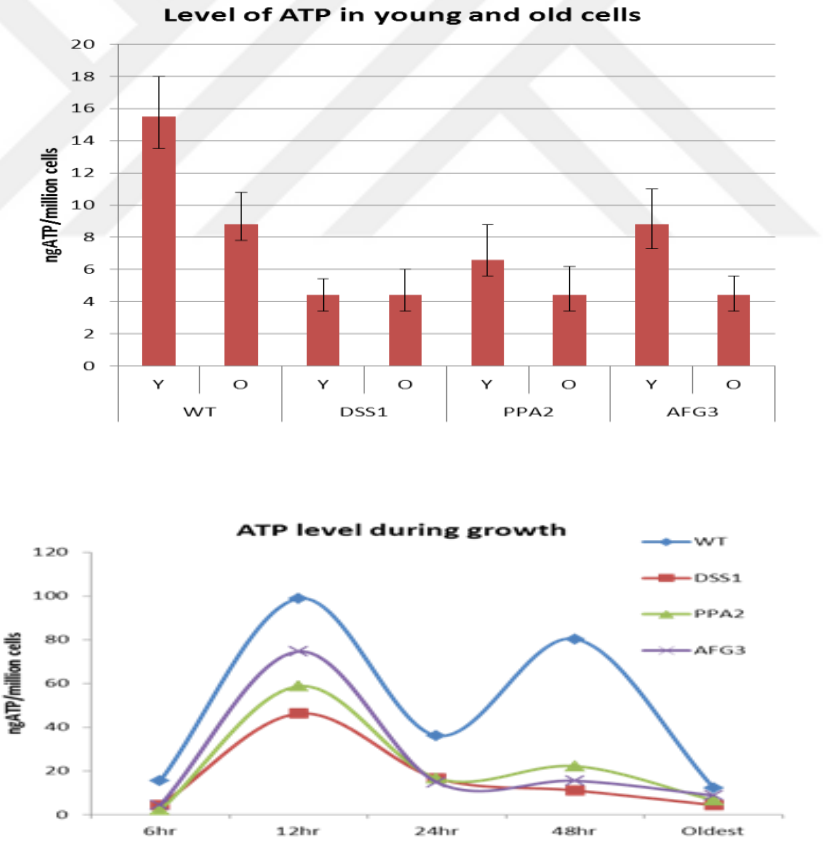


Figure 10. Relative level of cellular ATP. Long lived young and old cells have relatively less amount of ATP compare to thats of WT (upper figure). ATP level measured during growth exhibits similar result until glucose is present (lower figure).

## 3.8. TCA Cycle Activity

The biochemistry of most metabolic pathways is conserved. In both yeast and human, TCA cycle metabolites are used as macromolecular precursors. In this study, TCA cycle activity was tested by viewing the activity of mitochondrial Aconitase that roles in Citrate to İsocitrate synthesis in the TCA cycle. As expected, WT young cells had two fold higher TCA cycle activity than old. TCA cycle activity was slightly increased in young cells due to PPA2 and AFG3 deletion. While the activity was greatly reduced in long lived old cells (Fig.11)

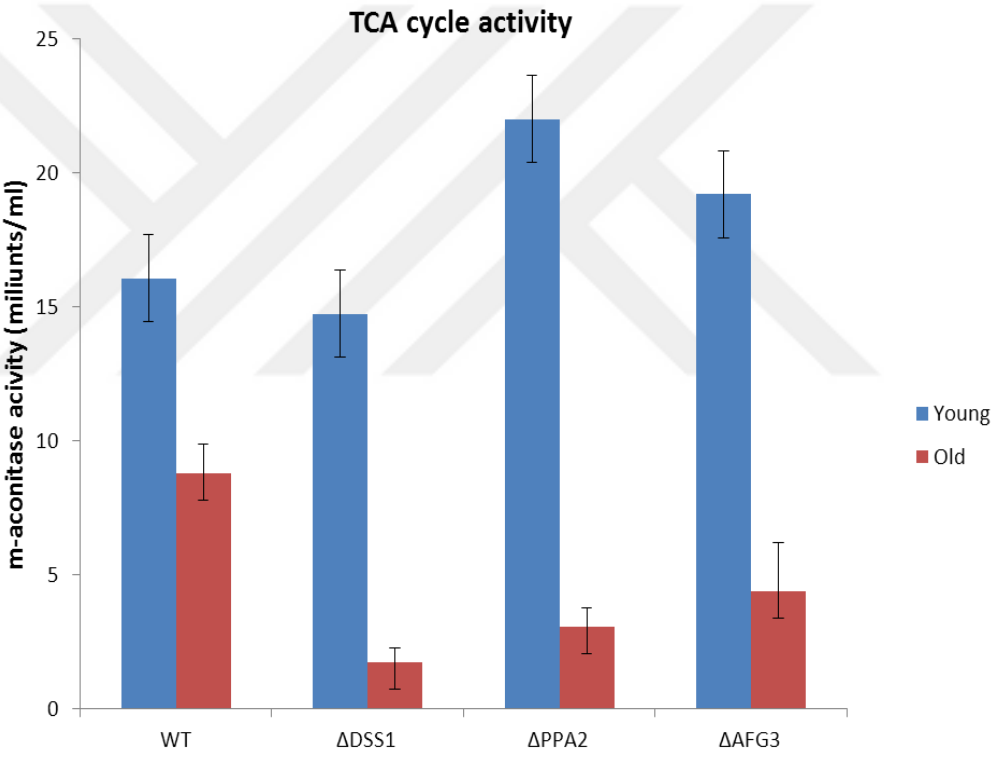


Figure 11. Aconitase activity showing the relative activity of TCA cycle in long lived cells compare to WT.

## 3.9. Cytoplasmic pH Determination

Recently the use of pH sensitive fluorescent proteins had made it possible to determine intracellular pH (Rick et.al 2011). Ratiometric pHluorin p413 TEF was used in this study to measure cytoplasmic pH of long lived cells. After transforming that plasmid, fluorescence spectrum was confirmed and emission ratio R390/470nm were plotted against corresponded known buffer of pH ranging from 5 to 9. Thus a calibration curve was obtained (Fig.12) that showed a gradual change of intensity with the change of pH. WT young cell exhibited slightly acidic cytoplasmic pH while replicaitvely old cell had slightly basic environment untill glucose is present in the medium. Interestingly, both young and old of all long lived cells used in this study rendered slightly acidic environment of the cytoplasm, pH ranged 6 to 6.7 (Fig.12).

