J Bioenerg Biomembr (2011) 43:483–491
DOI 10.1007/s10863-011-9377-0

Respiratory and TCA cycle activities affect *S. cerevisiae* lifespan, response to caloric restriction and mtDNA stability

Erich B. Tahara · Kizzy Cezário · Nadja C. Souza-Pinto · Mario H. Barros · Alicia J. Kowaltowski

Received: 18 May 2011 / Accepted: 27 June 2011 / Published online: 21 July 2011

Abstract We studied the importance of respiratory fitness in *S. cerevisiae* lifespan, response to caloric restriction (CR) and mtDNA stability. Mutants harboring mtDNA instability and electron transport defects do not respond to CR, while tricarboxylic acid cycle mutants presented extended lifespans due to CR. Interestingly, mtDNA is unstable in cells lacking dihydrolipoyl dehydrogenase under CR conditions, and cells lacking aconitase under standard conditions (both enzymes are components of the TCA and mitochondrial nucleoid). Altogether, our data indicate that respiratory integrity is required for lifespan extension by CR and that mtDNA stability is regulated by nucleoid proteins in a glucose-sensitive manner.

Keywords Aging · Calorie restriction · Mitochondria · Respiration · Yeast · Krebs cycle

Abbreviations

CR	calorie restriction
CLS	chronological lifespan
ETC	mitochondrial electron transport chain
mtDNA	mitochondrial DNA
TCA	tricarboxilic acid
YPD	yeast extract, peptone and glucose (dextrose) media
YPEG	yeast extract, peptone, ethanol and glycerol media

E. B. Tahara · N. C. Souza-Pinto · A. J. Kowaltowski (✉)
Departamento de Bioquímica, Instituto de Química,
Universidade de São Paulo,
Cidade Universitária, Av. Prof. Lineu Prestes, 748,
São Paulo, SP, Brazil 05508-000
e-mail: alicia@iq.usp.br

K. Cezário · M. H. Barros
Departamento de Microbiologia, Instituto de Ciências
Biomédicas, Universidade de São Paulo,
São Paulo, Brazil

Introduction

Aging is a complex, multifactorial, process in which biological systems undergo progressive changes in their metabolic functions, efficiency and behavior over time, generally associated with a decline in stress responses, fertility and, ultimately, increased age-dependent mortality (Kenyon 2001; Jazwinski 2002a, b). The use of simpler systems such as the budding yeast *Saccharomyces cerevisiae* has vastly added to the understanding of the more relevant hallmarks and molecular mechanisms involved in the aging process (Sinclair et al. 1998; Jazwinski 2000a, b; Bitterman et al. 2003; Fabrizio et al. 2005; Piper 2006; Barros et al. 2010).

S. cerevisiae has proven to be a convenient model organism for aging studies, and attracted intense interest after Jiang et al. (2000) and Lin et al. (2000) independently demonstrated that this yeast was responsive to calorie restriction (CR), a dietary intervention capable of increasing the lifespan of a large number of organisms (Fontana et al. 2010). The replicative lifespan of *S. cerevisiae*—i.e., the number of daughter cells generated by a single mother cell—was shown to be significantly enhanced by decreasing the initial glucose content in YPD media from the usual 2.0% to 0.5% (Jiang et al. 2000; Lin et al. 2000; Barros et al. 2010). Subsequent work indicated that this protocol was also capable of increasing chronological lifespan (CLS) in this yeast (Reverter-Branchat et al. 2004; Barros et al. 2004; Smith et al. 2007), or the period of time that a single *S. cerevisiae* cell remains metabolically active when in the stationary growth phase (Müller et al. 1980; MacLean et al. 2001; Fabrizio and Longo 2003).

S. cerevisiae is a Crabtree-positive yeast, capable of simultaneously fermenting and respiring under conditions of high glucose concentration (Gancedo 1998; Klein et al.

1998; Gombert et al. 2001). Interestingly, Oliveira et al. (2008) verified that *Kluyveromyces lactis*, a Crabtree-negative yeast in which respiratory carbon metabolism occurs independently of glucose availability (Schaffrath and Breunig 2000) is not responsive to CR. This gives rise to the idea that the effects of CR may be related to a phenotype promoted by the mitigation of glucose signaling in *S. cerevisiae* (Oliveira et al. 2008).

