



Caloric Restriction-Induced Extension of Chronological Lifespan Requires Intact Respiration in Budding Yeast

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Caloric restriction (CR) has been shown to extend lifespan and prevent cellular senescence in various species ranging from yeast to humans. Many effects of CR may contribute to extend lifespan. Specifically, CR prevents oxidative damage from reactive oxygen species (ROS) by enhancing mitochondrial function. In this study, we characterized 33 single electron transport chain (ETC) gene-deletion strains to identify CR-induced chronological lifespan (CLS) extension mechanisms. Interestingly, defects in 17 of these 33 ETC gene-deleted strains showed loss of both respiratory function and CR-induced CLS extension. On the contrary, the other 16 respiration-capable mutants showed increased CLS upon CR along with increased mitochondrial membrane potential (MMP) and intracellular adenosine triphosphate (ATP) levels, with decreased mitochondrial superoxide generation. We measured the same parameters in the 17 non-respiratory mutants upon CR. CR simultaneously increased MMP and mitochondrial superoxide generation without altering intracellular ATP levels. In conclusion, respiration is essential for CLS extension by CR and is important for balancing MMP, ROS, and ATP levels.

Keywords: caloric restriction, chronological lifespan, electron transport chain, mitochondria, respiration

INTRODUCTION

Caloric restriction (CR) extends lifespan in various species. So far, suggested mechanisms point to mitochondria as a major effector of CR action. Mitochondria play central roles in cellular metabolism by generating adenosine triphosphate (ATP) and reactive oxygen species (ROS) as energy and toxic byproducts, respectively. The mitochondrial electron transport chain (ETC) is responsible for producing 90% of cellular ATP (Pagliarini et al., 2005). The ETC produces ATP using proton-motive force through oxidative phosphorylation (OXPHOS) (Newmeyer and Ferguson-Miller, 2003). ROS are also generated during this process because of electron leakage from respiratory complexes during electron transfer (Turrens, 2003). These ROS are deleterious and can directly damage mitochondria. These damaged mitochondria can then release even more ROS (Kurihara et al., 2012; Scialo et al., 2013). In fact, mitochondrial dysfunction is responsible for various age-related diseases (Martin-Montalvo and de Cabo, 2013).

Although improvements in mitochondrial function upon CR have been identified, the detailed mechanism by which this occurs is still controversial. CR increases mitochondrial biogenesis by increasing levels of mitochondrial DNA and expression of mitochondrial mRNA and protein (Nisoli et al., 2005). Under CR, mitochondrial superoxide and oxidative damage are reduced by preventing proton leakage from the

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ETC, thereby maintaining cellular ATP levels (Choi and Lee, 2013; Martin-Montalvo and de Cabo, 2013). CR does not change mitochondrial abundance but it does maintain mitochondrial function to prevent cellular senescence by increasing antioxidant activity and reducing oxidative damage to DNA and protein (Lanza et al., 2012).

CR extends chronological and replicative lifespan in *Saccharomyces (S) cerevisiae* (budding yeast) and increases respiration (Barros et al., 2004; Lin et al., 2002). CR prevents replicative senescence in human mesenchymal stem cells and increases lifespan in *Caenorhabditis elegans*, while increasing respiration in both systems (Lo et al., 2011; Schulz et al., 2007). From our previous study, fourteen out of 33 ETC genes were identified as essential for *S. cerevisiae* lifespan (Kwon et al., 2015). We also found that lifespan is dependent on mitochondrial ATP production efficiency and reduced ROS using single ETC component deletion mutants in yeast (Choi and Lee, 2013; Kwon et al., 2015).

Mitochondrial respiration provides cellular energy when budding yeast is grown in a non-fermentable carbon source. Cellular respiration is influenced by the various environmental factors such as nutrient levels, aeration, and temperature. CR increases respiration in yeast and mice (Lin et al., 2002; Nisoli et al., 2005; Oliveira et al., 2008). Inhibiting respiration by deleting the *cyt1* (cytochrome c1) gene or treating cells with antimycin A (a cytochrome bc₁ complex inhibitor) does not extend chronological lifespan (CLS) (Ocampo et al., 2012). On the other hand, partially inhibiting respiration (approximately 40%) by deleting the *cox5a* gene or treating cells with oligomycin (a mitochondrial ATP-synthase inhibitor) extends lifespan in the W303 yeast strain (Ocampo et al., 2012). These studies suggest that mitochondrial respiration plays an important role in CR-induced CLS extension. Previously, we identified that gene expression of OXPHOS members had a positive correlation with CR strength in a microarray analysis (Lee and Lee, 2008). In this study, we further evaluated the role of individual genes in the mitochondrial ETC system under CR during the chronological aging process.

