IDENTIFICATION OF MITOCHONDRIAL ELECTRON TRANSPORT CHAIN MUTATIONS THAT EFFECT AGEING

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ABSTRACT

IDENTIFICATION OF MITOCHONDRIAL ELECTRON TRANSPORT CHAIN MUTATIONS THAT EFFECT AGEING

Aging can be defined as the loss of cell functionality by accumulation of deleterious effects. Mitochondrial electron transport chain (ETC) is the main site for reactive oxygen species (ROS) production. According to free radical theory of aging, free radicals produced by normal aerobic respiration accumulate by time and can cause aging. Although previous studies have identified that inner mitochondrial membrane complexes I and III are the major sites of ROS production, role of ETC genes in ROS production is a matter of debate. The purpose of the present study was to determine the ETC mutations that affect aging using *S.cerevisiae* as a model organism. Deletion mutants of *S.cerevisiae* lacking 73 genes of ETC were analyzed aging and we found out that nine mutants caused reduction in replicative lifespan. In addition to aging profiles, ROS production levels, respiratory competence and oxidative stress tolerance level of these deletion strains were also investigated. In order to verify lifespan modulation by these genes, they were all overexpressed in wild-type cells and aging profile of these cells was analyzed. Most of the cells lived longer than wild type control cells containing sham vector. Our results suggest that some of the ETC genes play important roles in mitochondrial functions and aging. We hope that our results will contribute to the field of aging studies.

ÖZET

YAŞLANMAYI ETKİLEYEN MİTOKONDRİ ELEKTRON TAŞIMA ZİNCİRİ MUTASYONLARININ TESPİTİ

Yaşlılık zararlı etkilerin birikmesine bağlı olarak hücrenin fonksiyonunu kaybetmesi olarak tanımlanabilir. Mitokondriyel elektron taşıma zinciri (ETC) reaktif oksijen türlerinin (ROS) asıl oluşum yeridir. Yaşlılığın serbest radikal teorisine göre normal aerobik solunumla oluşan serbest radikaller zamanla birikerek yaşlılık ve dejeneratif hastalıklara sebep olabilirler. Önceki çalışmalarda, iç mitokondriyel zar kompleksleri I ve III, ROS oluşum yerleri olarak tespit edilmesine rağmen yaşlılığın moleküler mekanizması hala tartışma konusudur. Çalışmanın amacı, model organizma olarak *S.cerevisiae* hücrelerini kullanarak ve bu hücrelerin ROS oluşturma potansiyelleri esas alınarak yaşlanmayı etkileyen mitokondriyel ETC mutasyonlarını tespit etmektir. 73 ETC geninden yoksun delesyon mutantları analiz edildi ve dokuz genin yaşlanma profillerine göre replikatif ömürlerinde azalma olduğu bulundu. Ayrıca, yaşlanma profillerinin yanı sıra, bu delesyon suşların ROS üretim seviyeleri, respirasyon kapasiteleri ve dış kaynaklı oksidatif stres toleransları incelendi. Yaşam süresinin bu genler tarafından düzenlendiğini doğrulamak için, genler yabani türde aşırı ifadelendirildi ve yaşlanma profilleri analiz edildi. Birçok hücre yalnızca vektörü bulunduran yabani tür kontrol hücrelerine göre daha uzun yaşadı. Çalışmamız bazı ETC genlerinin mitokondriyel fonksiyonlarda ve yaşlılıkta önemli bir rol aldığını öne sürmektedir. Bu sebeple çalışmamızın yaşlılık çalışma alanlarında katkı sağlamasını umuyoruz.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1. Aging

Aging is a natural process and defined as accumulation of deleterious alterations inside the cell. These alterations include telomere shortening, over-expression of oncogenes, and oxidative stress. In human, these alterations activate DNA damage response pathways which stimulate various signaling pathways resulting in senescence. Accumulation of senescent cells may cause aging in two ways. Firstly, accumulated senescent cells may reduce self-renewing capability of cells and cause aging as a result of decreased tissue function. Secondly, increasing levels of degradative enzymes, inflammatory cytokines and epithelial growth factors may cause tissue structure disruption and function, resulting in aging (Chen, et al. 2007). In other words excess amounts of reactive oxygen species (ROS), dysfunction of antioxidant defense systems, declining in DNA repair system induce DNA damage and aging process.

As a single-celled eukaryote, *S. cerevisiae* is the best model organism although there is no differentiation and specialization. Their short life cycle, small genome (about 6000 genes and completely sequenced), nuclear genome without introns and orthology with human genome make them attractive for aging studies (Gershon and Gershon 2000).

Two modes of aging are defined in *S. cerevisiae* which are replicative lifespan and chronological lifespan. Chronological lifespan is determined as a chronological time that a cell can remain stationary phase post-mitotically (Fabrizio and Longo 2003). The number of buds produced from mother cell (mitotically) gives the replicative lifespan of the yeast (Jazwinski 2004). Asymmetrical division of yeast cells is the adaptation to the wild in order to rapid growth of colonies. Human orthologs *RAS1* and

RAS2 modulate yeast replicative aging. *RAS1* deletion caused lifespan extension while, *RAS2* caused short lifespan (Sun et al. 1994). Mutations in *SIR* complex and reduction in autophagy-inducer Tor signaling resulted in replicative lifespan extension (Kennedy, Smith, and Kaeberlein 2005; Kaeberlein et al. 2005). Calorie restriction is another determinant of yeast replicative aging as it increases lifespan. Calori restriction inactivates Tor signaling, protein kinase A and Sch9 (nutrient responsive kinase) followed by increased respiration rate due to Msn2/4 (stress response proteins) activation. Msn2/4 also inhibits nicotinamide deaminase Pnc1. Both events activates Sir2 and result in lifespan extension (Steinkraus, et al. 2008). In *S. cerevisiae* telomere shortening is not observed over the life-time unlike human. Cell surface area expansion, deceleration of cell cycle, infertility, and accumulation of aging factors such as extra-chromosomal rDNA circles (ERCs), carbonylated proteins, and protein aggregates are associated with aged *S. cerevisiae* (Steinkraus, et al. 2008).

Besides these aging markers, elevated ROS production is the key point. According to free radical theory of aging by Denham Harman, ROS generation by mitochondria or other sites from the cell cause initiation of degradative processes related to mitochondrial damage (Harman 2003). Mitochondria have a central role in aging because ROS is mainly produced by electron transport chain. Short half-life of the ETC-derived ROS causes oxidative stress-mediated mitochondrial dysfunction. mtDNA is found in matrix and does not have histones. Because of this reason mitochondria can not protect their mtDNA from ROS-mediated impact (Cadenas and Davies 2000). Mitochondria have both antioxidant defense systems and DNA repair systems. However, elevated accumulation of the ROS blocks antioxidant systems and causes mtDNA lesions which can not be repaired (Cadenas and Davies 2000). Oxidative damage to the mitochondria reduces mtDNA turnover and results in mitochondrial impair dependent limitation of maximum lifespan. Oxidative damage to the nuclear DNA results in degenerative diseases and reduction in mean lifespan (Sastre, Pallardo, and Vina 2003).

1.2. Oxidative Stress and Reactive Oxygen Species Production Sites In Mitochondria

Oxidative stress is defined as imbalance between antioxidant defense system mechanisms and accumulation of free radicals. Highly reactive hydroxyl radical-mediated irreparable DNA lesions cause DNA repair mechanisms dysfunction (Turrens 2003). This process results in oxidative stress related neurodegenerative diseases, aging and cancer due to excess amounts of reactive oxygen species (ROS) accumulation (Perrone, et al. 2008; Imlay 2003). ROS are stable free radical intermediates which are derived from one unpaired electron participation to the molecular oxygen (Cadenas and Davies 2000). There are two main sources of ROS which are endogenous and exogenous sources. Endogenous sources are mitochondrial respiration, NADPH-oxidase activity, peroxizomes, lipoxygenase; while exogenous sources are UV, ionizing radiation, and xenobiotics that pick up an electron from a respiratory carrier and transfer it to molecular oxygen, stimulating free radical formation without inhibiting the respiratory chain (Turrens 2003).