A characteristic of batch cultures—in which aging studies using yeast are carried out—is the limited availability of substrates. Interestingly, in both standard and CR media, glucose is expected to be exhausted within the first culture day (Goldberg et al. 2009), while *S. cerevisiae* cells remain viable for several weeks (Sinclair et al. 1998; Reverter-Branchat et al. 2004; Fabrizio and Longo 2003). After glucose exhaustion, the remaining substrates present in the initial media, such as aminoacids, and those formed during the metabolism of glucose, such as ethanol, acetic acid and glycerol, can be metabolized only through aerobic pathways (MacLean et al. 2001; Frick and Wittmann 2005). Therefore, respiratory fitness is an expected requirement for *S. cerevisiae* survival during the stationary growth phase (MacLean et al. 2001; Fabrizio and Longo 2003; Samokhvalov et al. 2004). Currently, however, there is little information about the importance of specific aerobic bioenergetic pathways in the chronological aging of *S. cerevisae*, as well in its responsiveness to CR.

Here we describe the impact of tricarboxylic acid (TCA) cycle and mitochondrial electron transport chain (ETC) components in CLS and the responsiveness to CR in *S. cerevisiae*, since both are determinant for the aerobic utilization of glucose as an energetic substrate. We further verified the role of mitochondrial DNA (mtDNA) stability in these responses because, in this yeast, seven proteins encoded by this genome are involved in mitochondrial electron transport, proton pumping and oxidative phosphorylation (Foury et al. 1998). Our results demonstrate the importance of mtDNA integrity and functionality, as well long-term respiratory ability in *S. cerevisiae* responsiveness to CR. Finally, we uncover a concentration-dependent role of glucose in regulating proteins responsible for maintaining mtDNA integrity in this yeast.

Materials and methods

S. cerevisiae

S. cerevisiae parental cells (WT), *aco1Δ* (Gangloff et al. 1990), *icl1Δ* (Fernández et al. 1992), *kgd1Δ* (Repetto and Tzagoloff 1989), *lpd1Δ* (Dickinson et al. 1986), *sdh1Δ* (Chapman et al. 1992), *mdh1Δ* (McAlister-Henn and Thompson 1987), *cyt1Δ* (Sadler et al. 1984), *atp2Δ* (Saltzgaber-Muller et al. 1983), *abf2Δ* (Diffley and Stillman 1992; Newman et al. 1996) and ρ^0 mutants used in this study were BY4741 strains (MATa; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; Brachmann et al. 1998).

Media and cell culture

Media used for this study were liquid YPD (1.0% yeast extract, 2.0% peptone and 2.0% for standard or 0.5% glucose for CR) or solid YPD (standard liquid YPD supplemented with 2.0% bacteriological agar) and solid YPEG (1.0% yeast extract, 2.0% peptone, 2.0% ethanol, 2.0% glycerol and 2.0% bacteriological agar), sterilized for 20 min at 121 °C. Cell cultures (50–80 mL) were carried out in aseptic cotton-stopped 250 mL Erlenmeyer flasks with continuous orbital shaking at 200 rpm, at 30 °C. The number of pre-growth cells inoculated per mL of fresh media to initiate the cultures was set at $1 \cdot 10^5$ for all strains tested. Differences in colony counts at 16 h and later (as described below) therefore reflect changes in survival. Plates containing solid media were also incubated at 30 °C.

ρ^0 mutant identification, isolation and characterization

S. cerevisiae ρ^0 mutants were obtained spontaneously after growth of WT cells cultured for 20 h in 2.0% liquid YPD. 100 cells were plated onto solid YPD and after 72 h, this plate was replicated onto YPEG, a respiratory-selective medium. After 48 h of incubation, respiratory incompetent colonies were identified and isolated from the YPD plate. The ρ^0 phenotype of selected colonies was confirmed by mating them with *S. cerevisiae* mit$^-$ strains containing point mutations in the mitochondrial genes *cox1*, *cob1* and *atp6* (Slonimski and Tzagoloff 1976). After diploid selection based on heterozygous auxotrophy complementations, no reversion of respiratory incompetence was observed after mitotic segregation of the resultant diploids, confirming the ρ^0 phenotype. We then selected one isolated colony and further characterized it by following its growth curve (which did not exhibit pos-diauxic biomass formation) and by monitoring the exhaustion of aerobic metabolites from culture media. The elected ρ^0 mutant was not able to consume glucose-derived aerobic metabolites such as ethanol, acetic acid and glycerol and presented a decreased rate of growth when compared to WT cells (data not shown).

CLS determination

CLS was accessed through colony-forming ability over time. After 16 h and 7, 14, 21 and 28 days of growth, we transferred a 2 mL aliquot from each culture to a sterile

centrifuge conic tube and added 3 mL of sterile ultra-purified distilled water. The suspension was centrifuged for 1 min at 1000 × *g*, 25 °C, and the supernatant was discarded. The washing procedure was repeated. The cells were resuspended in 2 mL of sterile ultra-purified distilled water and the absorbance at 600 nm (Abs_{600}) was determined. Serial dilutions to a final Abs_{600} of 0.2, 0.02, 0.002 and 0.0002 were conducted and 50 μL of the last dilution (containing 100 cells) were added to YPD plates and incubated for 72 h to promote cellular growth, after which the number of colonies was counted (Tahara et al. 2007). Results are indicated as the absolute number of colonies counted, and were not corrected for survival percentages at 16 h, in order to reflect the true differences in behavior of each strain studied.

