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Identification of respiratory chain gene mutations that shorten replicative life span in yeast

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ABSTRACT

Aging is the progressive accumulation of alterations in cells that elevates the risk of death. The mitochondrial theory of aging postulates that free radicals produced by the mitochondrial respiratory system contribute to the aging process. However, the roles of individual electron transfer chain (ETC) components in cellular aging have not been elucidated. In this study, we analyzed the replicative life span of 73 yeast deletion mutants lacking the genes of the mitochondrial electron transfer chain system, and found that nine of these mutants (Δ*nde1*, Δ*tcm62*, Δ*rip1*, Δ*cyt1*, Δ*qrc8*, Δ*pet117*, Δ*cox11*, Δ*atp11*, Δ*fmc1*) had significantly shorter life spans. These mutants had lower rates of respiration and were slightly sensitive to exogenous administration of hydrogen peroxide. However, only two of them, Δ*nde1* and Δ*fmc1*, produced higher amounts of intrinsic superoxide radicals in the presence of glucose compared to that of wild type cells. Interestingly, there were no significant alterations in the mitochondrial membrane potentials of these mutants. We speculate that the shorter life spans of ETC mutants result from multiple mechanisms including the low respiration rate and low energy production rather than just a ROS-dependent path.

1. Introduction

Mitochondria are of great importance as being the site of energy production of eukaryotic cells (Schneider and Guarente, 1991). Oxidative phosphorylation that takes place in the mitochondria is crucial for the energy metabolism and implicated to be related with the aging process (Speakman, 2005; Yang and Hekimi, 2010). The yeast *Saccharomyces cerevisiae* has the ability to grow by fermentation in the presence of glucose or by mitochondrial respiration in the presence of respiratory substrates such as glycerol. However, it primarily prefers fermentation and can inhibit aerobic respiration in the presence of glucose (Skinner and Lin, 2010). Therefore, since *S. cerevisiae* can grow either by fermentation or respiration, it is a suitable model to study the effects of the loss of genes needed for respiration, which can be lethal in many other organisms.

The mitochondrial electron transport chain (ETC), couples electrons from oxidized metabolic fuels to ATP production (Dimroth et al., 2000). The ETC is composed of five enzyme complexes (complexes I–V) (Dibrov et al., 1998), of which are known to be highly regulated in yeast (Schneider and Guarente, 1991). The assembly of the complexes requires several proteins including chaperones that assist their formation. The electrons are transfered from NADH and succinate to oxygen by respiratory complexes I to IV and the electrochemical gradients of protons formed via this transfer are used by the complex V (ATP synthase) to produce ATP (Hamel et al., 1998). Yeasts do not have a multisubunit complex I, instead they have two NADH-dehydrogenases and one NADH-ubiquinone oxidoreductase (de Vries and Grivell, 1988; Melo et al., 2004). Reactive oxygen species (ROS) are produced mainly by the ETC in the inner mitochondrial membrane, especially from redox sites of complexes I and III (Boveris, 1977; Turrens, 2003; Drose and Brandt, 2008; Murphy, 2009; Brand, 2010; Cortes-Rojo et al., 2011).

Mitochondrial dysfunction is shown to be related with several pathological processes (Janssen et al., 2004; Seppet et al., 2009; Perry et al., 2011). The roles of the ROS and mitochondria in aging have been studied in yeast and other eukaryotes, and results vary depending on the model organisms studied. There is a continous increase of ROS formation and an age-dependent decline in mitochondrial function throughout the aging process (Harman, 1956; Shigenaga et al., 1994; Beckman and Ames, 1998; Laun et al., 2001; Navarro and Boveris, 2007; Lam et al., 2011). Calorie restriction seems to extend replicative life span (RLS) by decreasing the production of ROS and increasing the rate of the mitochondrial respiration (Barros et al., 2004). Dinitrophenol, an uncoupling agent, mimics the effect of calorie restriction on ROS production and life span modulation. On the other hand, antimycin A, an inhibitor of complex III,