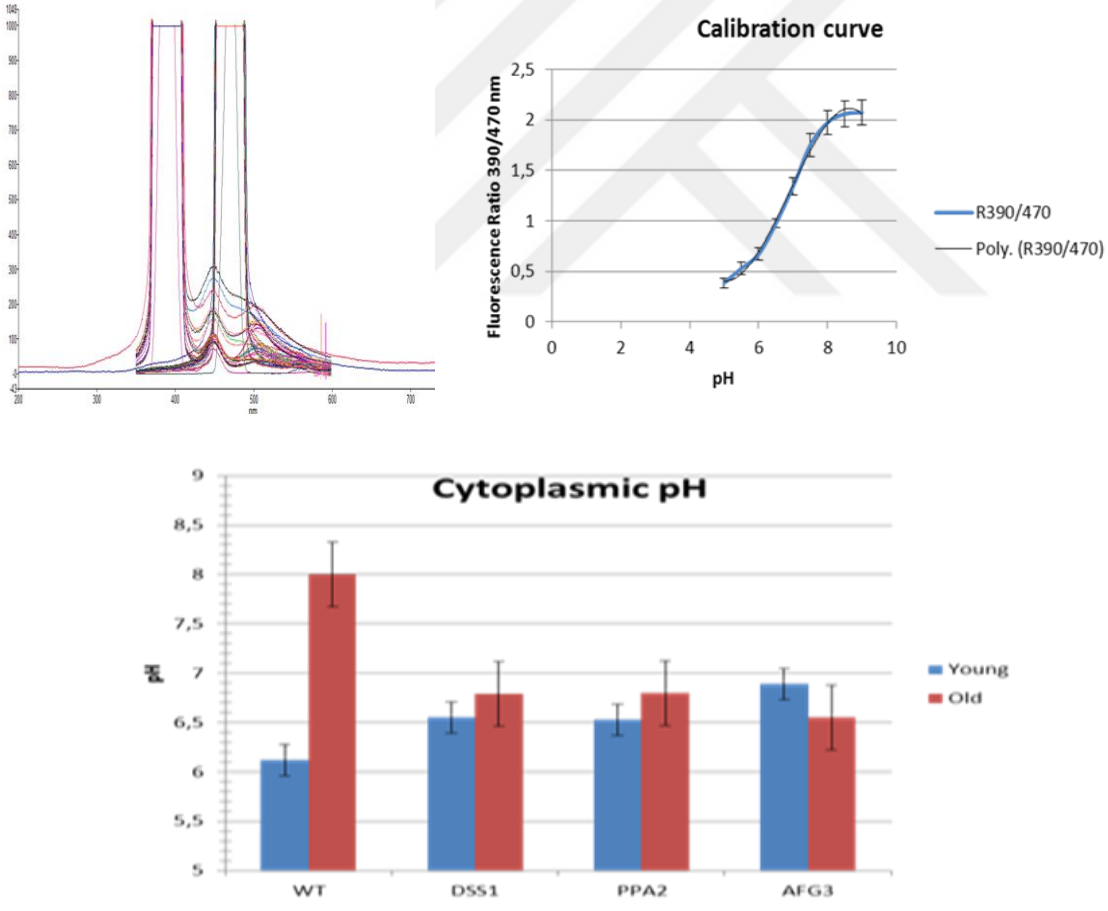


Figure 12. In vivo measurement of cytoplasmic pH. Fluorescence spectrum of GFP tagged plasmid p413 TEF showing two excitation at 390nm and at 470nm (upper left). Calibration curve was plotted against pH buffer (upper right). Long lived cells showed slightly acidic cytoplasm (lower figure).

## 3.10. Gene Expression Profiling of Long Lived Cells

whole genome microarray analysis of wild-type and long lived yeast cells was done with Agilent-One color-Yeast Expression to determine the expression levels profiling of potential genes that may commonly found and play a role in the extension of the replicative lifespan. Bioinformatic part was accomplished through LIMMA package of Bioconductor and the values were logarithmically transfomed where two fold up or down regulated genes were taken under consideration (Fig.13, Table 3). Fun Spec, a web-based cluster Interpreter for Yeast, analysis was done with MIPS (Munih Information Center for Protein Sequences) to identify the molecular and biological function of up and down regulated genes (Table 4).

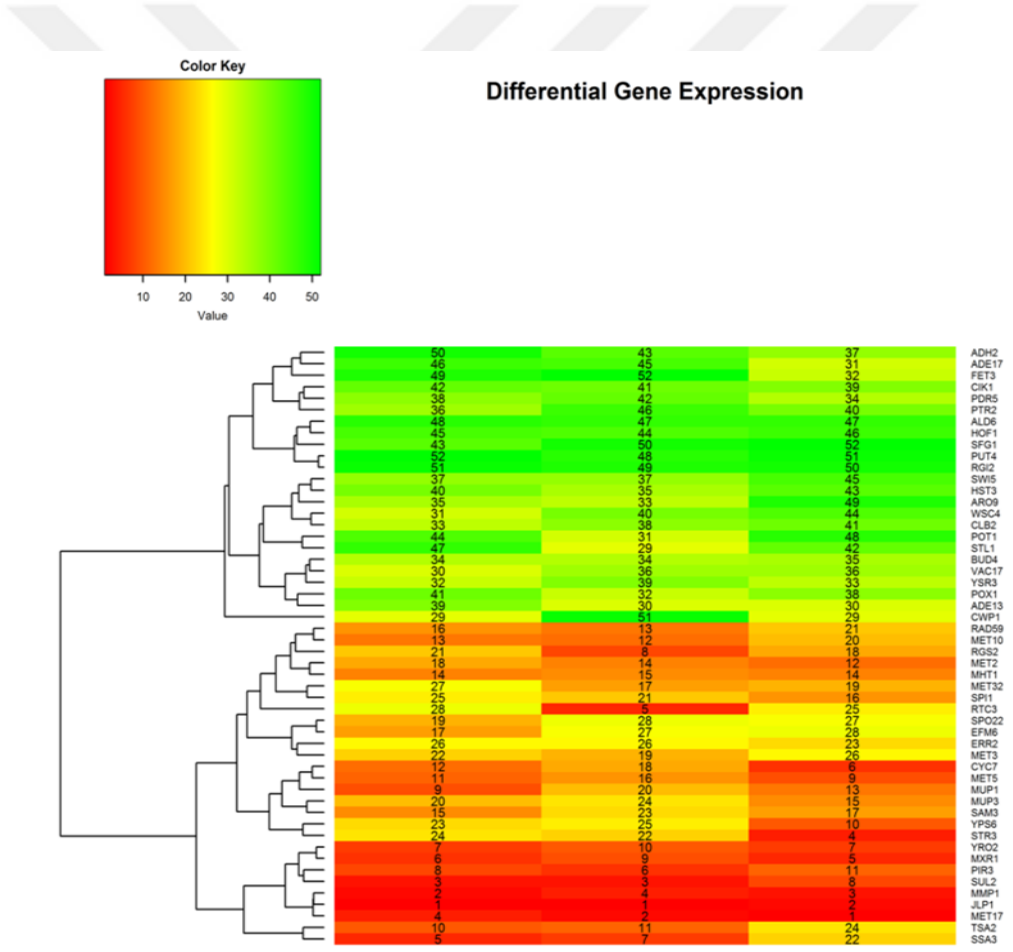


Figure 13. Heat map poduced from bioinfomaticaly processed data: Clustering of differential expressed genes that commonly upregulated and downregulated in long lived cells.