Morphologically, mitochondria are composed of four compartments including outer and inner membrane, inter-membrane space and matrix and different kinds of metabolic pathways are carried out such as Krebs cycle, oxidative phosphorylation, glycolysis and mitochondrial protein assembly. Outer membrane is composed of porins which allow the passage of small molecules (Pfanner and Meijer 1997) and nDNA-encoded mitochondrial proteins (Wallace 2005). Both outer and inner membrane includes translocases called as TOM and TIM, respectively. Pre-protein structures cross through these two general import pores and subsequently become mature proteins with the help of the mitochondrial chaperone Hsp70 and mitochondrial protein peptidases in the matrix region (Pfanner and Meijer 1997). Mitochondrial inner membrane is the site of oxidative phosphorylation which allows ATP production via the oxidation-reduction reactions (Scheffler 2001) and this site is responsible for the formation of most of the free radicals (Murphy 2009).

Superoxide, hydrogen peroxide and hydroxyl radical are important reactive oxygen species that are produced mainly in mitochondria. Superoxide is the product of the molecular oxygen reduction with one unpaired electron and precursor of other

oxidants. Superoxide is mainly produced from the leakage of electrons from the mitochondrial electron transport chain as the side product of aerobic respiration (Murphy 2009). Molecular oxygen has a tendency to produce superoxide ion because of its thermodynamic. Hydrogen peroxide is derived from two superoxide dismutation by superoxide dismutases or spontaneously. Although hydrogen peroxide seems to be harmless, it is the precursor of the hydroxyl radical and its formation is catalyzed by transition metals reduction with Fenton reactions, UV-irradiation and radiolysis of water by x-or γ-rays (Orrenius, et al. 2007). Small electron carriers like NADH, NADPH, ubiquinone, and glutathione (GSH) do not react with molecular oxygen therefore they do not have a tendency to generate superoxide (Imlay 2003). Mitochondrial superoxide production exists in redox active sites of the protein and when electron carriers are bound to proteins, superoxide production is favored by kinetic factors (Imlay 2003). Superoxide formation concentration is proportional to the enzyme (redox active) concentration and the molecular oxygen concentration. Previous studies show that superoxide generation increases if oxygen concentration raises with respect to normal atmospheric level of oxygen (Murphy 2009). It is thought that active sites which are facing with aqueous phase or membrane core are the superoxide production sites because of closer oxygen interaction (Imlay 2003). Superoxide generation in mitochondrial matrix is measured as the sum of hydrogen peroxide efflux from mitochondria, superoxide sinks, and degraded hydrogen peroxide concentration minus external hydrogen peroxide production concentration (Murphy 2009).

Electron transport chain is composed of five enzyme complexes which are Complex I (NADH-ubiquinone oxidoreductase), Complex II (succinate-ubiquinone oxidoreductase), Complex III (ubiquinol:cytochrome c oxidoreductase), Complex IV (cytochrome c oxidase) and ATP synthase (Complex V). NADH and $FADH_2$ enter the chain via complex I and complex II, proceeding with electron transfer through molecular oxygen and result in H_2O formation in complex IV (Liu, Fiskum, and Schubert 2002). H_2O formation changes proton gradient and membrane potential resulted in ADP phosporylation in complex V (Liu, et al. 2002; Wallace 2005).

The main source of superoxide in electron transport chain is Complex I and its subunits. Complex I (NADH-ubiquinone oxidoreductase) is the first place for electron entrance at the site of FMN (flavin mononucleotide). The entire complex composed of more than 40 subunits. *Saccharomyces cerevisiae* have rotenone-insensitive matrix-facing NADH-ubiquinone oxidoreductase which is encoded by the gene *NDI1* and

intermembrane space-facing NADH-ubiquinone oxidoreductase which is encoded by the genes *NDE1* and *NDE2* instead of complex I (Grandier-Vazeille et al. 2001).Complex I composed of FMN and up to seven non-heme iron-sulfur clusters on peripheral arm which faces with matrix site. The other structural portion of the complex is ubiquinone hydrogenase site which is embedded in the membrane. After passage of electrons to the complex III via ubiquinone four H^+ are pumped out to inter-membrane space and changes both membrane potential and proton gradient. Translocation of the protons through the intermembrane space FMN or ubisemiquinone is required (Hatefi 1985). However in yeast, there is no proton pumping mechanism in complex I (Fang and Beattie 2003).These are free radical form of FMN, F-site (NADH binding site for oxidation of NADH), R-site (NAD^+ binding site for univalent oxygen reduction). Once NADH/NAD^+ratio increases in the matrix, these subunits converted to electron leakage sites (Grivennikova and Vinogradov 2006; Kushnareva, et al. 2002). Previous studies about *S. cerevisiae* show that if cytosolic NADH is used as electron donor, it was found that high concentrations of hydrogen peroxide and superoxide produced by the external NADH dehydrogenases unlike human (Nde1p, Nde2p) (Herrero et al. 2008; Fang and Beattie 2003).

Complex II is referred as succinate-ubiquinone oxidoreductase which has a catalytic heterodimer containing covalent FAD and three non-heme iron-sulfur clusters which are [2Fe-2S], [3Fe-3S] and [4Fe-4S] (Lemire and Oyedotun 2002). Because of cysteine residues in iron sulfur clusters which have redox potential, electrons can pass through these clusters to the complex structure. Previous study with *Caenorhabditis elegans* shows that SdhC Gly69 mutation to glutamic acid residue exhibits *mev-1* mutations in *SDH* genes (coding for succinate dehydrogenase) causes a significant increase in superoxide production in Complex II (Cecchini 2003). The [3Fe-4S] cluster seems to the site of reactive oxygen species production site in the presence of quinones results with the enzyme inactivation in *E.coli* (Senoo-Matsuda et al. 2001). Coenzyme-Q related enzymes seem to be the ROS production sites like in succinate oxidation via complex II by reverse electron transport at complex I (Guzy et al. 2008). The yeast *SDH3* S94E (mimics *C. elegans mev-1* mutation) *SDH2* P190Q mutations near the Q_P site (ubiquinone-binding site) decrease the activity of the complex II and cause hypersensitivity to paraquat. This indicates that mutations are located near the Q_P site and this increases the possibility that Q_P site is the site of radical formation and the

mitochondrial succinate dehydrogenases can be significant sources of oxygen-free radicals (Guo and Lemire 2003).

Complex III (ubiquinol:cytochrome c oxidoreductase) composed of redox-active diheme cytochrome b (high-potential b_H and lower potential b_L), cytochrome c_1, [2Fe-2S]-containing Rieske iron-sulfur protein. The molecular function of the complex III is to transfer electrons from ubiquinol to cytochrome c by Q-cycle (Lenaz et al. 2007; Hunte et al. 2008). This transfer is coupled with proton translocation to the intermembrane space. Amino acid sequences of the cytochrome b display high homology between human and *S.cerevisiae*. They both have conserved histidine residues (Hatefi 1985). E272 (glutamate at position 272; highly conserved cytochrome b residue) has important role in prevention of short circuit reactions and correct binding and pH stabilization (Wenz et al. 2006). [2Fe–2S] domain mobility of the Rieske protein is required to transportation of electrons between ubihydroquinone oxidation at center P (Qo; electropositive site; toward intermembrane space) to cytochrome *c*1. The low-potential heme *b*L (*b*566) is closer to the inter-membrane space and center P, whereas the high-potential heme *b*H (*b*562) is close to center N (Qi; electronegative site; inner membrane facing matrix side) (Hunte et al. 2008). Electron transfer pathway is followed by passage of electrons through the high-potential chain consisting of ISP, cytochrome *c*1, and cytochrome *c*. The second electron is transferred through the low-potential chain via heme *b*L and heme *b*H to N center at which quinone is reduced to semiquinone (reaction intermediate). In order to transfer electrons through the cytochrome c requires oxidation of two ubihydroquinone which is reduced to semiquinone completely.

Complex III is the main site for ROS production in mitochondria (Turrens 2003), and blockade of electron flow by using rotenone and succinate dehydrogenase inhibitor upstream of complex III reduces net ROS production that are oxidizing complex I substrates (Chen et al. 2003). Q_i center produces superoxide through the matrix side however; Q_o center produces toward intermembrane space (Chen et al. 2003). Another study about Q_o superoxide production ability shows that myxothiazol usage as a Complex III inhibitor stimulates hydrogen peroxide formation at the site of Q_o (Starkov and Fiskum 2001).