MATERIALS AND METHODS

Yeast strains and culture conditions

Isogenic single gene deletion strains were obtained from the BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0, ura3Δ0*) genetic background of *S. cerevisiae*. The 33 mitochondrial ETC gene deletion strains (*nde1Δ, nde2Δ, ndi1Δ, sdh1Δ, sdh2Δ, sdh4Δ, cor1Δ, cyt1Δ, qcr2Δ, qcr6Δ, qcr7Δ, qcr8Δ, qcr9Δ, qcr10Δ, rip1Δ, cox5aΔ, cox5bΔ, cox6Δ, cox7Δ, cox8Δ, cox9Δ, cox12Δ, cox13Δ, atp1Δ, atp4Δ, atp5Δ, atp7Δ, atp15Δ, atp17Δ, atp18Δ, atp19Δ, atp20Δ* and *tim11Δ*) from the Open Biosystems Yeast library were genotyped using PCR-based methods with previously described primer sequences (Kwon et al., 2015).

Yeast strains were streaked onto YPD agar plates (2% Bacto agar, 1% Bacto yeast extract, 2% Bacto peptone, and 2% Difco dextrose (BD Diagnostics, USA)). Plates were incubated at 30°C until single colonies appeared. Isolated single colonies were inoculated into 10 ml of YPD medium containing 1% Bacto yeast extract, 2% Bacto peptone, and 2%

Difco dextrose (BD Bioscience, USA), followed by overnight culture. This overnight seed culture was inoculated into 10 ml of fresh 2% YPD medium and cultured for 10 min. Subsequently, the final seed culture was inoculated into 20 ml of 2% or 0.5% glucose-containing YPD medium for non-restricted (NR) and caloric restriction (CR) conditions, respectively. The cultures were adjusted to an initial OD₆₀₀ = 0.05. All yeast cultures were incubated in a 30°C orbital shaking incubator at 200 rpm.

Measurement of CLS using propidium iodide (PI) staining and flow cytometry analysis

Assessment of CLS using PI was performed as previously described (Choi et al., 2013; 2015; Kwon et al., 2015). Cells were harvested by centrifugation, resuspended with 1 ml phosphate-buffered saline (PBS) for washing, and incubated for 20 min at 30°C after adding 5 μl of PI solution (1 mg/ml, Sigma Aldrich, USA). Stained cells were analyzed with flow cytometry (FACS Verse; Becton Dickinson, USA). Excitation was performed using a blue laser at 488 nm and emission was detected at 585 nm in the FL2 channel. A total of 20,000 cells were analyzed for each sample, and data was analyzed using Cell Quest software (Becton Dickinson).

Measurement of mitochondrial membrane potential (MMP) and mitochondrial superoxide levels by flow cytometry

MMP and mitochondrial superoxide levels were measured by flow cytometry using 3,3-dihexyloxycarbocyanine iodide (DiOC₆) (Invitrogen, USA) and MitoSOX Red (Invitrogen), respectively. To measure MMP and mitochondrial superoxide, 2 × 10⁶ cells were harvested by centrifugation and washed in 10 mM HEPES buffer containing 5% glucose (pH 7.4) and PBS buffer (pH 7.4), respectively. Washed cells were incubated in 175 nM DiOC₆ or 5 μM MitoSOX Red for 20 min at 30°C. Before analysis, MitoSOX Red-stained cells were washed in PBS. Stained cells were measured by a BD FACSVerse flow cytometer (Becton Dickinson) using a 488-nm blue laser for excitation, and a 527-nm (MMP) or 585-nm (mitochondrial superoxide) filter for emission detection. Data were analyzed using BD FACSuite.