Table 3. Representative of the fold changes of the differentially expressed genes that were commonly found in all long lived backgrounds.

**A) Commonly Upregulated genes in long lived strains**

| Gene Name | Systematic Name | Dss1 log fold change | PPa2log fold change | Afg3 log fold change |
|---|---|---|---|---|
| STL1 | YDR536W | 5,48217553 | 4,77413265 | 5,29083803 |
| CWP1 | YKL096W | 3,45706532 | 2,02790519 | 3,34091234 |
| PUT4 | YOR348C | 3,121736 | 1,82095942 | 3,09890083 |
| RGI2 | YIL057C | 2,92300963 | 1,98491527 | 2,69790965 |
| Uncharactereized | YHR033W | 2,77516604 | 1,72780371 | 3,44042261 |
| ADH2 | YMR303C | 2,57487014 | 1,53108641 | 1,62263222 |
| Unknown | YML131W | 2,5189405 | 1,1617521 | 2,73950741 |
| FET3 | YMR058W | 2,47480705 | 2,93774844 | 1,14495018 |
| ALD6 | YPL061W | 2,46362316 | 1,70620218 | 2,59062659 |
| ADE17 | YMR120C | 2,28442789 | 1,64521944 | 1,1178377 |
| HOF1 | YMR032W | 1,98873712 | 1,55388089 | 2,24631026 |
| POT1 | YIL160C | 1,89652009 | 1,021525 | 2,63325636 |
| SFG1 | YOR315W | 1,85713036 | 2,01029026 | 3,30196293 |
| CIK1 | YMR198W | 1,81172874 | 1,36601506 | 1,85272229 |
| Uncharacterized | YHR093W.1 | 1,735031 | 1,01605615 | 2,17027 |
| POX1 | YGL205W | 1,54062198 | 1,08171237 | 1,72613725 |
| HST3 | YOR025W | 1,49504194 | 1,19146302 | 2,04299457 |
| ADE13 | YLR359W | 1,39097808 | 1,01542273 | 1,09239388 |
| PDR5 | YOR153W | 1,37258136 | 1,47791763 | 1,24579271 |
| SWI5 | YDR146C | 1,29190257 | 1,24228838 | 2,22122478 |
| PTR2 | YKR093W | 1,28335071 | 1,70577351 | 1,94585952 |
| ARO9 | YHR137W | 1,25717386 | 1,08280249 | 2,6619841 |
| BUD4 | YJR092W | 1,2179407 | 1,14302122 | 1,4755305 |
| CLB2 | YPR119W | 1,18186951 | 1,28151532 | 1,96849628 |
| YSR3 | YKR053C | 1,14412696 | 1,2857388 | 1,16843078 |
| WSC4 | YHL028W | 1,10382358 | 1,32420073 | 2,08868515 |
| VAC17 | YCL063W | 1,02122926 | 1,22307567 | 1,56445795 |

**(Cont. on next page)**

**Table 3. (Cont.)**

**B) Commonly downregulated genes in long lived strains:**

| | | | | |
|---|---|---|---|---|
| Gene Name | Systematic Name | dss1Δ log Fold Change | ppa2Δ log Fold Change | afg3 log Fold Change |
| JLP1 | YLL057C | -3,2168351 | -2,3815608 | -4,0290569 |
| MMP1 | YLL061W | -1,9495569 | -1,9571331 | -2,5856647 |
| Uncharacterized | YLR338W.1 | -1,9063748 | -1,6130281 | -2,2448375 |
| Uncharacterized | YOR338W | -1,9026605 | -1,4413453 | -2,6073412 |
| SUL2 | YLR092W | -1,9019166 | -1,9861274 | -2,1548053 |
| MET17 | YLR303W | -1,8472641 | -2,274599 | -9,3477614 |
| SSA3 | YBL075C | -1,8041816 | -1,7916466 | -1,3478907 |
| Uncharacterized | YCR007C | -1,7632073 | -1,0335515 | -3,1076095 |
| MXR1 | YER042W | -1,7235776 | -1,5512149 | -2,3657909 |
| YRO2 | YBR054W | -1,6855366 | -1,4836423 | -2,2633773 |
| PIR3 | YKL163W | -1,6514079 | -1,7949065 | -1,8743317 |
| MUP1 | YGR055W | -1,6151622 | -1,2765358 | -1,771177 |
| Uncharacterized | YOL163W | -1,5802937 | -1,0403506 | -2,1854037 |
| TSA2 | YDR453C | -1,5584513 | -1,4793565 | -1,2551263 |
| Uncharacterized | YOL014W | -1,5518722 | -1,1842617 | -1,1254494 |
| MET5 | YJR137C | -1,5000621 | -1,3249802 | -2,1422761 |
| Uncharacterized | YOR385W | -1,4928811 | -1,0088779 | -1,8065447 |
| CYC7 | YEL039C | -1,4129946 | -1,3120246 | -2,2989619 |
| MET10 | YFR030W | -1,4104522 | -1,4246983 | -1,4802004 |
| MHT1 | YLL062C | -1,3775848 | -1,3433981 | -1,7163554 |
| SAM3 | YPL274W | -1,3584896 | -1,1720021 | -1,66416 |
| RAD59 | YDL059C | -1,3382865 | -1,3779014 | -1,3539203 |
| EFM6 | YNL024C | -1,3111681 | -1,0363846 | -1,0770295 |
| Uncharacterized | YGL258W-A | -1,2940278 | -1,3960118 | -1,4247257 |
| MET2 | YNL277W | -1,282681 | -1,3494438 | -1,8194111 |
| SPO22 | YIL073C | -1,2638927 | -1,0062545 | -1,0783908 |
| Uncharacterized | YHL044W | -1,262233 | -1,3687908 | -1,2800493 |
| MUP3 | YHL036W | -1,261456 | -1,1351655 | -1,703475 |
| RGS2 | YOR107W | -1,2333895 | -1,5617379 | -1,5142262 |
| MET3 | YJR010W | -1,2119545 | -1,3025715 | -1,2132963 |
| YPS6 | YIR039C | -1,1271991 | -1,1205254 | -1,9209553 |
| STR3 | YGL184C | -1,1210927 | -1,1884586 | -2,4317897 |
| SPI1 | YER150W | -1,089921 | -1,2105988 | -1,6658809 |
| ERR2 | YPL281C | -1,0405045 | -1,0656792 | -1,2989793 |
| MET32 | YDR253C | -1,0276655 | -1,3151694 | -1,4960959 |
| RTC3 | YHR087W | -1,0092572 | -1,8657514 | -1,214034 |