Complex IV is the final enzyme of the electron transport chain and called as cytochrome c oxidase and catalyses the transferring of the electrons from the cytochrome c to molecular oxygen, converting molecular oxygen to two molecules of

water. Cytochrome c oxidase is composed of 13 subunits in human and 11 subunits in *S.cerevisiae.* Large three subunits are encoded by mitochondrial genome and the others which are found in core protective shield encoded by nuclear genome (Fontanesi, et al. 2008). Complex contain two hemes that are *a* and a_3, and two copper centers. Except that components complex IV contains Mg and Zn metal centers which are not redox centers. Their role appears to stabilizing three-dimensional structure, proton translocation process and the direction of the electron transfer (Ludwig et al. 2001). Electron transfer path in complex IV begins with electron transfer from cytochrome c to Cu_A center to heme a, then heme a_3/Cu_B and finally to the molecular oxygen which is used to water production coupled with proton translocation process (Varotsis et al. 1993). Normally ROS do not produced from Complex IV. According to the second mechanism of respiratory control is independent from the membrane potential unlike the first mechanism of respiratory control in which at high membrane potentials complexes I, III, IV are inhibited. Second mechanism proposes that without allosteric ATP-inhibition stimulation of ADP is inhibited because ADP uses membrane potential to convert ADP to ATP. Dephosphorylation of Complex IV by phosphatases results in blockade of allosteric ATP-inhibition and causes high membrane potential which is excited state and stimulates ROS production, apoptosis and degenerative diseases (Kadenbach, et al. 2009).

Proton translocation to the intermembrane space by complex I, III and IV results in membrane potential variation and proton gradient forces ATP synthase to produce energy. ATP synthase is composed of two main parts which are insoluble F_0 and soluble F_1. The F_0 part provides a channel for proton translocation from its high potential on the outside. Mitochondrial F_1F_0 ATP synthases contain eight subunits which are consisted of five different subunits α, β, γ, δ and ε in F_1 domain including central stalk and three different subunits named a, b and c in F_0 domain (Dudkina et al. 2006). *Saccharomyces cerevisiae* has mitochondrial specific ε subunit located in the central stalk. Remaining d, e, f, g, h, i and k are located in the F_0 domain closer to the peripheral stalk portion. Previous studies show that these small subunits are involved in dimerization process (Dudkina et al. 2006). As it is well known that there is no indication about ROS production from ATP synthase.

1.3. Aim of the Study

All of studies about electron transport chain and ROS production capacity are a matter of debate and not enough to answer the questions that which specific mutations cause electron leakage-mediated ROS production, where they are produced and how they are related to aging process. Because of these question marks we wanted to study; electron transport chain mutations that affect aging process. In addition, enlightening the mechanisms of human genetic disorders related to electron transport chain dysfunction is the main scope of all aging research including our project.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

A detailed list of commonly used media, solutions, and their compositions are presented in Appendix A.

2.2. Methods

2.2.1. Yeast Strains

Wild-type (BY4741; MATa, *his3*, *leu3*, *met15*, *ura3*) and isogenic 73 different deletion mutants (derived from BY4741) of *Saccharomyces cerevisiae* were examined (Table 3.1.). For the further analysis ***nde1***Δ (MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*nde1*:KAN_R), ***tcm62***Δ (MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*tcm62*:KAN_R), Δ***rip1*** (MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*rip1*:KAN_R), ***qcr8***Δ (MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*qcr8*:KAN_R), ***cyt1***Δ (MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*cyt1*:KAN_R), ***pet117***Δ (MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*pet117* :KAN_R), ***cox11***Δ (MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*cox11*:KAN_R), ***atp11***Δ (MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*atp11*:KAN_R), ***fmc1***Δ (MATa, *his3*, *leu2*, *met15*, *ura3*, Δ*fmc1*:KAN_R) were used.

2.2.2. Identification of ETC Mutations That Lead to Aging

Yeast strains were grown on YPD rich medium (2% glucose, 2% peptone, 1% yeast extract and 2% agar) for determination of short-lived mutants and YNB minimal medium (0.17% yeast nitrogen base without amino acids, 5% ammonium sulfate, 2% glucose supplemented with appropriate amino acids and bases) for determination of strains that have over-expressing genes. Before analysis, cells were kept for 2 days incubation at 30ºC. Cells were inoculated onto agar plates as a single line. For each strain, average 40 individual cells were selected and removed from yeast colonies by using a micromanipulator. 20 of 40 individual daughter cells were collected and selected as starting mother cells. Newly formed daughter cells from these virgin cells were removed and discarded. Every 90 minutes intervals plates were controlled and newly formed daughters were removed until cells stopped dividing. During night periods plates were stored at 4°C. Replicative life span was determined as the total number of daughter cells that each mother cell generated.

2.2.3. Oxidative Stress Tolerance of Short Living ETC Mutants

Short-living mutants compared to the WT were analyzed for oxidative stress tolerance with using diamide and hydrogen peroxide as oxidizing agents. Diamide acts on thiol-containing groups and oxidizes them via increasing oxidizing glutathione levels which means that redox imbalance in vivo and oxidizes cysteine residues of the proteins in vitro (Flattery-O'Brien and Dawes 1998). Hydrogen peroxide is the product of dismutation of the two superoxide anion and exogenous H_2O_2 increase the intracellular reactive oxygen species levels (Lu and Gong 2009). In order to determine the endogenous oxidative effect of the diamide spotting assay and for hydrogen peroxide Halo assay were performed, respectively.

For spotting assay; yeast cells were grown over-night and diluted to OD_{600} 0.2. Four dilution series were prepared as 0.2, 0.02, 0.002 and 0.0002. From each dilution 5 µl of sample was dropped onto YPD media which include no diamide, 2mM diamide,

2.5mM diamide. YPD plates were incubated at 30ºC approximately 2 days and growth behavior was examined.

For Halo assay; strains were grown over night in liquid YPD medium at 30°C OD_{600} values were equalized to 0.2 with sterile H_2O. 400µl of sample was plated and 5µl 8.8M hydrogen peroxide was dropped at the middle of the plate and incubated overnight at 30ºC in order to observe sensitivity which is determined as a diameter of the growth inhibition zone.

2.2.4. Determination of Respiratory Deficient Strains in Short Living ETC Mutants

In order to determine respiratory deficient strains mutants that have short lifespan were grown on YPG media including 3% glycerol. Usage of glycerol as a carbon source forces mitochondria to respire. Glycerol as a carbon source inhibits ethanol formation and induces pyruvate conversion to acetyl-CoA through Krebs cycle and oxidative phosphorylation in order to produce ATP via respiration activation.

For determination of respiratory deficient strains spotting assay was performed. For spotting assay; yeast cells were grown over-night on liquid YPD media and diluted to OD_{600} 0.2 with dH_2O. Four dilution series were prepared as 0.2, 0.02, 0.002 and 0.0002. From each dilution 5 µl of sample was dropped onto YPG (3% glycerol) media. YPG plates were incubated at 30ºC approximately 2 days and growth behavior was examined.

2.2.5. Measurement of Intracellular Superoxide Levels of the Short Living ETC Mutants

Relative intracellular superoxide levels were analyzed using the superoxide sensitive fluorescent probe MitoSOX Red (Molecular Probes) which is superoxide indicator in living cells. It is oxidized by superoxide and displays red fluorescence in the mitochondria. Strains were grown in YPD media to an OD_{600} of 0.5. At that OD_{600}

value MitoSOX added to the YPD to a concentration of 5μM and negative control (not treated with MitoSOX) was prepared. Cells were incubated for 1h in the fluorescent dye at 30°C for shaking at 180 rpm. After incubation, cells were centrifuged and washed twice with phosphate saline buffer (PBS) and resuspended in 200μL PBS. The fluorescence intensity of cells for each strain was measured by Varioskan spectrophotometer with 510/580 nm excitation/emission wavelengths.

2.2.6. Overexpressing ETC Genes that are Important for Aging

Over-expression constructs of *NDE1, TCM62, RIP1, CYT1, QCR8, PET117, COX11, ATP11, FMC1* were made with Gateway® Cloning System. System is composed of two main reactions. BP recombination reaction was performed with PCR products (as a template, yeast genomic DNA; primers of each of nine gene) and pDONR221 was used to generate an entry clone. LR recombination reaction was performed by using pAG423-GPD (2μ high copy plasmid; HA-tagged; GPD promoter; Addgene) and pAG413-GPD (low copy plasmid; GPD promoter; Addgene) as destination vectors and entry clone for each plasmid. BP and LR reaction mixtures were transformed to the OmniMax competent cells for the selection of antibiotic resistant colonies which include desired gene in plasmids. Recombination occurs between *att*B and *att*P sites give rise to *att*L and *att*R sites. Homolog recombination between these sites allows insertion of desired gene into the expression vector (Gateway® Technology; Invitrogen). All constructs were made for this study verified by sequencing.