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increases ROS production and shortens the chronological life span (CLS) in yeast (Barros et al., 2004). In *C. elegans*, impairment of mitochondrial functions or treatment with antimycin A increases the life span (Lee et al., 2003; Anson and Hansford, 2004; Ventura et al., 2005). Mutation in the iron sulfur protein Isp-1, which is a component of complex III, results in lower respiratory rate and longer life span (Feng et al., 2001). In *Drosophila*, complex IV inhibitors such as KCN and sodium azide increase the production of hydrogen peroxide and decrease cytochrome c oxidase activity. Flies with deficiency in complex II (*sdhB*) suffer from elevated oxidative stress and age faster (Walker et al., 2006). Additionally, inactivation of five genes encoding components of respiratory complexes I, III, IV, and V by RNAi can prolong life span (Copeland et al., 2009). In human cell line 143B, administration of ETC inhibitors results in increased ROS production (Indo et al., 2007). Thus, the roles of the elecron transport chain components or chemicals that modulate electron transfer show contradictory results on life spans and ROS levels.

In this study, we analyzed the RLS of ETC gene mutants to elucidate the roles of these genes in the aging process of the budding yeast *S. cerevisiae*. Out of 73 yeast deletion mutants of non-essential ETC genes, deletion of *NDE1*, *TCM62*, *RIP1*, *CYT1*, *QCR8*, *PET117*, *COX11*, *ATP11*, and *FMC1* were found to be important in replicative life span determination. Short living ETC gene mutants had lower rates of respiration and showed different levels of sensitivity to exogenous administration of hydrogen peroxide. Moreover, they did not have significant alterations in their mitochondrial membrane potentials. Thus, our results suggest that short living ETC mutants neither experience severe oxidative stress nor have significant differences in means of their membrane potentials compared to wild type cells.

2. Materials and methods

2.1. Yeast strains and growth

WT strain BY4741 (*MATa his3 leu2 met15 ura3*) and its isogenic deletion mutants were obtained from the yeast deletion library (Invitrogen). Cells were grown on YPD agar (1% yeast extract, 2% peptone, 2% dextrose and 2% agar) media, if otherwise not stated.

2.2. Life span analyses

WT strain BY4741 (*MATa his3 leu2 met15 ura3*) and its isogenic deletion mutants were obtained from the yeast deletion library (Invitrogen). Cells were grown on YPD agar (1% yeast extract, 2% peptone, 2% dextrose and 2% agar) media for 2 days before analysis. For each strain, 25 daughter cells (starter mothers) were collected and lined up by a micromanipulator on agar plates. New buds (daughters) from these virgin cells were removed and discarded as they formed. This process continued until cells ceased dividing. The life span was determined as the total number of daughter cells that each mother cell generated. For crude reduction of sample numbers, we analyzed the life span of five cells per strain for the initial screening and identified the mutants with possible short life spans. In the second and third rounds of aging analyses, 25 cells for each strain were followed. For further analyses of the respiratory mutants, 0.1% glucose containing YPD media was used. For the chronological life span analysis, yeast strains were grown in 2 ml YPD media overnight and were suspended in 25 ml fresh YPD in 250 ml flasks and incubated at 30 °C for 15 days at 180 rpm. The survival rate of cells was measured by counting the colony forming units every 72 h. Each analysis was performed three times.

2.3. Hydrogen peroxide sensitivity

A halo assay was performed to assess the sensitivity of the cells to hydrogen peroxide. Briefly, cells were grown in liquid YPD overnight and their OD_{600} values were adjusted to 0.2. 400 μl aliquot from each culture was transferred to YPD plates and dried for 30 min. Then, 5 μl 8.8 M hydrogen peroxide was administered to the center of the plates and the plates were incubated at 30 °C overnight. Radius of the clear zone in the center of each plate was measured by a ruler. The assay was performed three times in duplicates for each strain.