Measurement of ATP level

Extraction of ATP from yeast cells was performed as previously described (Kwon et al., 2015). Yeast culture samples were taken and cells were washed three times with distilled water. Washed cells were frozen immediately using liquid N₂. Frozen samples were stored at -70°C until use. ATP was measured by following the manufacturer's protocol (ATP colorimetric/Fluorometric kit, Biovision, USA), using 535 nm for excitation and 587 nm for emission in a multilabel plate reader (Victor3, Perkin Elmer, USA).

RESULTS AND DISCUSSION

CR successfully extends CLS in respiratory mutants but not in non-respiratory mutants

We exposed 17 respiratory mutant strains (*nde1Δ, nde2Δ, ndi1Δ, sdh1Δ, sdh2Δ, sdh4Δ, qcr6Δ, qcr9Δ, qcr10Δ, cox5aΔ, cox5bΔ, cox8Δ, cox13Δ, atp18Δ, atp19Δ, atp20Δ*

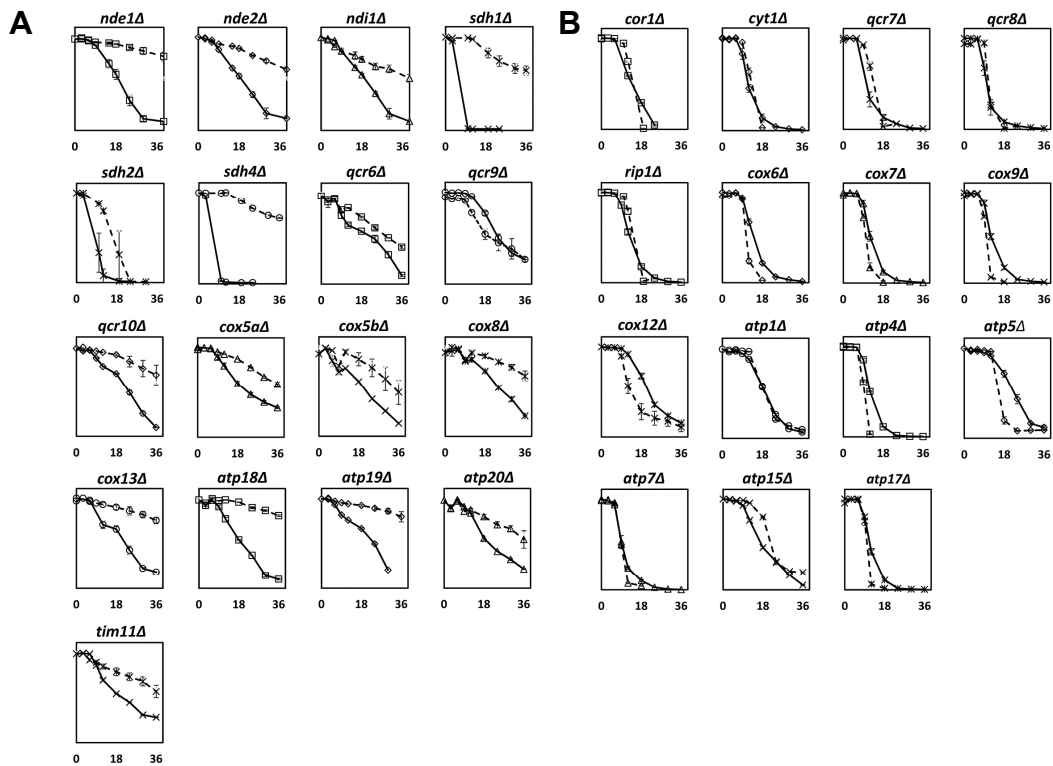


Fig. 1. Chronological lifespan (CLS) of single-gene deletion mutants for electron transport chain (ETC) components under non-restricted (NR: 2% glucose, solid line) and caloric restriction (CR: 0.5% glucose, dashed line) conditions. (A) Respiratory strains. (B) Non-respiratory strains. Error bars indicate standard deviation.

and *tim11Δ*) and 16 non-respiratory mutant strains (*cor1Δ*, *cyt1Δ*, *qcr2Δ*, *qcr7Δ*, *qcr8Δ*, *rip1Δ*, *cox6Δ*, *cox7Δ*, *cox9Δ*, *cox12Δ*, *atp1Δ*, *atp4Δ*, *atp5Δ*, *atp7Δ*, *atp15Δ* and *atp17Δ*) to CR conditions. CR clearly extended CLS in respiratory mutant strains except *qcr9Δ* (Fig. 1A), and CR failed to extend CLS of all non-respiratory mutants (Fig. 1B). Although the *cox5aΔ* strain showed a respiration rate reduced to 4% of that of the wild type rate (Kwon et al., 2015), its CLS was extended by CR. Results of the present study correspond well with the results of the *rho⁰* (mitochondrial DNA deficient mutant) and the *cox5aΔ* strain (partially respired mutant) from previous studies (Ocampo et al., 2012). Altogether, respiration is required for CR-induced CLS extension.