2.2.7.Transformation of Cells Overexpressing ETC Genes that are Important for Aging

In order to perform transformation of yeast strains lithium-acetate method was used with a few modifications (Amberg et al. 2005). Yeast wild-type strain was grown in liquid YPD media over-night at 30°C. Grown cells was centrifuged in 10sec and

washed with sterile dH_2O. Pellet was resuspended and washed twice with 0.1M lithium-acetate. Then pellet was resuspended with 240µl PEG (50%), 36µl 1M lithium-acetate, 7µl single-stranded salmon sperm carrier DNA, 5µl expression vector (including the gene that was wanted to be over-express), 10µl DTT (Dithiothreitol; 0.3M) for high transformation efficiency and 62µl sterile dH_2O in order. Transformation mix was incubated for 30 min at 30°C shaker incubator. After that, transformation mix was allowed to heat shock at 42°C for 15 min and spreaded onto YNB synthetic media (without histidine). Each expression vector including the gene that was wanted to be over-express was transformed into the wild-type strain.

CHAPTER 3

RESULTS

3.1. Identification of ETC Mutations That Lead to Aging

Since electron leakage at mitochondrial ETC is the major cause of ROS production, we thought that disruption of electron flow by genetic manipulations may elevate the level of ROS production and leads to premature aging. To analyze the role of all the nonessential ETC genes (Table 3.1.) in life span, 73 deletion mutants were first screened in the fashion of 5 replicas. For the second screening, 40 replicas were analyzed for each mutant and repeated at least twice. We found out that mutations in nine ETC genes shortened lifespan enormously (Figure 3.1). These genes were *NDE1, TCM62, RIP1, CYT1, QCR8, PET117, COX11, ATP11* and *FMC1*.

The *NDE1* which encodes mitochondrial external NADH dehydrogenase that catalyzes the oxidation of cytosolic NADH. Previous studies about *NDE1* showed that deletion of it was resulted in shorter replicative lifespan (Lin et al. 2004). Another study proposed that deletion of *NDE1* was resulted in extension of chronological lifespan (Li et al. 2006).Consistent with previous report (Lin et al. 2004), we determined 40% reduction in lifespan of *nde1*Δ mutants (Table 3.2.).

TCM62 plays role in the assembly and stability of the mitochondrial succinate dehydrogenase complex and is required for essential mitochondrial functions at high temperature (Klanner, Neupert, and Langer 2000). Previous studies showed that Complex II has a role on aging process studied in C.elegans harbors *mev-1* mutation. This mutation found in the succinate dehydrogenaseC (*SdhC*) causes reduction in lifespan and accumulation of aging markers (Cecchini 2003). There is no indication about molecular function of the *TCM62* as a cell aging process in the literature. Our

study showed that deletion of *TCM62* was resulted in lifespan reduction in 28% compared to the wild-type (Table 3.2.).

Aging assays showed that 3 genes were determined related to Complex III. First one is the *RIP1* which encodes ubiquinol-cytochrome-c reductase, a Rieske iron-sulfur protein of the mitochondrial Complex III and transfers electrons from ubiquinol to cytochrome c1 during aerobic respiration. Its biological role only includes aerobic respiration and electron transfer (Beckmann et al. 1987). Second one is the *CYT1* which encodes cytochrome c1 protein which transfers electrons from ubiquinol to cytochrome c (Ahmad and Sherman 2001). Third one is the *QCR8* encodes subunit 8 which is a member of ubiquinol cytochrome-c reductase complex (component of the both Qo and Qi sites) and its function is the electron transfer from ubiquinol to the cytochrome c (Bruel, Brasseur, and Trumpower 1996). According to our aging assays, percent reduction in lifespan of Δrip1, Δcyt1 and Δqcr8 mutants were 55%, 31% and 33% compared to the wild-type strain (Table 3.2.).

PET117 and *COX11* encode proteins that are essential for assembly of the multi-subunit enzyme Complex IV, which catalyzes the conversion of molecular oxygen to water for cellular respiration. *COX11* is required in the copper addition to the Cu(B) site of complex IV (Hiser et al. 2000). *PET117* encodes protein that has a role in assembly of the Complex IV (McEwen et al. 1993) and molecular function of it is not known (*Saccharomyces* Genome Database). Deletion of *PET117* and *COX11* reduced lifespan by 34% and 31%, respectively (Table 3.2.).

Two other genes that were identified as aging genes were ATP11 and FMC1 which encode the assembly proteins in F1 sector of F1-F0 ATPase (Ackerman 2002; Lefebvre-Legendre et al. 2001). Aging assay results showed that deletion of ATP11 and FMC1 (with unknown molecular function) which are encodes subunits of Complex V was resulted in reduction of life span by 44% and 26%, respectively (Table 3.2.).

Table 3.1. Non-essential ETC genes that were investigated in this study

	ORF (gene)	Description
Complex I	***NDE1***	Mitochondrial external NADH dehydrogenase
	NDI1	NADH:ubiquinone oxidoreductase
	NDE2	Mitochondrial external NADH dehydrogenase
Complex II	***SDH2***	Iron-sulfur protein subunit of succinate dehydrogenase
	SDH4	Membrane anchor subunit of succinate dehydrogenase
	TCM62	Assembly of the mitochondrial succinate dehydrogenase complex
	SDH1	Flavoprotein subunit of succinate dehydrogenase
	YJL045W	Minor succinate dehydrogenase isozyme
Complex III	***QCR7***	Subunit 7 of the ubiquinol cytochrome-c reductase complex
	QCR9	Subunit 9 of the ubiquinol cytochrome-c reductase complex
	QCR2	Subunit 2 of the ubiquinol cytochrome-c reductase complex
	CBP4	Required for assembly of ubiquinol cytochrome-c reductase
	BCS1	ATP-dependent chaperone, required for the assembly of the cytochrome bc(1) complex
	QCR6	Subunit 6 of the ubiquinol cytochrome-c reductase complex, required for maturation of cytochrome c1
	CBP3	Mitochondrial protein required for assembly of ubiquinol cytochrome-c reductase complex
	RIP1	A Rieske iron-sulfur protein of the mitochondrial cytochrome bc1 complex; transfers electrons from ubiquinol to cytochrome c1 during respiration
	QCR1	Core subunit of the ubiquinol-cytochrome c reductase complex (bc1 complex)
	CYT1	Cytochrome c1, component of the mitochondrial respiratory chain
	QCR8	Subunit 8 of ubiquinol cytochrome-c reductase complex
	QCR10	Subunit of the ubiqunol-cytochrome c oxidoreductase complex, involved in aerobic respiration

(cont. on next page)

Table 3.1. (cont) Non-essential ETC genes that were investigated in this study

Complex IV	***MBA1***	Protein involved in assembly of mitochondrial respiratory complexes
	COX8	Subunit VIII of cytochrome c oxidase, which is the terminal member of the electron transport chain
	COX15	Protein required for the hydroxylation of heme O to form heme A, which is an essential prosthetic group for cytochrome c oxidase
	COX6	Subunit VI of cytochrome c oxidase, which is the terminal member of the mitochondrial inner membrane electron transport chain
	COX12	Subunit VIb of cytochrome c oxidase is required for assembly of fully active cytochrome c oxidase
	PET100	Chaperone that specifically facilitates the assembly of cytochrome c oxidase
	PET117	Protein required for assembly of cytochrome c oxidase
	COX5B	Subunit Vb of cytochrome c oxidase is predominantly expressed during anaerobic growth
	COX11	Mitochondrial inner membrane protein required for delivery of copper to the Cox1p subunit of cytochrome c oxidase
	PET161	Required for cytochrome c oxidase activity, respiration deliver copper to cytochrome c oxidase
	SCO2	A redundant function with Sco1p in delivery of copper to cytochrome c oxidase
	COX10	Required for cytochrome c oxidase activity; human ortholog is associated with mitochondrial disorders
	SHY1	Required for normal respiration, possible chaperone involved in assembly of cytochrome c oxidase
	PET191	Protein required for assembly of cytochrome c oxidase
	CYC1	Cytochrome c, isoform 1; electron carrier of the mitochondrial intermembrane space
	COX14	Mitochondrial membrane protein, required for assembly of cytochrome c oxidase
	COX17	Copper metallochaperone that shuttles copper from the cytosol to the mitochondrial intermembrane space
	COX5A	Subunit Va of cytochrome c oxidase
	CYC7	Cytochrome c isoform 2 ; transfers electrons from ubiquinone-cytochrome c oxidoreductase to cytochrome c oxidase during cellular respiration
	COX16	Required for assembly of cytochrome c oxidase
	COX18	Required for export of the Cox2p C terminus from the mitochondrial matrix to the intermembrane space during its assembly into cytochrome c oxidase;
	COX20	Required for proteolytic processing of Cox2p and its assembly into cytochrome c oxidase
	COX9	Subunit VIIa of cytochrome c oxidase
	COX7	Subunit VII of cytochrome c oxidase
	CYB2	Cytochrome b2 (L-lactate cytochrome-c oxidoreductase), required for lactate utilization
	COX19	Protein required for cytochrome c oxidase assembly that delivers copper to cytochrome c oxidase