2.4. Determination of respiratory deficient strains

Yeast strains with shorter replicative lifespans were grown in YPG (3% glycerol). Spotting assay was performed with cells with adjusted OD_{600} values of 0.2. Serial dilution was performed to obtain OD_{600} values of 0.02, 0.002, and 0.0002. 5 μl of cell solutions was dropped onto YPG-agar plates and incubated at 30 °C for 48 h.

2.5. Determination of intracellular superoxide levels

The intracellular superoxide production rates of the yeast strains were measured with the fluorometric dye MitoSOX™ red (Invitrogen) as described by the manufacturer. Briefly, yeast cells were grown in either 2% glucose or 0.1% glucose containing YPD up to a OD_{600} value of 0.5. Cells were pelleted and resuspended in YPD containing 5 μM of MitoSOX red dye. After 1 h of incubation at 30 °C, cells were washed with PBS twice and analyzed by a fluorometric spectrometer (Thermo VarioScan).

2.6. Measurement of oxygen consumption

The cells were grown in liquid YPD overnight and OD_{600} values were adjusted to 0.3. They were incubated at 30 °C until reaching OD_{600} of 0.8. The cells were washed with dH_2O, suspended in glycerol media (YPG) and incubated at 30 °C for 30 min. As a control of electron transport chain involvement in oxygen consumption, the cell cultures were treated with 5.8 mg/l antimycin A (Sigma-Aldrich) for 45 min before measurements. The dissolved oxygen values were measured using a HI9146 oximeter with an HI7640714 probe (Hanna Instruments) and readings were recorded every minute for a period of 15 min.

2.7. 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. OD_{600} 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, cells were analyzed with a fluorescent spectrometer (Thermo VarioScan).

2.8. Statistical analysis

The JMP® statistical software (SAS Institute Inc.) was used for the statistical analysis. Dunnet's test was performed for the statistical analysis. For the life span analysis, a nonparametric Median test was performed. 95% significance confidence level was used for the analyses.

3. Results

3.1. Replicative life span analyses of ETC mutants

The electron transport chain is the major site for ROS production due to electron leakage that occurs during electron flow through the ETC. We reasoned that the disruption of the electron transfer chain by genetic manipulations may increase ROS production, decrease

respiration and modulate life span. Starting from this point, mutants for all the non-essential ETC genes were analyzed for their relative RLS compared to the wild type. Genes whose products play role in ETC were identified by MitoP2 database (Andreoli et al., 2004) and are listed in Table S1. Yeast has about 90 genes related to the ETC function and 8 of them are encoded by mtDNA and few of them are essential for life. We used 73 deletion mutants (Table S1), produced by the yeast deletion project, which did not include the mutants for mtDNA encoded genes and the essential ones.

For the initial life span analyses, we first analyzed five cells for each of 73 ETC mutants to reduce the number of samples. After the initial screening, mutants with altered life spans were analyzed again using a higher number of cell counts. At the end, we found that the deletion of *NDE1*, *TCM62*, *RIP1*, *CYT1*, *QCR8*, *PET117*, *COX11*, *ATP11*, and *FMC1* genes reduced RLS at significant rates (Fig. 1).

We were able to identify only one deletion mutant with reduced RLS for complex I and complex II (Fig. 1). Deletion of *NDE1*, external NADH dehydrogenase, decreased the RLS by 40%. On the other hand, deletion of *TCM62* which encodes for a chaperonin required for the assembly of succinate dehydrogenase complex (complex II) resulted in 28% decrease in the RLS.

The cytochrome bc1 complex (complex III) is represented by three short living mutants, Δ*rip1*, Δ*cyt1* and Δ*qcr8* in our analyses (Fig. 1). Rip1 and Cyt1 are the catalytic subunits of complex III, and their deletion leads to 55% and 32% decrease in the RLS, respectively. QCR8 encodes for a subunit for the complex III and its absence caused a 35% decrease in the RLS.