Among the ETC single gene deleted strains, 14 ETC mutants, *sdh1Δ*, *sdh2Δ*, *sdh4Δ*, *cor1Δ*, *cyt1Δ*, *qcr7Δ*, *qcr8Δ*, *rip1Δ*, *cox6Δ*, *cox7Δ*, *cox9Δ*, *atp4Δ*, *atp7Δ*, and *atp17Δ*, were determined to be short-lived strains during the chronological aging process in our previous report (Kwon et al., 2015). Among them, mutating succinate dehydrogenase showed markedly reduced CLS under NR. Interestingly, CR successfully increased CLS for succinate dehydrogenase mutants (*sdh1Δ*, *sdh2Δ*, and *sdh4Δ*) to the same lifespan as wild type. The results indicate that the succinate dehydrogenase complex may play an essential role under NR, but the enzyme complex may not participate in the CR-induced CLS extension mechanism.

General growth phenotypes of the ETC mutants under NR and CR conditions

Because respiration was required for CR-mediated lifespan extension, we further investigated differences in general growth phenotypes between the respiratory and non-respiratory mutant strains. First, we measured growth rates during the exponential phase and the maximum OD₆₀₀ values of these mutants. Most strains showed doubling times similar to the wild type strain doubling time, except *atp5Δ* and *atp15Δ* strains, and CR meagerly increased doubling times of most mutant strains similar to the doubling time of the wild type strain (Figs. 2A, 2B, and 2C). We also determined maximum cell mass when OD₆₀₀ values reached the stationary phase. Respiratory strains showed two-fold higher maximum OD₆₀₀ values than those of non-respiratory strains (Figs. 2D, 2E, and 2F). CR led to maximum OD₆₀₀ values that were significantly lower by approximately 60% for the respiratory strains and by 75% for the non-respiratory strains than for the NR condition (Figs. 2E and 2F). Maximum cell mass was significantly higher in respiratory mutant strains than in non-respiratory mutant strains in NR conditions, except for respiratory mutant strains *sdh1Δ*, *sdh2Δ*, and *sdh4Δ*. The effect of CR on maximum cell mass was also significantly higher in the respiratory group than in the non-respiratory group.

Exceptionally, CR-induced CLS extension was failed with respiration in the *qcr9Δ* strain. The *qcr9Δ* strain could respire