(cont. on next page)

Table 3.1. (cont) Non-essential ETC genes that were investigated in this study

ATP Synthase	***ATP5***	Subunit 5 of the stator stalk of mitochondrial F1F0 ATP synthase, required for ATP synthesis
	COQ6	Putative flavin-dependent monooxygenase, involved in ubiquinone (Coenzyme Q) biosynthesis
	COQ1	Catalyzes the first step in ubiquinone (coenzyme Q) biosynthesis
	COQ5	Involved in ubiquinone (Coenzyme Q) biosynthesis
	ATP1	Alpha subunit of the F1 sector of mitochondrial F1F0 ATP synthase, required for ATP synthesis
	ATP4	Subunit b of the stator stalk of mitochondrial F1F0 ATP synthase, required for ATP synthesis
	ATP1	Subunit of the mitochondrial F1F0 ATP synthase, required for ATP synthesis
	ATP11	Molecular chaperone, required for the assembly of alpha and beta subunits into the F1 sector of mitochondrial F1F0 ATP synthase
	ATP15	Epsilon subunit of the F1 sector of mitochondrial F1F0 ATP synthase
	ATP12	Molecular chaperone, required for the assembly of alpha and beta subunits into the F1 sector of mitochondrial F1F0 ATP synthase
	ATP13	Mitochondrial protein, likely involved in translation of the mitochondrial OLI1 mRNA
	ATP14	Subunit h of the F0 sector of mitochondrial F1F0 ATP synthase, required for ATP synthesis
	COQ2	Catalyzes the second step in ubiquinone biosynthesis
	FMC1	Required for assembly or stability at high temperature of the F1 sector of mitochondrial F1F0 ATP synthase
	INH1	Protein that inhibits ATP hydrolysis by the F1F0-ATP synthase
	COQ3	Catalyzes two different O-methylation steps in ubiquinone (Coenzyme Q) biosynthesis
	ATP17	Subunit f of the F0 sector of mitochondrial F1F0 ATP synthase, required for ATP synthesis
	ATP10	Mitochondrial inner membrane protein required for assembly of the F0 sector of mitochondrial F1F0 ATP synthase
	COQ4	Protein with a role in ubiquinone (Coenzyme Q) biosynthesis
	ATP2	Beta subunit of the F1 sector of mitochondrial F1F0 ATP synthase, required for ATP synthesis
	ATP7	Subunit d of the stator stalk of mitochondrial F1F0 ATP synthase, required for ATP synthesis
	CIR2	Strong similarity to human electron transfer flavoprotein-ubiquinone oxidoreductase
	CAT5	Involved in ubiquinone biosynthesis, essential for respiration and gluconeogenic gene activation
	TFP1	Vacuolar ATPase V1 domain subunit A containing the catalytic nucleotide binding sites
	ATP20	Subunit g of the mitochondrial F1F0 ATP synthase, required for ATP synthesis
	STF1	Protein involved in regulation of the mitochondrial F1F0-ATP synthase
	STF2	Protein involved in regulation of the mitochondrial F1F0-ATP synthase

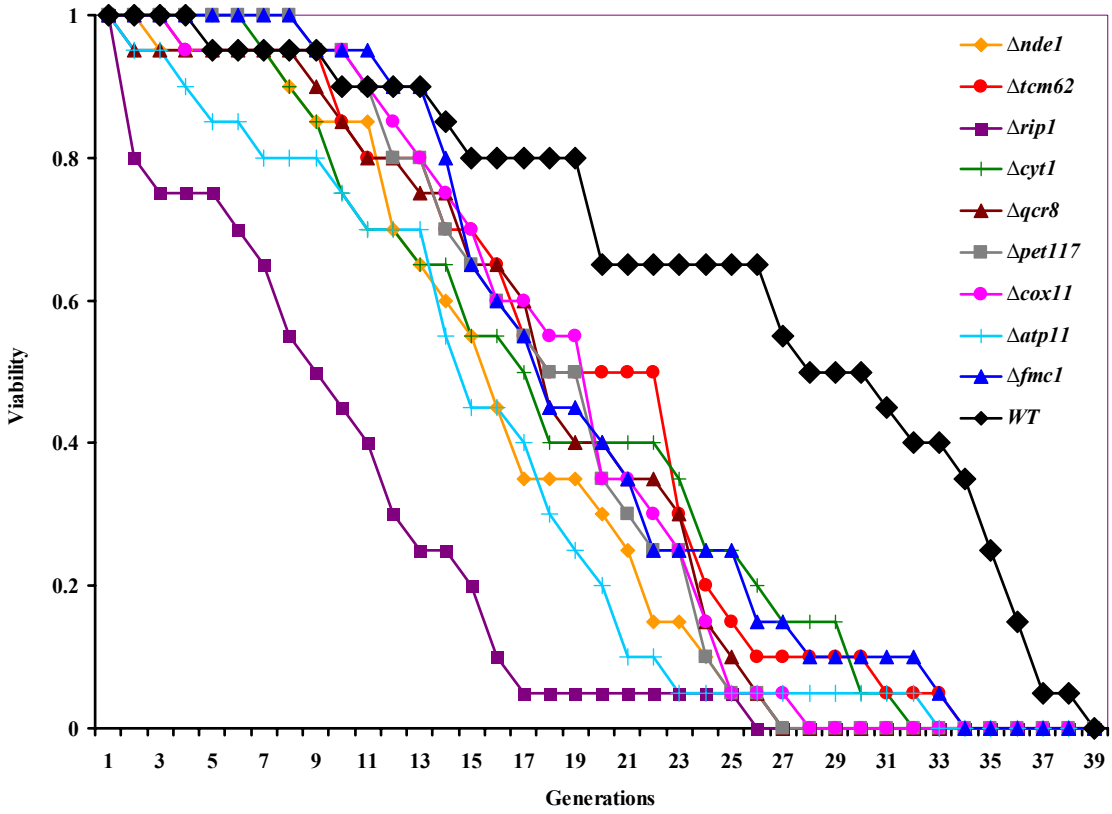

Figure 3.1. Replicative life span analysis of WT and mutant cells which grown in 2% glucose media (YPD).

Table 3.2. Percent reduction of lifespan of the mutant cells compared to the WT

Mutants	Average Lifespan	% reduction in replicative lifespan
*nde1*Δ	16.3	39.6
*tcm62*Δ	19.4	28.1
*rip1*Δ	12.0	55.4
*cyt1*Δ	18.4	31.8
*qcr8*Δ	17.9	33.5
*pet117*Δ	17.5	34.7
*cox11*Δ	18.5	31.2
*atp11*Δ	15.0	44.0
*fmc1*Δ	19.7	26.5
WT	26.9	

3.2. Oxidative Stress Tolerance of Short Living ETC Mutants

In order to test whether these short living mutants were sensitive to oxidants, we treated them with diamide and hydrogen peroxide (H_2O_2). Exogenous treatment of hydrogen peroxide and diamide determines possible superoxide sites in ETC. These oxidants accept electrons without blocking electron transport resulting with increase in steady-state concentration of superoxide and cytotoxicity levels. Determining mutations that cause sensitivity to these oxidants gives the possible electron leakage sites. In this study we wanted to determine diamide and hydrogen peroxide sensitive mutants which could be the reason of shorter lifespan. To compare the oxidant sensitivity of the short living ETC mutants, dose-responses to diamide were generated by spotting cells onto YPD with 2mM and 2.5mM diamide. The result of this study showed that *pet117*Δ exhibited strong sensitivity both concentrations of diamide. Other mutants which were *cox11*Δ, *atp11*Δ, and *fmc1*Δ exhibited sensitivity only to 2,5mM diamide. However, *nde1*Δ, *tcm62*Δ, *rip1*Δ, *cyt1*Δ, and *qcr8*Δ tolerated both concentrations (Figure 3.2).