Absence of two of the complex IV genes resulted in shorter RLS (Fig. 1). Cells lacking *PET117*, which is required for the assembly of cytochrome c oxidase, lived 35% shorter than wild type cells. *COX11* encodes a protein that is essential for the assembly of the cytochrome c oxidase, which catalyzes the last step of the electron transport chain. Deletion of *COX11* gene leads to a 31% decrease in the RLS.

Related to complex V, the deletion of *ATP11*, which encodes a protein required for the assembly of F1 sector of mitochondrial F1F0 ATP synthase resulted in 44% decrease in the RLS. Absence of the *FMC1* gene which encodes a protein required for assembly and high temperature stability of the F1 sector of F1F0 ATP synthase resulted in 27% decrease in the RLS (Fig. 1).

3.2. ROS production and tolerance levels of short living ETC mutants

In order to understand the mechanisms of shorter life span of the mutants, we challenged them with hydrogen peroxide to see whether their oxidative stress tolerance mechanisms are impaired or overloaded. The mutants showed only a small increase in their sensitivity to hydrogen peroxide (Fig. 2A). We also analyzed superoxide production rates to see whether there is a correlation between superoxide levels and aging phenotype of the mutants. Interestingly, only two

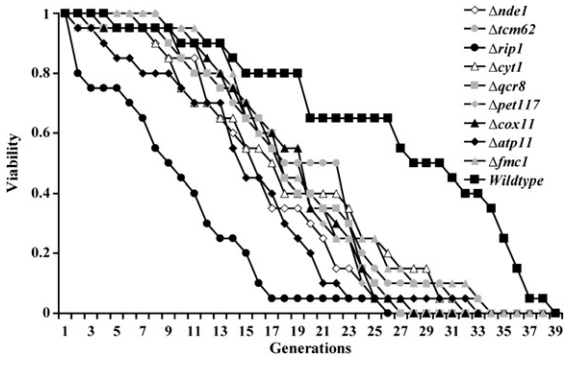

Fig. 1. Replicative aging pattern of ETC mutants with short life span. The assay was performed for three times and a representative one is shown in the figure.

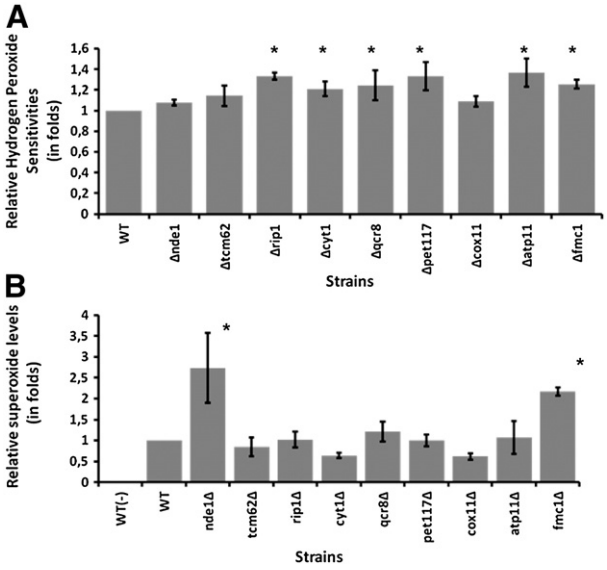

Fig. 2. Oxidative stress status of the mutants. A) Relative hydrogen peroxide sensitivity levels. A halo assay was performed to measure the sensitivity level of the mutants. B) The intracellular superoxide levels of mutants in the presence of 2% glucose. Cells were stained with MitoSOX dye and analyzed by a fluorescent spectrometer. Experiments were repeated at least 3 times. Bars represent mean and SEM values.

of the mutants, Δ*nde1* and Δ*fmc1*, harbored elevated levels of superoxide radicals (2.8- and 2.2- fold, respectively) compared to that of wild type cells (Fig. 2B).