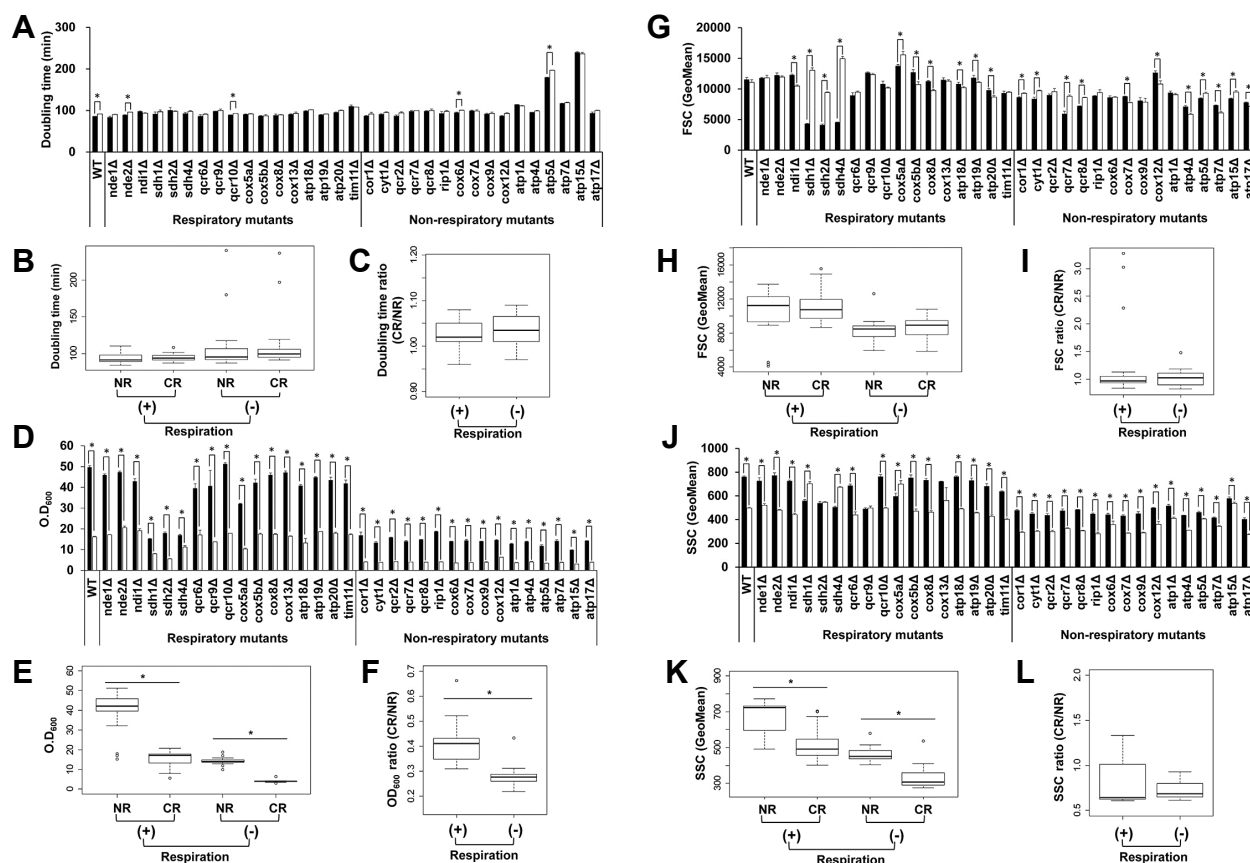


Fig. 2. Growth phenotype alteration in ETC single-component deletion strains upon CR. (A) Cell doubling time of ETC knock-out (KO) strains was calculated in the exponential phase under both NR and CR conditions. (B, C) Distribution of doubling time and ratio of CR/NR is shown in box plots for respiratory strains (+) and non-respiratory strains (-). (D) Maximum cell mass (OD_{600}) of ETC KO strains was determined when the cells reached the stationary phase during the chronological aging process in batch culture under NR and CR. (E, F) Distribution of maximum cell mass and CR/NR ratio is shown in box plots for respiratory strains (+) and non-respiratory strains (-). (G) Forward scatter (FCS) data of flow cytometry at day 12 was used to estimate cell size. (H, I) Distribution of cell size and CR/NR ratio was shown in box plots for respiratory strains (+) and non-respiratory strains (-). (J) Side scatter (FCS) data of flow cytometry at day 12 were used to estimate cell complexity. (K, L) Distribution of cell complexity and CR/NR ratio is shown in box plots for respiratory strains (+) and non-respiratory strains (-). Error bars indicate standard deviation of 3 biological replicates. * $P < 0.05$

weakly and showed a unique growth phenotype including a prolonged period of diauxic shift. When glucose was exhausted in the culture medium, most respiratory strains shifted their metabolism from fermentation to respiration and regrew until they attained the stationary phase. For most respiratory strains, this metabolic shift occurred within 2 h and strains regrew until around day 3 in the NR condition. However, the *qcr9Δ* strain shifted its metabolism phase after 24 h and slowly regrew until around day 12. This discrepancy was also observed in CR conditions. Because *Qcr9p* was essential for making the functional structure of the cytochrome bc1 complex (Phillips et al., 1990), this discrepancy may be caused by the malfunction of the cytochrome bc1 complex.

In addition, we analyzed cell size and cell complexity using forward- and side-scatter (FSC and SSC) data, respectively, using FACS. Cell size was generally unchanged by CR (Figs.