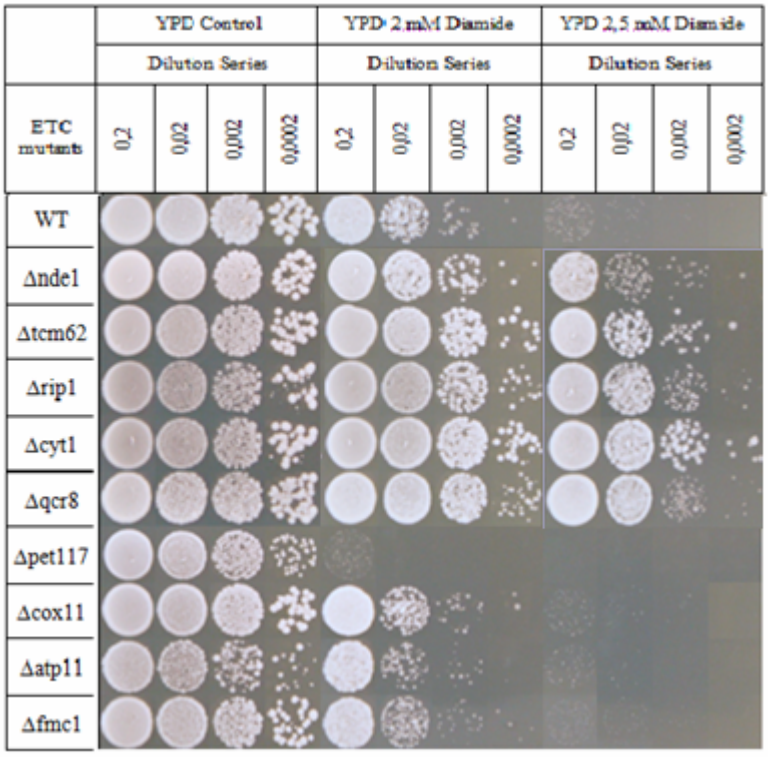

Figure 3.2. Diamide resistance of short living ETC mutants

In order to see the effect of exogenous H_2O_2 treatment, mutants treated with 8.8M hydrogen peroxide which determines which mutations related to aging. The more sensitivity amount showed more ROS accumulation and premature aging. In this study sensitivity amount was measured by Halo assay (measuring diameter of the zone) (Figure 3.3). According to Halo assay results sensitivity to H_2O_2 changed from 8% and 37% compared to the wild-type cells (Table 3.3).

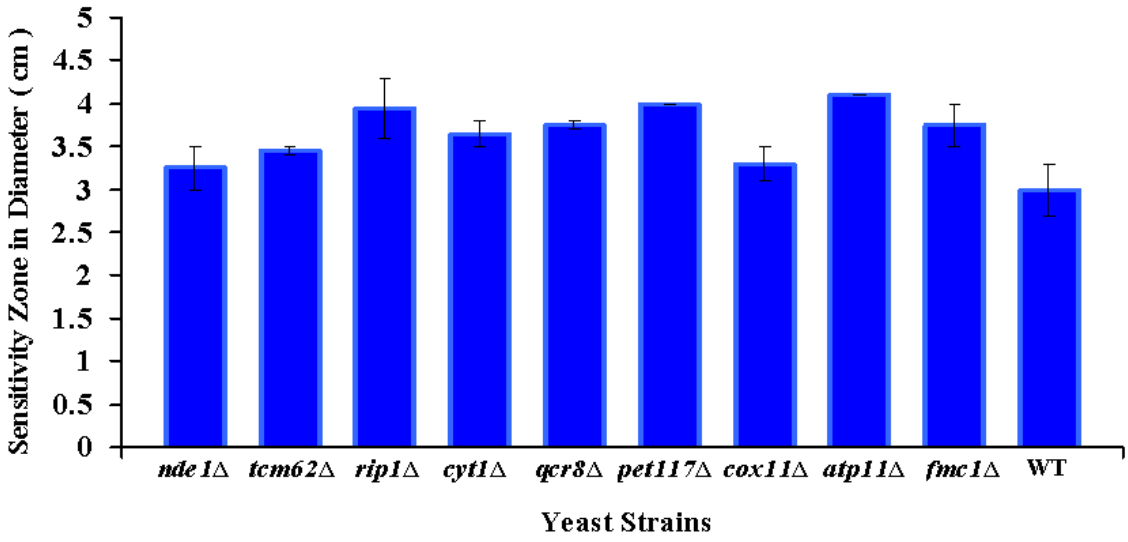

Figure 3.3. Hydrogen peroxide resistance of short living ETC mutants.

Table 3.3. Hydrogen Peroxide Sensitivity in %

Mutants	Average	% of sesitivity
*nde1*Δ	3.3	8.3
*tcm62*Δ	3.5	15.0
*rip1*Δ	3.9	31.7
*cyt1*Δ	3.6	21.7
*qcr8*Δ	3.7	25.0
*pet117*Δ	4.0	33.3
*cox11*Δ	3.3	10.0
*atp11*Δ	4.1	36.7
*fmc1*Δ	3.7	25.0
WT	3.0	

3.3. Determination of Respiratory-Deficient Strains in Short Living ETC Mutants

In YPD media yeast cells do not use their mitochondria actively because of the tendency to producing ethanol via fermentation. When YPG used as a carbon source yeast cells can use their mitochondria actively. Besides determining which mutants use their mitochondria actively for respiration and which mutations essential when glycerol used as a carbon source were the third part of our study. In order to determine respiratory-deficient strains, mutant strains were grown on YPG media (3% glycerol) and spotting assay were performed with serial dilutions. According to spotting assay results, four mutants could grow on glycerol media which means that they could use their mitochondria actively and these mutations were not essential for growing in YPG media. These mutants were *nde1*Δ, *tcm62*Δ, *qcr8*Δ, and *fmc1*Δ. Other mutants which could not be grown on YPG showed that short lifespan of these mutants may be that reason (Figure 3.4).

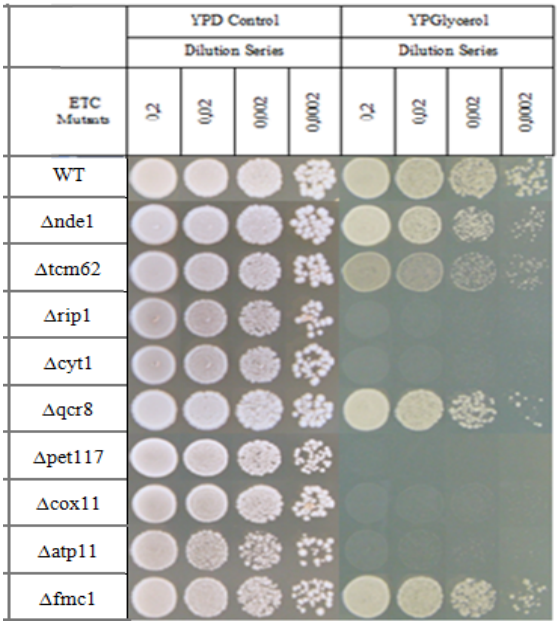

Figure 3.4. Determination of the respiratory deficient mutants by spotting assay.

3.4. Measurement of Intracellular Superoxide Levels

The relative levels of cellular ROS in mitochondria were determined following exposure to fluorescent probe MitoSOX Red. Superoxide specific MitoSOX react with it and show which mutation cause elevated level of superoxide and facilitates the indication that which mutation in ETC cause shorter lifespan. In this part of the study we expected to see that respiratory deficient strains produced low level of superoxide and results confirmed our analysis. In *pet117*Δ, *rip1*Δ, *cox11*Δ, *atp11*Δ and *cyt1*Δ strains, superoxide production was relatively low compared to wild-type cells, as we expected. Analysis of cells revealed that *nde1* and *fmc1* mutations were resulted in elevated levels of mitochondrial superoxide compared to wild-type (with MitoSOX red) strain (Figure3.5). These results showed that *nde1, fmc1* and *qcr8* mutations could related to aging due to detected superoxide.