Table 4. Fun Spec analysis for the MIPS functionai classification of A) Commonly Upregulated genes and B) Commonly down regulated genes obtained from micro array analysis. (The letter K indicates the number of genes shown to function in corresponding specific functional category while the letter f represents the total number of genes in that specific functional category).

**A) Commonly upregulated**

| **Category** | **p-value** | **In Category from Cluster** | **k** | **f** |
|---|---|---|---|---|
| C-2 compound and organic acid catabolism [01.05.06.07] | 0.0004117 | ADH2 ALD6 | 2 | 9 |
| oxidation of fatty acids [02.25] | 0.0004117 | POX1 POT1 | 2 | 9 |
| fatty acid metabolism [01.06.05] | 0.003058 | POX1 POT1 | 2 | 24 |
| purine nucleotide/nucleoside/nucleobase anabolism [01.03.01.03] | 0.004451 | ADE13 ADE17 | 2 | 29 |
| metabolism of alkaloids [01.20.17.09] | 0.006955 | ARO9 | 1 | 2 |
| hyphae formation [43.01.03.06] | 0.006955 | SFG1 | 1 | 2 |
| transcription activation [11.02.03.04.01] | 0.009183 | SWI5 SFG1 | 2 | 42 |

**(Cont. on next page)**

**Table 4. (Cont.)**

**B) Commonly downregulated**

| Category | p-value | In Category from Cluster | k | f |
|---|---|---|---|---|
| metabolism of methionine [01.01.06.05] | 1.266e-06 | MET32 MET3 MHT1 MET17 | 4 | 21 |
| biosynthesis of homocysteine [01.01.06.05.01.01] | 2.111e-06 | MET10 STR3 MET5 | 3 | 7 |
| sulfate assimilation [01.02.03.01] | 3.369e-06 | MET10 MET3 MET5 | 3 | 8 |
| amino acid metabolism [01.01] | 1.654e-05 | MXR1 MUP1 MUP3 SUL2 | 4 | 39 |
| amino acid/amino acid derivatives transport [20.01.07] | 2.947e-05 | MUP1 MUP3 MMP1 SAM3 | 4 | 45 |
| metabolism of cysteine [01.01.09.03] | 0.0005696 | MET32 MHT1 | 2 | 9 |
| oxygen and radical detoxification [32.07.07] | 0.001036 | TSA2 MXR1 | 2 | 12 |
| nitrogen, sulfur and selenium metabolism [01.02] | 0.001316 | STR3 JLP1 MET17 | 3 | 54 |
| stress response [32.01] | 0.003944 | SSA3 YRO2 CYC7 PIR3 | 4 | 162 |
| conjunction of sulfate [01.02.03.04] | 0.004089 | MET3 | 1 | 1 |
| NAD/NADP binding [16.21.07] | 0.009312 | MET10 MET5 | 2 | 36 |

## 3.11. Mitochondrial Retrograde Signaling

In this study, three representative genes CIT2, RTG2 and Hsp60 were chosen for a quantitative and precise investigation through realtime PCR. The primer were deigned and cDNA synthesis were performed before analysis. The relative expression of RTG2 and HsP60 were not sigificantly different from WT expression. But CIT2 expressed 2 fold higher in AFG3 mutant, 3 fold higher in DSS1 mutant and 5 fold higher in PPA2 mutant.

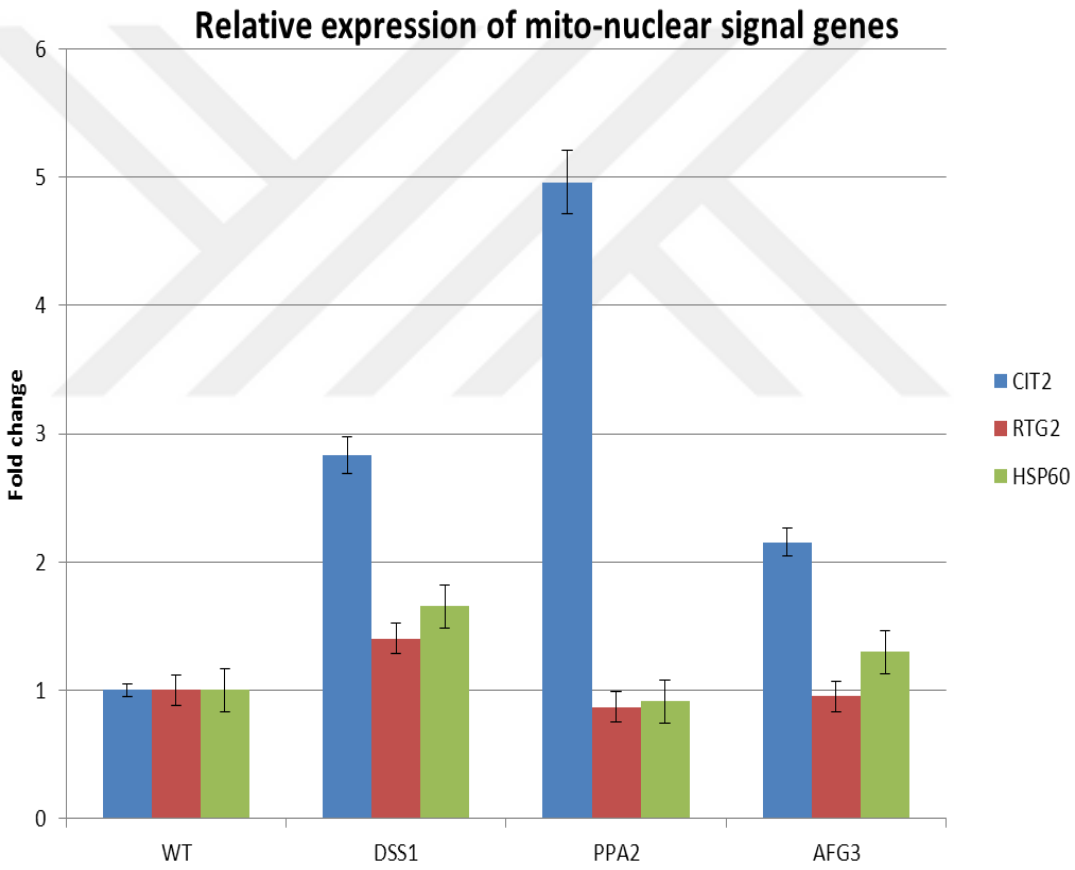


Figure 14. Quantitative Real time PCR analysis of mitochondria to nuclear signal genes. Retrograde response pathway gene CIT2 has relaively higher expression among all long lived mutants.