In order to make sure that our biochemical comparisons include cells that are in the same stage of the growth, we analyzed the growth curves of the mutants. As seen in Fig. S1, we observed that all the mutants showed similar growth dynamics to that of wild type cells. Overnight cultures switched into the stationary phase after 16 h and into the diauxic shift after 32 h of incubation. Thus, our comparisons contain cells only coming from the log phase of the growth.

3.3. Respiration status of short living mutants

Cells with defective mitochondria cannot utilize respiratory substrates such as ethanol, glycerol or lactic acid. We tested whether ETC mutants with short life spans are defective in respiration by growing them on a glycerol containing media. Among the mutants, Δ*rip1*, Δ*cyt1*, Δ*pet117*, Δ*cox11*, and Δ*atp11* cells were not able to utilize glycerol as the carbon source to derive energy, but the others were respiration competent (Fig. 3A).

We also checked the cells for their ability to consume oxygen from the growth media. Cells with defective ETC are expected to have lower rates of oxygen intake. As expected, all of the mutants showed a decrease in their capacity to use oxygen (Fig. 3B). Complex III inhibitor antimycin A blocked oxygen usage of all the mutants (data not shown). The complex III mutants, Δ*rip1* and Δ*cyt1*, completely lost their ability to uptake oxygen. Thus, the short living mutants had lower rates of oxygen uptake compared to that of wild type cells.

We analyzed the RLS of mutants on growth media that contain 2% glucose as the carbon source. It has been shown that lower amount of glucose in growth media increases respiratory rate and extends life span (Jiang et al., 2000; Lin et al., 2002). Since several of the mutants that showed decreased RLS are respiratory competent, we tested whether induction of respiration by growing them in the presence of lower amount of glucose (0.1%) would alter the life span of these cells (Δ*nde1*, Δ*tcm62*, Δ*qcr8* and Δ*fmc1*). As seen in Fig. S2.A, reducing the amount of glucose to 0.1% shortened the RLS of all the cells. When compared to wild type cells, Δ*nde1*, Δ*qcr8*, Δ*tcm62* and Δ*fmc1*

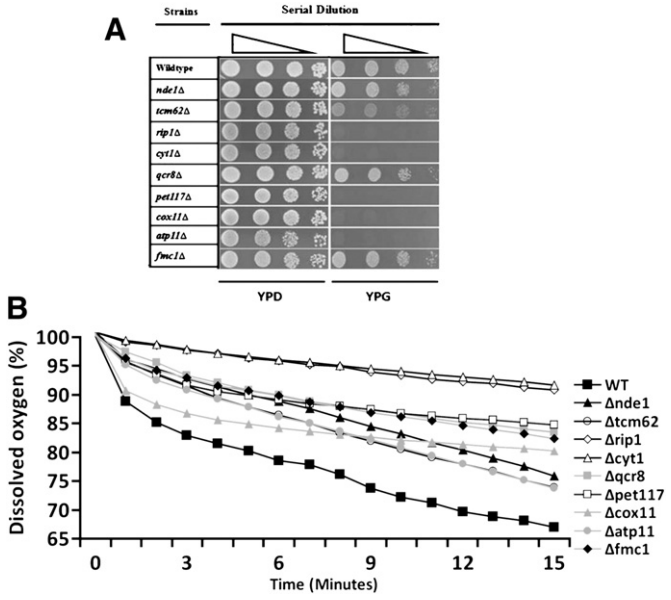

Fig. 3. Mutants are deficient in respiration. A) Glycerol utilization of the mutants. The figure shows the growth pattern of strains both in glucose rich YPD media and YPGlycerol (YPG) media. Serial dilution was performed to obtain cell densities 0.2, 0.02, 0.002, 0.0002 of OD600 values. B) Dissolved oxygen measurements of the strains in respiratory media. The experiments were performed for three times.