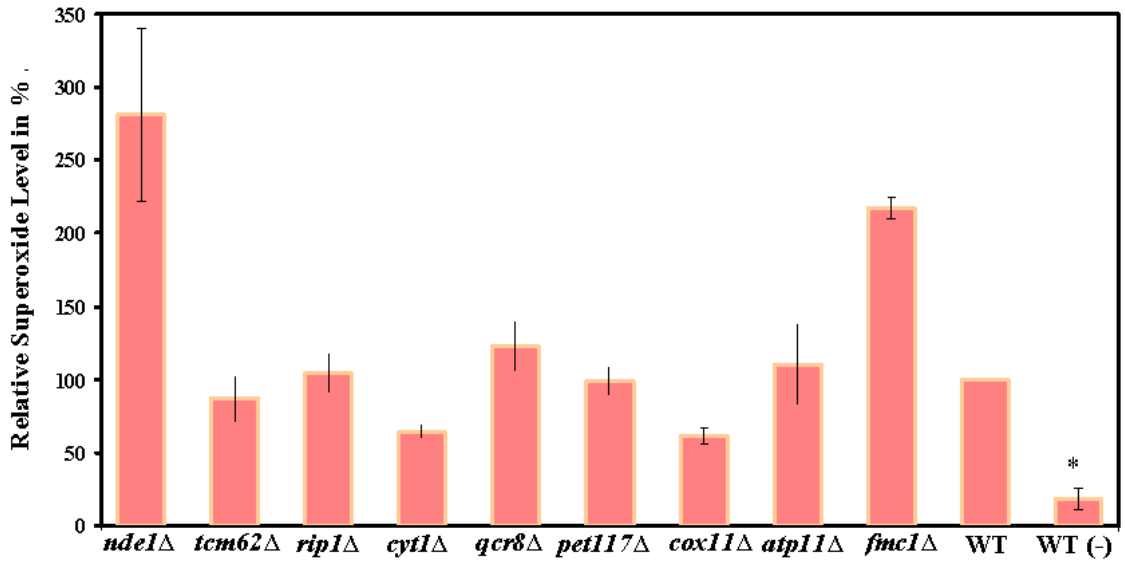

Figure 3.5. Flourometric analysis of ROS levels in mutants. Cells were incubated with MitoSOX Red to assess the levels of mitochondrial superoxide levels.

3.5. Aging Analyses of Cells Over-expressing ETC Genes that are Important for Aging

In this part we wanted to confirm that if deletion of ETC genes resulted in shorter lifespan, overexpression of these ETC genes should be resulted in longer lifespan. Thus, electron transport chain genes that we thought to be important for aging

were overexpressed by Gateway cloning system. In order to confirm overexpression either cause longer lifespan or not aging assay performed again. According to aging assay results, aging profile of these strains exhibited longer lifespan as we expected except *RIP1* and *PET117*. Cells overexpressing *COX11, CYT1* and *QCR8* exhibited a longer lifespan as 55%, 51% and 48% (Table 3.4) compared to the wild-type including empty vector (pAG423) (Figure 3.6). Cells overexpressing *NDE1* exhibit slightly increase in lifespan which were previously proposed by Lin (Lin et al. 2004).

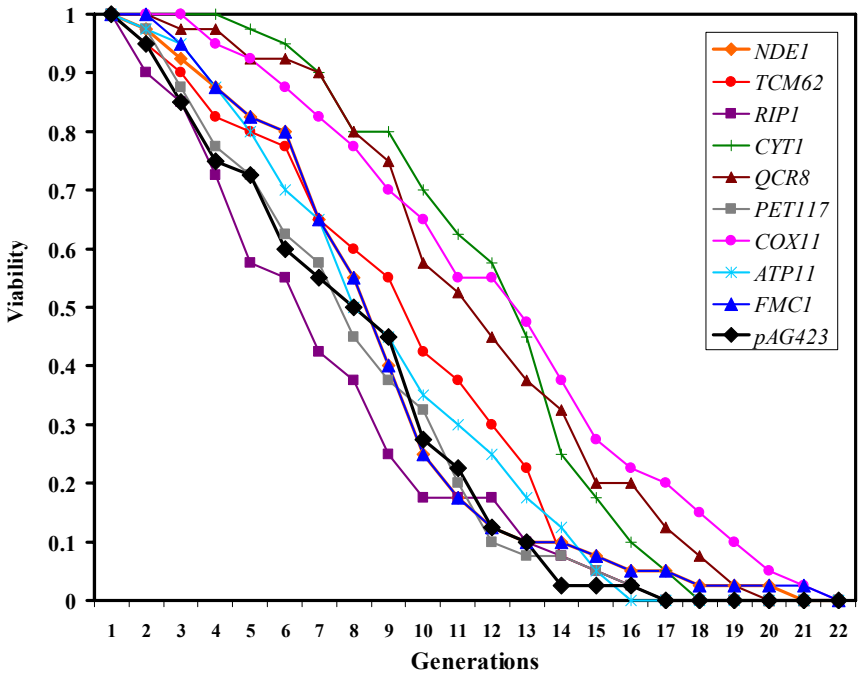

Figure 3.6. Repicative life-span analysis of wild-type strain that over-expressing *FMC1, COX11, TCM62, CYT1, QCR8, ATP11, NDE1, PET117* and *RIP1* grown in 2% glucose containing YNB-His selective media. Wild-type strain carrying control vector (pAG423).

Table 3.4. Lifespan increase of the ETC over-expressed genes compared to the WT (pAG423 only)

ETC over-expressed genes	Average Lifespan	% increase compared with WT (pAG423 only)
FMC1-ove	9.4	15.9
COX11-ove	12.6	55.0
TCM62-ove	9.2	12.5
CYT1-ove	12.3	51.0
QCR8-ove	12.1	48.3
ATP11-ove	9.1	11.9
NDE1-ove	9.3	13.7
PET117-ove	8.2	0.6
RIP1-ove	7.4	-9.1
WT (pAG423 only)	8.1	

In order to confirm the idea that high copy plasmids cause lifespan reduction and compensation of that problem with low copy plasmids, ETC genes were overexpressed by using pAG413 vector which is a low copy plasmid. According to aging assay results overexpression of *COX11, NDE1* and *QCR8* cause lifespan extension compared to the WT including sham vector (Figure 3.7) approximately 22%, 9% and 7% respectively (Table 3.5). In addition average lifespan of strains extended unlike in strains which include high copy plasmid (Table 3.5). Overexpression of other 5 genes with pAG413 expression vector did not cause lifespan extension (Table 3.5). Overexpression ATP11 was not implemented.

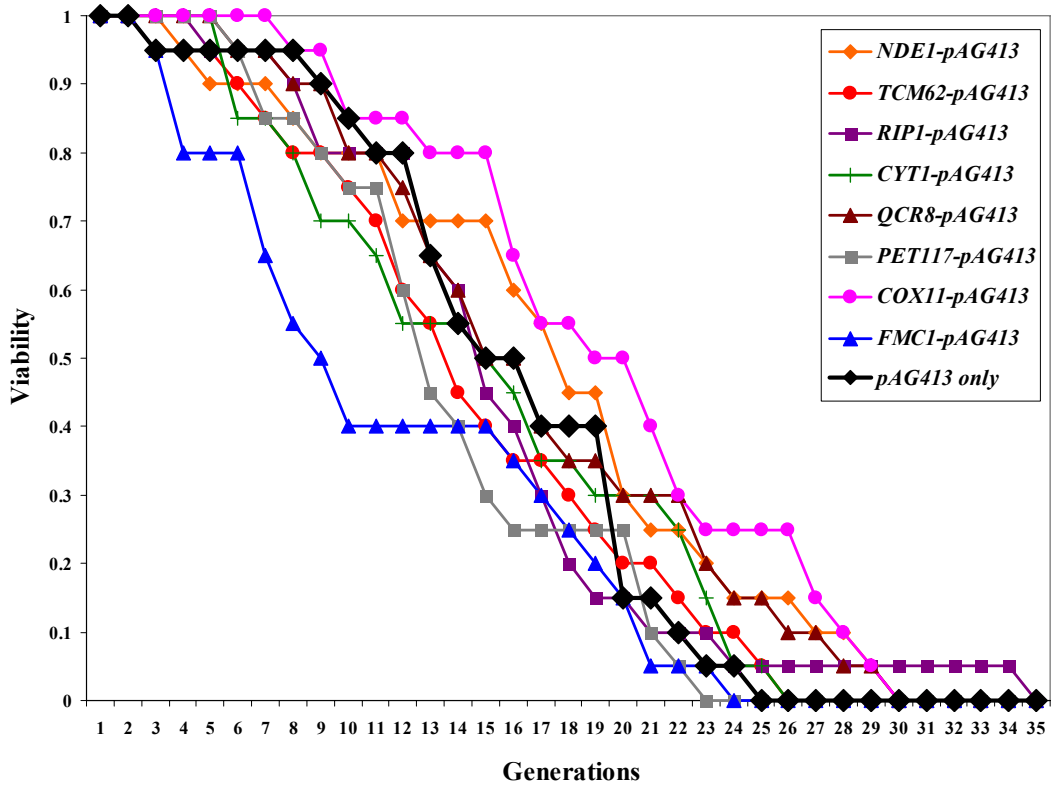

Figure 3.7. Repicative life-span analysis of wild-type strain that over-expressing *FMC1, COX11, TCM62, CYT1, QCR8, NDE1, PET117* and *RIP1* grown in 2% glucose containing YNB-His selective media. Wild-type strain carrying control vector (pAG413).