## 3.12. Cloning and Overexpressing the Genes DSS1, PPA2 and AFG3

To overexpress the target genes, Gateway cloning system was followed in the present study. Gate way cloning is a sofisticated and fast technology widely used for the efficient transformation of gene or DNA fragment into vector. The target genes DSS1, PPA2 and AFG3 were successfully cloned with the help of BP clonase (invitrogen) into pDONR211 vector in order to make an entry clone and confirmed through analysis of the sequences that are restricted to BsrGI (Fig.15.A). Then the generated entry clone was replaced into a Yeast expression vector, pAG423 GPD, through LR recombinase and confirmed by analysis of those gene sequences showed EcoRV specific restriciton (Fig.15.B). Expression vector cotaining selectable marker His- along with the target genes were also selected by growing them in the media lacking His. Plasmid were isolated and then transformed into WT cells that allowed them to overexpress.

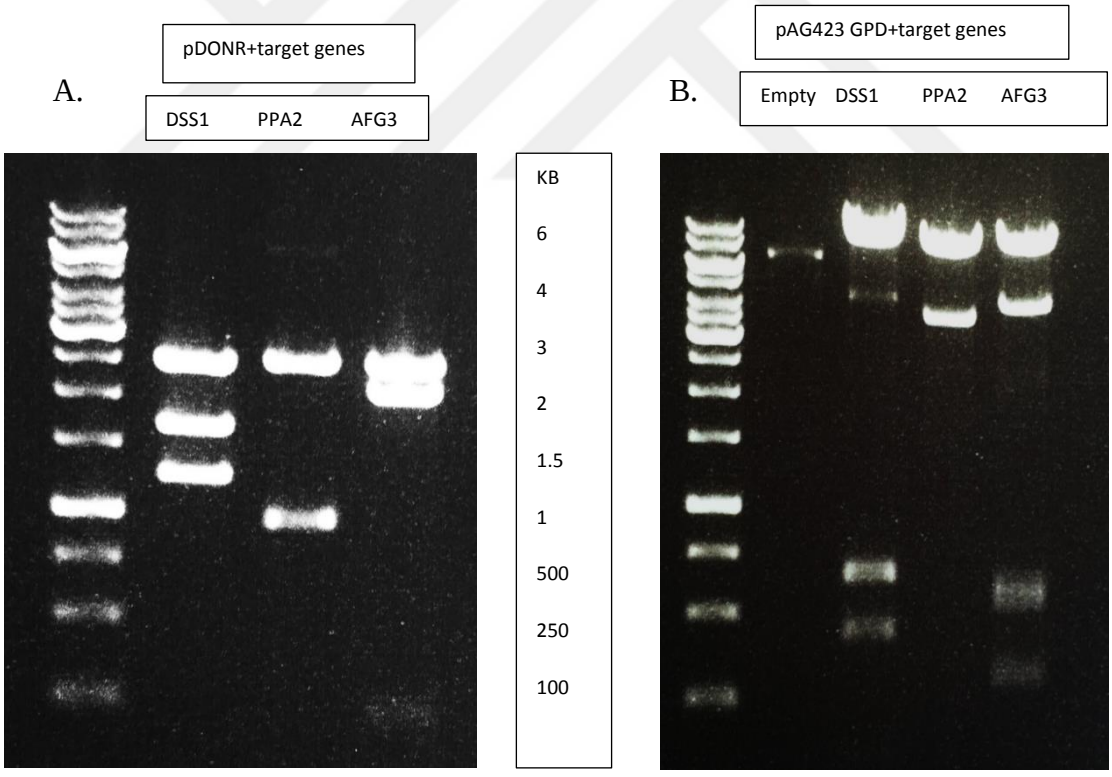


Figure 15. Cloning confirmation. A. Target genes were sucessfully cloned into pDONR221 vector confirmed by BsrGI digestion mathched with the restriciton map provided by yeast geneome database, B. Confirmation of gene replacement from donor vector to pAG423 GPD expression vector by Eco-RV digestion.

## 3.13. Effect of Overexpression on Life Span

WT cells transformed with the empty vector, pAG423 GPD, that contains only vector backbone without any inserted gene was taken as control. WT cells containing overexpressed genes DSS1, PPA2 and AFG3 along with pAG423 GPD expression vector were directly subjected to replicative life span analysis. In this investigation, it is observed that the overexpression of these target genes did not affect replicative life span in yeast (neither extended nor shortend) (fig. 16).