2G, 2H, and 2I), except in the case of *sdh1Δ*, *sdh2Δ*, and *sdh4Δ* strains, where cell size was greatly increased upon CR. Cell complexity was significantly reduced by CR in both respiratory and non-respiratory mutants (Figs. 2J, 2K, and 2L). CR reduced cell complexity in most strains regardless of respiration capability.

CR increases MMP more strongly in non-respiratory strains than in respiratory strains

To understand differences in mitochondrial activity between respiratory and non-respiratory groups, we measured MMP in several growth phases: exponential, post-diauxic, and stationary phases. We did not observe clear MMP differences in the exponential phase between two groups (respiratory vs. non-respiratory) or two conditions (CR vs. NR) (Figs. 3A and 3D), but, we observed clear differences in MMP at the post-diauxic phase between NR and CR conditions in

almost all strains, except *sdh4Δ* (Figs. 3B and 3D). MMP was significantly higher at the post-diauxic phase in CR conditions than MMP in NR conditions. This CR vs. NR difference in MMP was maintained at the stationary phase for most respiratory strains, and further increased for most non-

respiratory strains (Figs. 3C and 3D). Distribution of the MMP ratio between CR and NR highlighted the effect of CR on the MMP increase, which was stronger in non-respiratory strains than in respiratory strains at the stationary phase (Fig. 3E). Accumulated MMP through ETC drives ATP formation by ATP synthase and maintenance of intracellular ATP is essential for cell survival (Kwon et al., 2015). Strangely, although MMP was dramatically increased by CR in non-respiratory mutants, CR failed to extend CLS for these mutants.

ATP levels are increased by CR in respiratory mutant strains but not in non-respiratory mutant strains

In general, MMP results in ATP production in healthy cells. To determine the fate of the increased MMP upon CR at the stationary phase, we measured intracellular ATP levels in all strains under NR and CR conditions. Intracellular ATP levels were increased in most respiratory strains under CR conditions proportionally to the MMP increase in these strains; however, there were no changes in ATP upon CR for most non-respiratory strains with the notably increased MMP for these strains (Figs. 4A, 4B, and 4C). Interestingly, intracellular ATP levels were much lower in most non-respiratory strains than in respiratory strains, regardless of the media conditions (Fig. 4B). In addition, we found dramatic decreases in ATP level for all three strains containing complex II mutants in NR conditions but dramatic increases in ATP were observed upon CR in these strains. Increased MMP by CR resulted in a corresponding increase of ATP levels in respiratory strains, but not in non-respiratory strains.