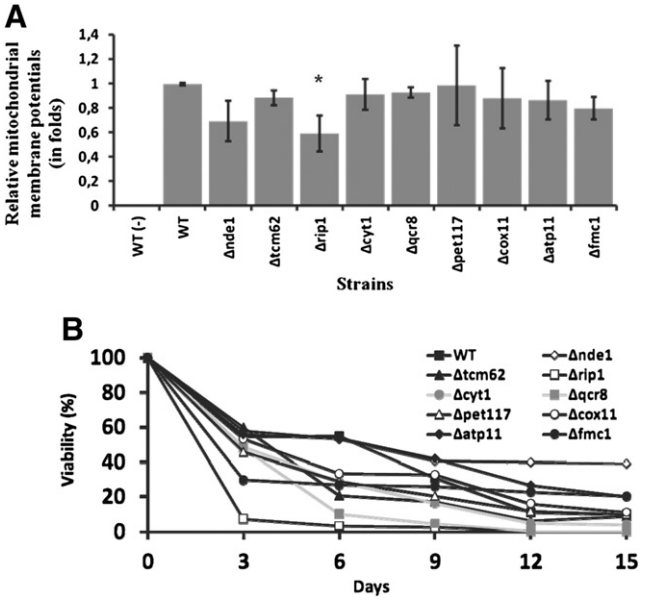

Fig. 4. Mitochondrial membrane potential and chronological life span of the cells. A) Relative ratios of mitochondrial membrane potentials determined by JC-1 assay. The experiments were performed for three times and bars represent mean and SEM values. B) Chronological life span analyses of the cells. The assays were performed for three times and the viability rates are represented in percentages.

mutants showed a 27%, 21%, 16% and 11% reduction in their RLS, respectively.

Surprisingly, superoxide profiles of these mutants were different in 0.1% glucose media when compared to those of 2% glucose, only Δ*qrc8* mutants showed statistically significant ($p = 0.003$) elevation in 0.1% glucose (Fig. S2.B).

3.4. Mitochondrial membrane potential and chronological lifespan analyses of mutants

As well as deficiencies in oxygen usage, the impaired function of electron transport chain is also expected to lead to a decrease in mitochondrial membrane potential. In order to analyze the mitochondrial membrane potentials of the mutant strains, cells were stained with JC-1 dye. Most of the mutants showed a wild type level of membrane potentials, except for Δ*rip1* cells whose membrane potential was decreased by 41% ($p = 0.0452$) (Fig. 4A).

Replicative and chronological life spans are not linked to each other and genetic manipulations may result in different outcomes for both life span analyses. Therefore, we wondered if the ETC mutations that lead to reduction in RLS would also lead to reduction in CLS. Cells lacking the *NDE1* gene were able to survive better than wild type cells in the stationary phase. On the other hand, cells missing the *RIP1*, *CYT1*, *QCR8*, *TCM62* genes lost their viability faster than wild type cells ($p = 0.028$). The rest of the mutants showed more or less similar viability patterns to that of wild type cells (Fig. 4B).

4. Discussion

Mitochondria are the energy source of eukaryotic cells in which ATP production takes place for the function of the cellular metabolism (Greaves et al., 2010). Superoxide, which is the precursor of several ROS, is mainly produced from electron leakage from the mitochondrial electron transport chain (Murphy, 2009).

In this study, nine electron transport chain mutations, Δ*nde1*, Δ*tcm62*, Δ*rip1*, Δ*cyt1*, Δ*qrc8*, Δ*pet117*, Δ*cox11*, Δ*atp11*, Δ*fmc1* were found to lead to replicative life span reduction in the yeast *S. cerevisiae*. All of these mutants showed reduced amounts of oxygen intake. Most of them showed normal levels of mitochondrial membrane potentials and increase in their hydrogen peroxide sensitivity, and only two mutants (Δ*nde1* and Δ*fmc1*) contained higher amounts of superoxide radicals.