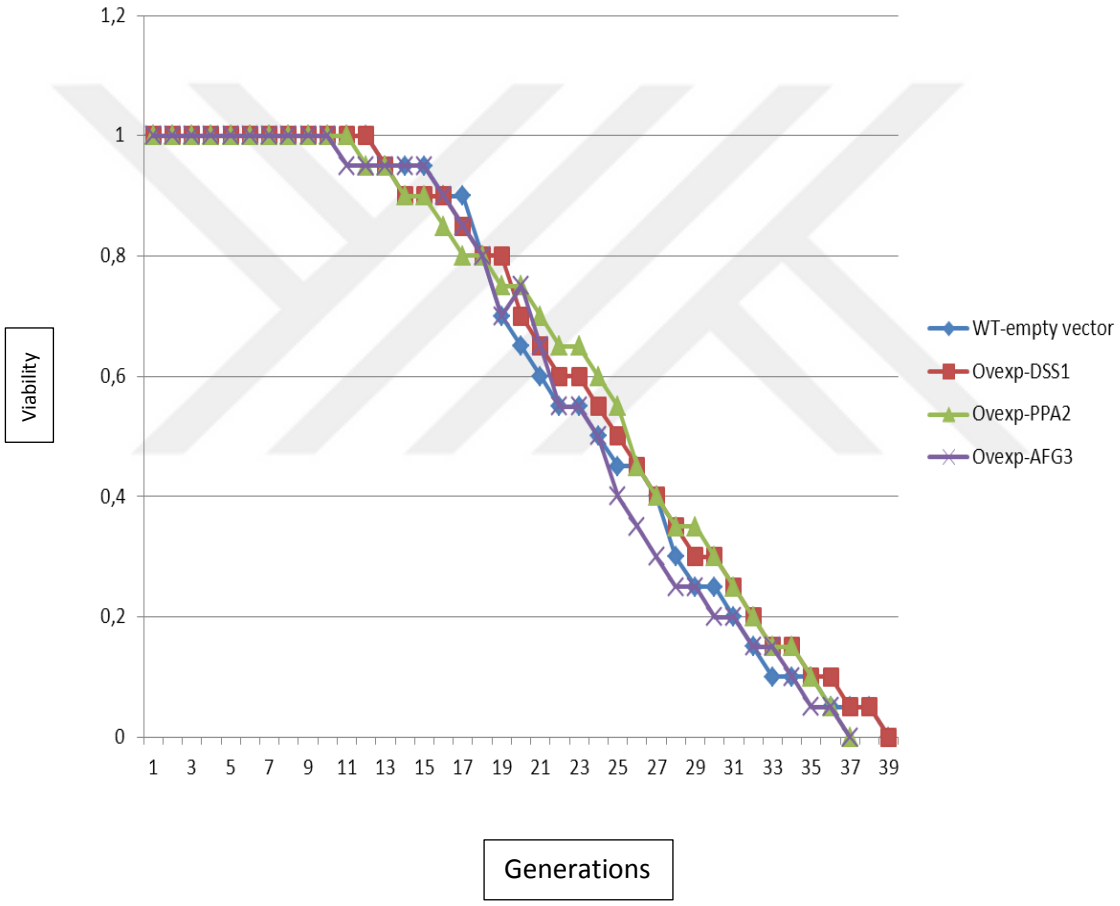


Figure 16. The overexpression of DSS1, PPA2 and AFG3 genes have no regulatory effect on WT replicative life span.

# CHAPTER 4

# DISCUSSION

The present study is pioneered to identify PPA2 within nucleic acid pathway gene as longevity gene. Protein folding pathway gene AFG3 and Heme-Ubiquinone pathway gene DSS1 were also investigated and confirmed their longevity role in this study using diploid cells (BY4743) (Fig.1). Among 144 gene deletion mutants, only three mutants (dss1Δ, ppa2Δ and afg3Δ) from three distinct pathways, were confirmed as long lived strains. When these genes DSS1, PPA2 and AFG3 were overexpressed in WT cells, life span was not affected (Fig.15,16) while the mutants dss1Δ, ppa2Δ and afg3Δ had 20%, 30% and 40% life span extension respectively (Fig.1), which implies that deletion of that genes has regulatory role for life span determination. When we screened the other genes of the same pathways, they do not showed longevity; this suggested that affect of deletion on life span is gene specific.

Andrzej et al.2003 identified DSS1 as mitochondrial degradosome that role in mitochondrial RNA metabolism by degrading aberrant RNAs and the RNase activity of this gene is necessary for mitochondrial biogenesis. They also reported that deletion of DSS1 strongly inhibit mitochondrial translation. Mitochondrial inorganic pyrophosphatase PPA2 is required for mitochondrial DNA synthesis and mitochondrial function (Maria Lundin et.al 1990). Joe R. Delaney et al. 2013, identified mitochondrial AFG3 (BY4742) as highly stress resistance mutant.The gene AFG3 acts on mitochondrial inner membrane as m-AAA protease (ATPase Associated Activity) that degrades missfolded or unassembled protein (Heike Arlt et al.1998), thus involved in mitochondrial protein folding pathway. So disruption of any of the gene was suspected to affect mitochondrial morphology and functions.

In this present investigaiton, long lived cells' mitochondria were seen as aggregated and colonized instead of chain networking form and the content of mitochondria remained intact in the course of time, young to old (Fig.2). This finding suggested that long lived cells developed an alternate morphology for functioning. The mechanism of breaking down of mitochondrial network was not investigated in this current study but Dimitry et al.2014 reported that the regulation of mitochondrial

architecture is mediated by fusion (join)/fission (separation) events and mitochondria are selectively removed by mitophagy that degrades imperfectly functional mitochondria. It has been shown that older mother cells have a tendency to segregate dysfunctional mitochondria to their daughters (Michal Jazwinski. 2004). Similarly all the young cells in this study showed almost equal amount of mitochondrial mass compared to old (Fig.3) which suggested that mitochondria were not destroyed, rather than it functioned in a remodeled way. Michal jaz 2012 reported that the mitochondrial fission–fusion cycle plays a role in mitophagy, since deletion of DNM1, which is required for fission, attenuates mitophagy without entirely eliminating it (Kanki et al., 2009). Interestingly, deletion of this gene extends yeast replicative lifespan (Scheckhuber et al., 2007). Another possible explanation is viewd from the yeast genome data base (http://www.yeastgenome.org/) where the genes DSS1, PPA2 and AFG3 were shown to be interacted genetically with DNM1, MDM, and ARD1complex that strongly role in mitochondrial localization, fision, fussion and early mitophagy.

AFG3 genetically interact with MIC family genes (MIC12, MIC26, MIC19, MIC26, MIC27, MIC60) and these genes acts for setting focal adhesion of mitochondrial membrane and network (GOsource: Yeast genome database). Taken together these data, it is assumed 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. And the colonized pattern of aggregation may be a protective morphology against destruction. Further investigation is required to reveal this hypothesis.

However, the most common spontaneous events that result in altered mitochondrial function in budding yeast are either presence of mitochondrial DNA with reduced expression of mitochondrial genome [ρ−], or total loss of the mtDNA [ρ0] which abolishes the complete production of mitochondria-encoded proteins. Saccharomyces cerevisiae rho0cells, which lack mtDNA, were defective in G1- to S-phase progression and thus, the cell cycle progression defect followed by growth defect in rho0 cells is caused by loss of DNA within mitochondria (David et.al.2012). Presence of mtDNA in young long lived cells and absence of mtDNA in old cells were observed in this study. When relative amount of mitochondrial DNA was quantified by amplifiying Cox2 in a qRealtime PCR, relatively less amount of mtDNA in long lived cells were found while mtDNA was highly decreased in old cells compared to wild type (Fig.4).