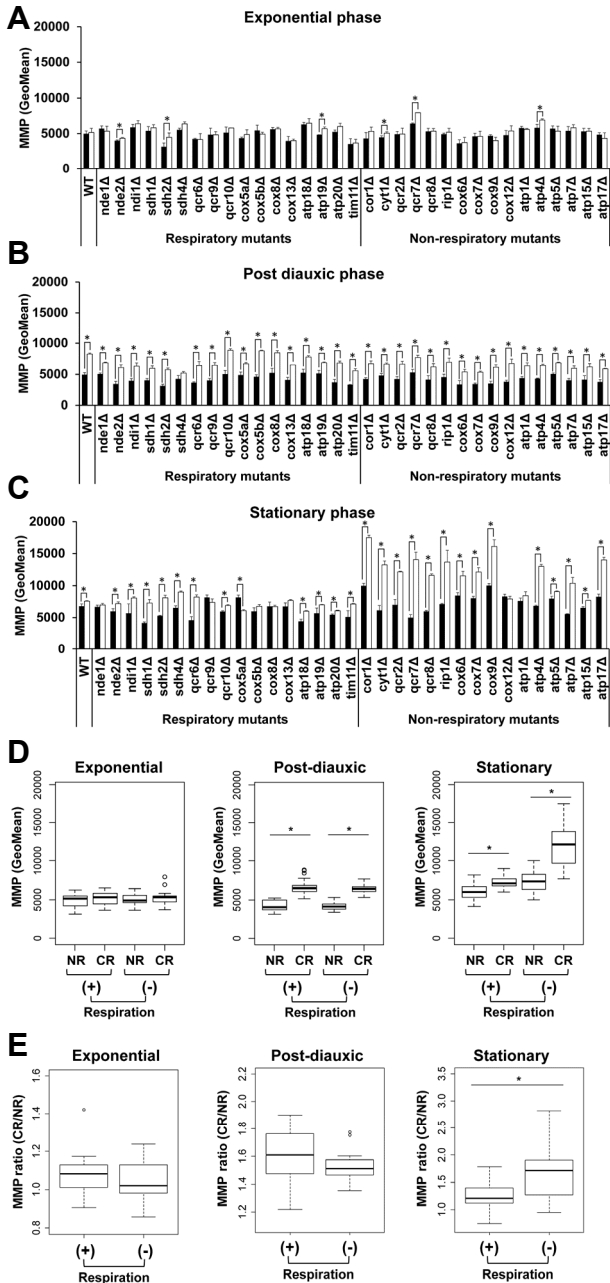


Fig. 3. Alteration of mitochondrial membrane potential (MMP) of ETC single-component deletion strains by CR. (A-C) MMP of ETC KO strains at exponential, post-diauxic, and stationary phases, respectively. (D, E) Distribution and CR/NR ratio of MMP were shown in box plots for respiratory strains (+) and non-respiratory strains (-). Error bars indicate standard deviation of 3 biological replicates. * $P < 0.05$

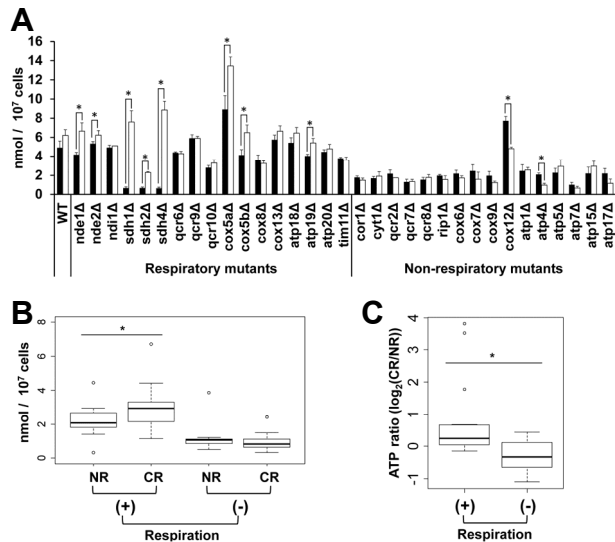


Fig. 4. Alteration of intracellular ATP by CR in ETC single-component deletion strains. (A) Intracellular ATP was measured at the stationary phase in NR and CR conditions. Filled bars and open bars represent NR and CR, respectively. (B, C) Distribution and CR/NR ratio of ATP content are shown in box plots for respiratory strains (+) and non-respiratory strains (-). Error bars indicate standard deviation of 3 biological replicates. * $P < 0.05$

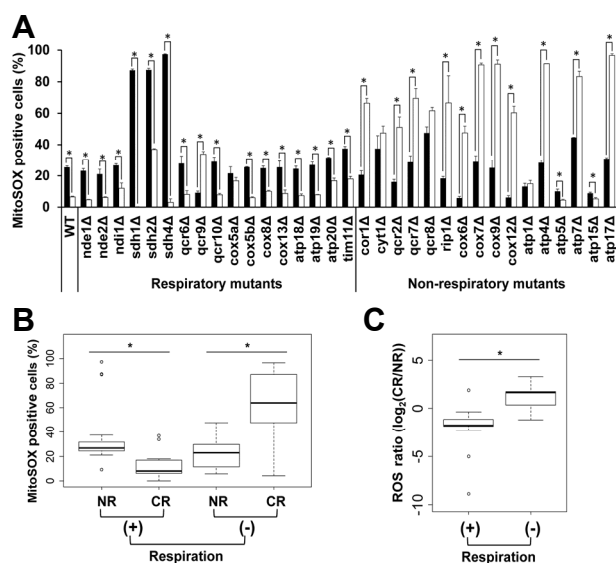


Fig. 5. Alteration of mitochondrial superoxide level by CR in ETC single-component deletion strains. (A) Mitochondrial superoxide levels were measured at the stationary phase (day 12) in NR and CR conditions. Filled bars and open bars represent NR and CR, respectively. (B, C) Distribution and CR/NR ratio of mitochondrial superoxide were shown in box plots for respiratory strains (+) and non-respiratory strains (-). Error bars indicate standard deviation of 3 biological replicates. * $P < 0.05$