Table 3.5. Lifespan increase of the ETC over-expressed genes compared to the WT (pAG413 only).

ETC-ove genes	Average Lifespan	% increase compared with WT
***NDE1*-pAG413**	17.45	9.404388715
***TCM62*-pAG413**	14.7	-7.836990596
***RIP1*-pAG413**	15.7	-1.567398119
***CYT1*-pAG413**	15.25	-4.388714734
***QCR8*-pAG413**	17.1	7.210031348
***PET117*-pAG413**	14.1	-11.59874608
***COX11*-pAG413**	19.6	22.88401254
***FMC1*-pAG413**	11.85	-25.70532915
pAG423 only	15.95	

CHAPTER 4

DISCUSSION

The current model for reactive oxygen species production by electron transport chain explains Complex I, II, III are the possible sites. However, which mutations induce ROS production and aging is the question mark. Although there are several studies about this issue, there is no valuable evidence. We have characterized electron transport chain mutations in order to learn more about which mutations are important for aging process.

In order to do this, we analyzed all nonessential ETC mutations and found out that nine of them were related to lifespan reduction. These were shown in Table 3.1 marked with red. According to aging assay results we proposed that besides Complex I, II and III; Complex IV and V could be responsible for aging process.

The second step of our study was identification of hydrogen peroxide and diamide sensitivity in ETC mutants, in order to test whether they were sensitive to oxidative stress or not. Thus, sensitivity phenotypes allowed indication about age-related sites in ETC. Although we tested short living ETC mutants against a range of oxygen radical-generating compounds, we only observed sensitivity in *pet117*Δ, *cox11*Δ, *atp11*Δ and *fmc1*Δ which were displayed hypersensitivity phenotype when treated with diamide. *rip1*Δ, *cyt1*Δ, *pet117*Δ, *atp11*Δ and *fmc1*Δ mutants exhibited hypersensitivity to hydrogen peroxide treatment. These sensitivities to diamide and hydrogen peroxide appear to be linked to the respiratory deficient characteristics of *rip1*Δ, *cyt1*Δ, *pet117*Δ, and *atp11*Δ mutants (no growth on YPG) which can not protect themselves against oxidative stress. Aging profile of those mutants including *cox11*Δ showed lifespan reduction on YPD media. After glucose was consumed, cells could not respire and lifespan was reduced could be the one possible explanation. Relative levels of superoxide in these mutants were much lower than the wild-type. Low levels of mitochondrial superoxide production in these mutants supported the idea that mutants which could not use respiratory chain efficiently and produce low level of superoxide.

Our results therefore suggest that hypersensitivity to exogenous oxidative stress, elevated levels of superoxide production in mitochondria may cause reduction in lifespan profile. These indications were not enough to say that these mutations cause shorter lifespan. In order to prove that the mutations of genes that cause shorter lifespan should be overexpressed to see the lifespan extension. Our results showed that overexpression of these genes to the wild-type cells with high copy plasmid exhibited increase in replicative lifespan compared to the wild-type strain carrying sham vector as we expected. Unexpected result of this study although deletion of these genes caused respiratory deficient phenotype; *COX11*, *CYT1* and *ATP11* genes were increased the lifespan 55%, 51% and 12%, respectively. Instead of completely deleting these genes, site directed mutations to specific residues can be the solution. Because, they are seem to be the important in aging according to their increased lifespan due to overexpression results. However; another respiratory deficient strains *PET117* and *RIP1* and their overexpression did not caused lifespan extension, this result showed that these two genes did not related to aging process. Previous report showed that the *NDE1* overexpression largely promote the replicative lifespan extension in 2% glucose (Lin et al. 2004). Consistent with previous report about *NDE1* overexpression, it increased replicative lifespan approximately 13% (Table 3.4). Another study about the *NDE1* showed that *nde1* mutation resulted in reduction of chronological lifespan (Li et al. 2006). These two indications exhibit contradictions each other. However, they study two different aging mechanisms and their interpretations about that chronological aging and replicative aging mechanisms different from each other. Overexpression of other genes *FMC1* (encode ATP synthase subunit) and *TCM62* (encode Complex II subunit) resulted with extension in lifespan which is unique for *FMC1* and complex V because; there is any information about its relation to aging. Another gene *QCR8* which encodes Complex III subunit 8 exhibited 48% lifespan extension as a result of overexpression and confirming the idea that Complex III is the site of ROS production. Surprisingly, average lifespan of all strains which include overexpressed genes were very short including wild-type strain with empty vector. Previous studies about this issue proposed that high copy plasmids are the reason for reduction in lifespan. High copy plasmids inside the cell may behave like extra-chromosomal rDNA circles which are responsible for premature aging due to loss of asymmetry. Autonomously replicating sequence elements with or without rDNA locus reduce replicative lifespan (Steinkraus, Kaeberlein, and Kennedy 2008). Because of this reason, we transformed ETC genes to

low copy plasmid. The results showed that low copy plasmids did not cause reduction in the average lifespan that overcome the premature aging problem. This result exhibited consistency about the inducing effect of high copy plasmids in lifespan reduction.

CHAPTER 5

CONCLUSION

Electron transport chain seems to be the largest production site of reactive oxygen species. This feature makes it very attractive to study oxidative stress and aging relation. Our study aimed to find which mutations in ETC related to aging process. For this reason we first screened all nonessential ETC mutants and found out nine of them exhibited shorter lifespan from 26% to 55%. After finding that, deletion of those genes concluded with short lifespan, we treated them with diamide and hydrogen peroxide in order to determine is there any relation between reduction of lifespan and oxidant hypersensitivity phenotypes of mutants. Consistent with previous studies about oxidative stress and free radical theory of aging, we found out that reactive oxygen species oriented hypersensitivity of mutants might be the reason for reduced lifespan. However, these strains were respiratory deficient and they could not use their mitochondrial respiratory chain efficiently. Thus, weak possibility of those genes was responsible for a reduction in lifespan. The measuring relative levels of superoxide produced in mitochondria showed that mutants have hypersensitivity to oxidizing agents, those produced low levels of superoxide. Other mutants that have ability to grown on YPG which were exhibited less diamide and hydrogen peroxide sensitivity increase the possibility of their role in aging process. After combining those data we decided to overexpress the genes that cause reduction in lifespan. If the absence of those genes caused lifespan reduction, then we expected that overexpression of them might increase the lifespan. According to all data, we proposed that deletion of genes may induce the electron leakage not only from complex I, II, III but also from complex IV and V; thus, lifespan may reduce due to reactive oxygen species accumulation. In order to say that which specific residue in subunits of complexes (encoded by those nine genes) causes electron leakage, random or site-directed mutagenesis can be performed. It brings information about ETC-derived genetic disorders, mechanism of the premature aging and age-related degenerative diseases.

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APPENDIX

COMPOSITION of MEDIA and STOCK SOLUTION

1. Media

a. Glucose (YPD) medium, per liter

1% yeast extract, 2% peptone, 2% glucose (Sterilization by autoclaving at 121 °C for 15')

b. Glucose (YPD) agar medium, per liter

1% yeast extract, 2% peptone, 2% glucose, 2% agar (Sterilization by autoclaving at 121 °C for 15')

c. Glycerol (YPG) medium, per liter

1% yeast extract, 2% peptone, 3% glycerol (v/v) (Sterilization by autoclaving at 121 °C for 15')

d. Glycerol (YPG) agar medium, per liter

1% yeast extract, 2% peptone, 3% glycerol(v/v), 2% agar (Sterilization by autoclaving at 121 °C for 15')

e. YNB Media, per liter

6.7 g Yeast Nitrogen Base with ammonium sulfate, 2% Glucose, w/wo 2% agar, CSM (complete synthetic media) without histidine (20ml/L)

2. Solutions

a. 1XPBS (Phosphate Buffered Saline), per liter

8g of NaCl**,** 0.2g of KCl**,** 1.44g of Na_2HPO_4**,** 0.24g of KH_2PO_4 (pH 7.4; sterilization by autoclaving at 121 °C for 15')