David et al. 2012 also showed that mtDNA itself affects cell cycle. They studied rho− cell in which all mtDNA-encoded genes are replaced with noncoding DNA. Hence that mtDNA has no expressed gene; the cell is respiratory incompetent but free from cell cycle progression defect. Thus rho- cells can not grow in YPG and thus respiratory deficient but cell cycle progression increased due to presence of mitochondrial DNA. A slow growth rate in petite culture could result from a high frequency of cell death, cell-cycle arrest, and/or a slower cell cycle. So, we checked the growth rate and colony formation of long lived cells in YPD media and found that unlike petite rho° mutants, these long lived mutants have similar growth rate (until glucose is present in the media) and healthy colonies compared to wild type (Fig. 5,6) suggested that these long lived cells got similarities with rho- characteristics and behave differently from petite rho°.

Gottchling 2009, reported that in Saccharomyces cerevisiae, loss of mtDNA leads to nuclear genome instability, through a process of cell-cycle arrest. which indicates that the relative content of mtDNA is important for cellular proliferation. Our studied long lived young cells comprise mtDNA but relatively less in amount compared to wild type and the presence of certain amount of mtDNA is enough for effective proliferation (Fig.6). Thus it is hypothesized that the reduction of mtDNA copy number may pass a signal to nucleus to create a pleiotropic phenotype on mitochondrial morphology and function thus in turn facilitated longevity. However, mtDNA loss overtime by a distinct process that remain unknown.

In Saccharomyces cerevisiae, the mtDNA encodes components of the mitochondrial translational apparatus, as well as protein subunits of respiratory complexes III, IV and V (Contamineet al. 2000). It has been known for many years that the loss of expression from the mtDNA in yeast results in pleiotropic phenotypes that are not completely explained by the loss of respiration (Gottch-Dujon, 1981). Specifically, cells that lack, or have severely rearranged, mtDNA display different mitochondrial morphologies (Church et al.1998), as well as different genetic requirements for viability (Joshura et al.2009) than respiration deficient cells with intact mtDNA.

Joshura et al. 2009 also observed whether mtDNA loss was a consequence of respiratory deficiency by deleting three different nuclear genes required for respiration at different steps of the electron transport chain. Consistent with previous reports, they found that there was no defect in growth in any of these three respiratory mutants,

despite the fact that each one eliminates the ability to respire (Francis et al., 2007). We also, in the present investigation, tested the long lived cells whether they can grow on non-fermentable media and found their inability to grow on Glycerol media indicating that they are respiratory deficient or incompetent. (Fig. 5). Moreover, though the long lived young cells had relatively less content of mtDNA and old cells had no mtDNA, they could proliferate as like as wild type. These observations show that loss or reduction of the mtDNA copy number has additional effects beyond respiration, could affect mitochondrial signaling pathways other than respiration.

Researchers have observed that the rate of mtROS production is comparatively less in the tissues of long-lived than in those of short-lived species (Barja et al.2007, Reinald 2011). In the present investigation, the long lived mutants showed reduced oxygen consumption compared to wild type cells (Fig. 7). In addition the endogenous ROS level were measured and found that wild type aging process is following ROS dependency while complete absence of ROS was observed both in long living young and old cells (Fig. 8). Mutation or reduced function in nuclear genes encoding electron transport chain (ETC) components has been found to extend lifespan in yeast, C. elegans, Drosophila, and mice (Jenni et al.2011). However, reduced ETC modulates the aging process in tissue-specific and temporal-specific manners. (Katharine 2012).

In the electron transport chain, electrons from reduced substrates are passed from complexes I , II and through complexes III and IV to oxygen, thus forming water moleculae. A major side reaction in this event is that electrons may leak from the respiratory chain complexes and may react with oxygen to produce the free radical superoxide (Montgomery et al.2011). Together all these data it is hypothesised that the deletion of the three genes, AFG3, PPA2 and DSS1, affected their interactive network with COA (Cytochorome oxydase Assembly factor) and cytochrome oxydase at complex IV of mitochondrial electron transport system (GO source:yeast genome databse), that may lead to restricting the consumption of oxygen and thus generated a ROS free state within the long lived cells.

The question arises from the mitochondrial theory of aging, by which endogenous ROS is the key modulator. Martin 2014 take the opposite tack, examining the evidence from long- and short-lived animal species, calorie restriction and genetically modified animals. They argue that the mitochondrial free radical theory of aging is in crisis because recent studies have shown no relationship between free radicals and longevity, and instead emphasize a role for mitochondrial reactive oxygen

species as intracellular messengers. Dimitry et al. 2014 showed that low doses of ROS could actually promote longevity while high doses, in contrast, shorten the lifespan of C. Elegans. Our studied long lived cells still grow old and dye in the absence of ROS indicates that aging is a genetic program while a threshold level of endogenous ROS may act as aging accelerator. However, rats, which are shorter-lived, show increased oxidative damage to fatty acids (Katharine 2012). The wild type senescence cells have higher degree of ROS that accumulated over time and previous extensive studies were performed to show the ROS dependent aging mechanisms. However, the long lived mutants may get benefit from this no-ROS state, such as reduction of endo-cyto toxicity, which in turn may facilitate longevity.

Ian et al.2010 described that Oxygen depletion commonly controls the switch from respiration to fermentation; illustrate that manipulating a single step can alter the mode of metabolism. While alcoholic fermentation is acknowledged as the catabolism of glucose to ethanol. According to the stoichiometry of alcoholic fermentation two molecules of ATP are produced per molecule of glucose converted into ethanol functioning as the main energy supply for maintenance and growth. In this study, the long lived cells have decreased O2 consumption, impaired respiration and complete absence of ROS status that implies that they utilize fermentation for the metabolic purpose. Karin et al. 2004 also described aerobic organisms respire pyruvate completely to CO2 with oxygen (O2) as the terminal electron acceptor, thereby making maximal use of energy transformations for ATP production. However, facultative aerobic organisms may add fermentation for fast energy production.

In this present investigation, we also measured the relative level of ATP both in young and old cells of these three mutants and compared with wild type. We observed that ATP level become doubled in Wild type young cells compared to old whereas no significant difference was found between long lived young and old cells ($P \geq .05$) (Fig. 10 A). But the long lived cells, both young and old, maintain a certain level of ATP that seems to be sufficient for their cellular function. Next, we also tested the time basis ATP level in the YPD culture media and observed the same fluctuation of ATP level until glucose is present (Fig. 10 B). 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 (Gorkem et al. 2013).