Mitochondrial superoxide generation is efficiently suppressed by CR in respiratory strains but increased in non-respiratory strains

Accumulated MMP can also turn into ROS as a byproduct of OXPHOS (Hamanaka and Chandel, 2009). In particular, a strong increase in MMP oftentimes can trigger ROS generation (Korshunov et al., 1997; Pozniakovsky et al., 2005). Taken together, increased MMP in non-respiratory mutants might turn into ROS instead of ATP. Indeed, the protons that accumulated in the mitochondrial intermembrane space upon CR were not utilized to produce ATP in most non-respiratory mutants. Therefore, we measured mitochondrial superoxide generation in these mutants. For the respiratory mutants, mitochondrial superoxide was dramatically reduced by CR in the stationary phase (Figs. 5A, 5B, and 5C). However, most non-respiratory strains showed dramatic increases in mitochondrial superoxide upon CR except *atp14*, *atp5A*, and *atp15A* strains (Fig. 5A). In particular, strains with mutations in complex II produced the greatest mitochondrial superoxide levels under NR conditions; however, CR remarkably suppressed the generation of mitochondrial superoxide in these mutant strains. Taken together, our CLS, ATP, and ROS data recapitulate the importance of the mitochondrial free radical theory of aging, which describes mitochondrial ROS as deleterious molecules that induce cellular senescence and cell death (Harman, 1972). The increased mitochondrial ROS production in non-respiratory mutants might be a cause of a failure to extend CLS by CR.

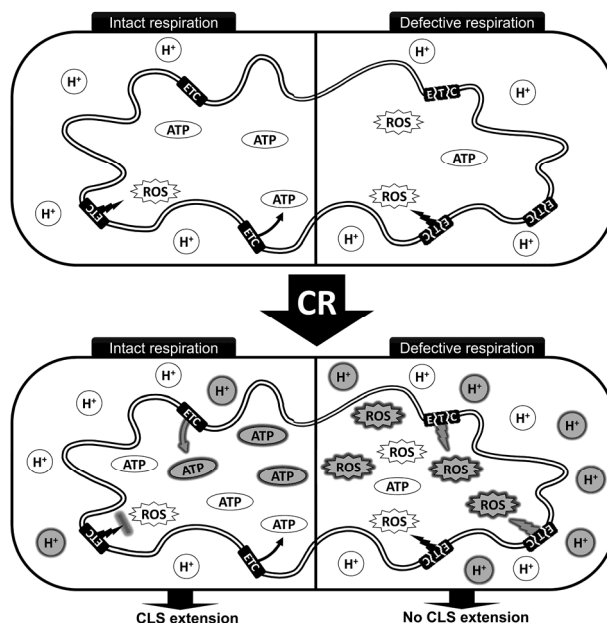


Fig. 6. Mechanism of CR-induced CLS extension when respiration is intact (left) or defective (right). Number of circles represents the level of H^+ , ATP, and ROS under NR and CR conditions, respectively. White marks represent the basal level of H^+ , ATP, and ROS under NR conditions, and gray marks represent additional H^+ , ATP, and ROS by CR.

In conclusion, using yeast strains with single gene deletions in ETC members, CR extended CLS for respiratory mutants but not for non-respiratory mutants. In all mutants examined, CR increased MMP. Interestingly, MMP was significantly higher in non-respiratory strains than in respiratory strains upon CR. Because respiration remained intact, increased MMP was coupled to increased ATP levels in respiratory strains. However, although MMP was greatly increased in non-respiratory strains upon CR, ATP was not increased at all due to compromised respiration. Compromised respiration could not handle high MMP levels, and MMP leakage increased mitochondrial ROS. This uncoupling of MMP and ROS in non-respiratory strains may be responsible for nullifying CR-mediated lifespan extension (Fig. 6). Therefore, MMP utilization by intact respiration pathways could be a key mediator of the effect of CR on lifespan extension. Overall, our results support the hypothesis that mitochondrial respiration might be a key factor for CR-induced lifespan extension. Further studies are required to ascertain how MMP, ATP, and ROS control CR-mediated CLS.

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