Deletion of the *NDE1* gene leads to a significant reduction in RLS on glucose rich media, however, absence of this gene increased the CLS of the cells. Previously, the roles of the *NDE1* gene in CLS and ROS production have been studied. Cells lacking *NDE1* gene lived longer chronologically, produced less ROS and had a normal mitochondrial membrane potential (Li et al., 2006). Our results partially agree with the previous knowledge; we observed that Δ*nde1* cells stay viable for a longer period of time in the stationary phase, have a normal level of mitochondrial membrane potential, yet, produced higher amount of ROS. The difference between distinct ROS production rates of Δ*nde1*cells in our study could be attributed to the method of detection. Li et al. (2006) assayed ROS levels by rhodamine 123 staining in SD medium, however we used MitoSOX dye, which is more specific to superoxide radicals, for cells growing in rich media.

Our results suggest that the complex II gene *TCM62* is also an important determinant of yeast RLS. There is no previous data about the involvement of this gene in RLS of yeast. Cells lacking *TCM62* did not show any significant phenotypes regarding the ROS production rate and mitochondrial membrane potential, but their respiration rate was lower compared to that of wild type cells.

In a previous study, Rip1 deficiency was shown to result in the functional loss of electron transport chain complex III (Zara et al., 2009). Here, we found that the absence of the *RIP1* gene completely blocked oxygen consumption, and growth in glycerol which confirms the previous results. Moreover, Δ*rip1* cells had the shortest RLS among the all ETC mutants tested. The Δ*cyt1* mutants showed 32% reduction in their RLS when compared to wild type cells. Previously, Kaeberlein et al. showed that the deletion of the CYT1 gene results in 36% reduction in RLS, which supports our findings (Kaeberlein et al., 2005). Similar to Δ*rip1*cells, Δ*cyt1* mutants were also deficient in oxygen consumption and glycerol utilization, while Δ*qcr8* cells were proficient in both. These mutants showed more hydrogen peroxide sensitivity ($p(\Delta rip1) = 0.0001$, $p(\Delta cyt1) = 0.0082$ and $p(\Delta qcr8) = 0.0021$).

Absence of complex IV genes, *PET117* and *COX11*, also resulted in shorter life span. Respiratory deficiency and hydrogen peroxide sensitivity of Δ*cox11* cells have been noticed earlier (Carr et al., 2002; Banting and Glerum, 2006). On the other hand, two of the complex V genes, *ATP11* and *FMC1*, were also required for normal RLS. These mutant cells did not show any significant phenotypes of ROS production or disrupted membrane potentials compared to that of wild type cells. Similar to other mutants, they showed decreased rates of respiration and hydrogen peroxide resistance.

Cells lacking the individual *RIP1*, *CYT1*, *QCR8*, *TCM62* genes lived shorter chronologically, however we did not investigate the mechanisms of short CLS in these cells. Recently, it has been shown that production of acetic acid and subsequent acidification of media is toxic during the course of the CLS assay (Burtner et al., 2009). Thus, further studies are necessary to elucidate these mechanisms.

Overall, our results suggest that several genes of ETC play important roles in RLS determination. The basis of short life span in these mutants is not clear currently. There might be specific reasons for each mutant such as the higher rate of superoxide production in Δ*nde1* and Δ*fmc1* cells, or more general reasons such as lower rate of respiration. The link between respiration rate and the RLS has been previously established (Lin et al., 2002; Barros et al., 2004; Koc et al., 2004). We speculate that lower rates of respiration in these cells might be one of the leading causes of their short RLS. However, it is likely that multiple mechanisms regulate the replicative life spans of these mutants, since the mutant cells exhibit different phenotypes under tested conditions. On the other hand, the data does not exclude the possibility that the short RLS of ETC mutants may be an indirect effect of mutation. Thus, further studies need to be performed to assess the roles of these genes in the modulation of the RLS.

Supplementary materials related to this article can be found online at doi:10.1016/j.exger.2011.11.009.

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