In respiring cells the ΔΨ is normally generated through the reactions of electron transport and oxidative phosphorylation. The activity of the mitochondrial electron transport chain drives the synthesis of ATP that generates the mitochondrial membrane potential that is utilized to power the transport of biosynthetic precursors out of the mitochondrion. The ATP is used among others in the maintenance of mitochondrial membrane potential. This occurs through the exchange of mitochondrial ADP for ATP by the action of the ADP–ATP translocator in the inner mitochondrial membrane. (Joshura et.al.2009). In addition to loss of respiration, the loss of mtDNA in mammalian (Jazayeri et al., 2003) or yeast cells (Dunn and Jensen, 2003) results in a reduction of the inner mitochondrial membrane electrochemical potential (ΔΨ). Following mtDNA loss or damage, a reduction in the mitochondrial membrane potential leads to decreased protein import into the mitochondria (Joshura et.al.2009).

But In our present investigation, we found that long lived senescent cells maintained mitochondrial membrane potential though their mitochondrial DNA was decreased. On the other hand, young cells of PPA2Δ and AFG3Δ have higher membrane potential compared to wild type young cells as well as their own old cells (Fig.9). When we compared the mean life span among the long lived mutants, the above mentioned two mutants were found to live longer than DSS1Δ (Fig.1). Gorkem et al.2013 stablished that deletion of the protein phosphatases raise ΔΨmito, and one feasible mechanism for increased ΔΨmito is a change in ion mobility and distribution across the mitochondrial inner membrane. The maintenance of ΔΨmito appears to have a predominant role in determining yeast longevity. Thus, mitochondria determine life span in a rather direct way in yeast.

Michal Jazwinski (2004) highlighted the importance of the maintenance of mitochondrial membrane potential, which drives the transport of biosynthetic precursors derived from the Krebs cycle. In both yeast and human, TCA cycle metabolites are used as macromolecular precursors. TCA pathway activity is maintained during fermentation for primary fuel biosynthetic reactions. In this study, TCA cycle activity was tested by viewing the activity of mitochondrial Aconitase that roles in Citrate to İsocitrate synthesis in the TCA cycle. TCA cycle activity was maintained slightly increased in young cells due to PPA2 and AFG3 deletion. While the activity was greatly reduced in long lived old cells (Fig.11). It has recently been shown that reductive carboxylation of α-ketoglutarate derived from glutamine, which yields both acetyl-coenzyme A and the

four-carbon TCA cycle intermediates that are used in various biosyntheses (Mullen et al., 2012).

In yeast, the glyoxylate cycle can be induced. Masa et al. 2011 explained that due to impaired mitochondrial functions by activaitng an evolutionary conserved communicaiton pathway from mitochondria to nucleus reffered as retrograde response. CIT2 , peroxisomal isoform of Citrate synthase that functions in glyoxylate cycle and RTG2 that encodes regulatory proteins. Another pathway, mitochondrial unfolded protein response (HsP60), was also known to mediate longevity in ETC deletion mutant (Jenni durieux et al. 2011). In this study, three representative genes CIT2, RTG2 and Hsp60 were relatively investigated through quantitative realtime PCR and found that CIT2 expressed 2 fold higher in AFG3 mutant, 3 fold higher in DSS1 mutant and 5 fold higher in PPA2 mutant while RTG2 and HsP60 had similar expression level compare to WT (Fig.14). Thus taken together with these data, it is suggested that a metabolic remodeling, so called “Glyoxylate cycle”, may occurred in our studied long lived cells.

In terms of mitochondrial function and morphology, similar pleitropic phenotype was observed among the long lived strains due to deletion DSS1, PPA2 and AFG3 genes, indicating that they may share a common gene expression pattern that confer longevity. In order to investigate the whole genome expression, microarray was performed with Agilent-One color-Yeast Expression and LIMMA package of Bioconductor was used to process the data.

In this sinvestigation, a set of genes was found commonly upregulated and sets of clustered genes were observed as commonly down regulated among all the long lived cells (Fig.13 , table 3,4). More surprisingly, STL1 (C-2 compund/H+ symporter) gene rendered very high level expression (16-32 fold over) among all genes. Joana et al.(2010) reported that osmotolerant yeast cells exhibit STL1 transporter activity in order to maintain intracellular glycerol content. Glycerol biosynthesis has also important roles in budding yeast by maintaining cytosolic redox balance, especially under anaerobic conditions, while under fermentive condition, ethanol is produced from pyruvate and glycerol is formed from glycolytic dihydroxyacetone phosphate (DHAP) and excreted in the medium. (Zheng et al.2001). Joana et al. 2010 also found that under gluconeogenic condititon STL1 gene was over expressed and yeast cells accumulate glycerol via transporter Stl1p while excrete via Gup1p and Gup2p for cellular homeostasis; glycerol is the precursor of fatty acid that can enter into glyoxylate cycle for the induction of gluconeogenesis. In this present study, CIT2 gene was quantified

and found as indicator of retrograde response that generates peroxisomal glyoxylate cycle activity for gluconeogenesis (Fig.14) Thus taken all together, it is strongly suggested that CIT2 derived retrograde signaling is aslo correlated with glycerol/proton symport activity.

In addition, this proton accumulation may confer acidic cytoplasm and to test this hypothesis, in vivo measurement of cytoplasm was performed by using a pH sensitive GFP plasmid p413 TEF. WT young cells showed slightly acidic and old cells had basic cytoplasmic environment which is also mathces with the data provided by daughter and old mother specific pH value reported by (Adam et al.2012). All young and old cells of long lived mutants rendered slightly acidic cytoplasm (pH 6.3-6.7) (Fig.12) indicaitng a regulatory role of cytoplasmic pH on life span determination. Chemical reactions in living cells are pH sensitive and under strict enzyme control that conserved from bacteria to humans. (Ian et al.2010).

# CHAPTER 5

# CONCLUSION

In addition with retrograde response, the genetic data also showed that purine biosynthesis genes have higher level of expression compared to WT, especially for the synthesis of IMP (Table 4) which is the precursor of nucleic acids, AMP,GMP,cAMP (widely known to act as secondary messanger). CLB2, SWI5, CIK1, SFG1, HOF1, BUD4 and CWP1 genes also upregulated that play role in cell divison, cytoskeleton and budding (Table 4). Cyclin B (CLB2), SWI factor 5 and SFG1 activates cyclin dependent kinase cdc28 in Yeast (CDK1 in mammal) which is known as the master regulator of cell divison . Thus these data provide links among retrograde response, Glycerol/proton symporter activity and cell divison regulators that together may facilitated longevity.

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