Long-term respiratory growth capacity

Long-term respiratory growth capacity was determined after 7 days of growth over solid media. *S. cerevisiae* were cultured for 16 h in standard YPD media and the same procedures described above were repeated, substituting a series of final dilutions described above with Abs_{600} of 1.0, 0.1, 0.01, 0.001 and 0.0001. 5 μL of each dilution were added to YPD and YPEG plates.

mtDNA stability

The loss of *S. cerevisiae* mtDNA leads to a lack of respiratory ability (Tzagoloff et al. 1975) and can be accessed through respiratory competence. To do so, YPD plates obtained from CLS determinations were replicated onto YPEG solid media in the manner described above, and the percentage of respiratory-competent or -incompetent colonies (ρ^+ and ρ^0, respectively) was determined. This determination provides a snapshot of the presence of ρ^0 over time in culture, although it does not determine the cumulative numbers of ρ^0 cells formed during chronological aging. However, it should be noted that ρ^0 cells are capable of surviving extended periods in the absence of added glucose, as demonstrated in Fig. 1, Panel c. Since *lpd1Δ* mutants exhibit marked respiratory incompetence due to the absence of pyruvate and α-ketoglutarate dehydrogenase activities, the YPEG plates where these mutants were replicated contained a layer of the ρ^0 tester strain αKL14ρ^0 cells (Foury and Tzagoloff 1976) previously grown in YPD liquid media for 16 h, in order to provide short-term respiratory ability to the resulting diploids and allow for colony counts after 48 h.

Graph generation and statistical analysis

Graphs were generated and statistical analysis was performed using GraphPad Prism 5.00 software. The results are expressed as means ± standard errors. Student's *t*-test (for paired comparisons) or Two-Way ANOVA (for multiple comparisons) were used.

Results

TCA cycle enzymes are not essential for CR effects and long-term respiratory growth capacity in *S. cerevisiae*

During the diauxic shift, *S. cerevisiae* drastically change the expression of TCA cycle enzymes and components of the mitochondrial electron transport chain (DeRisi et al. 1997). Genes related to aerobic metabolism become derepressed as glucose is consumed, and aerobic metabolism prevails during the stationary phase (MacLean et al. 2001; Fabrizio and Longo 2003; Samokhvalov et al. 2004). In order to evaluate the importance of TCA cycle activity in both CLS and the response to CR, as well in long-term respiratory growth capacity, we selected *S. cerevisiae* mutants harboring inactivations in aconitase (*aco1Δ*), dihydrolipoyl dehydrogenase (*lpd1Δ*)—a subunit of α-ketoglutarate dehydrogenase complex—and the flavoprotein subunit of succinate dehydrogenase (*sdh1Δ*; see Scheme 1).

Interestingly, although *lpd1Δ* and *sdh1Δ* mutants presented decreased CLS compared to the WT strain, neither of these mutants had their response to CR affected (Fig. 1, Panels a, c and d). Moreover, *aco1Δ* cell CLS was similar to WT, and also responded to CR (Panel B). In addition, *kgd1Δ* and *mdh1Δ* mutants—lacking α-ketoglutarate and malate dehydrogenase activities—presented statistically significant increases in CLS when cultured under CR conditions (results not shown). *S. cerevisiae* TCA mutants usually show impaired growth in media containing only non-fermentable carbon sources (Tzagoloff and Dieckmann 1990). Probably due to small amounts of TCA intermediates in rich media, NADH and $FADH_2$ can be slowly generated in the TCA cycle reactions up or downstream of the disruptions, or even during glycolysis and conversion of pyruvate into acetyl-CoA, and oxidized by the intact ETC. Therefore, in long-term incubations, TCA cycle mutants were capable of growing on solid YPEG media, even though they exhibited a lower growth capacity than WT cells (Fig. 2).

Loss of mtDNA suppresses CR-mediated CLS extension and long-term respiratory growth capacity in *S. cerevisiae*

Next, we used ρ^0 and *abf2Δ* mutants to investigate if electron transport chain and ATP synthase integrity were essential toward CR effects. Since seven proteins encoded by *S. cerevisiae* mtDNA are components of the oxidative phosphorylation system, namely cytochrome *c* subunits I, II and III, ATP synthase subunits 7, 8 and 9, and apocyto-

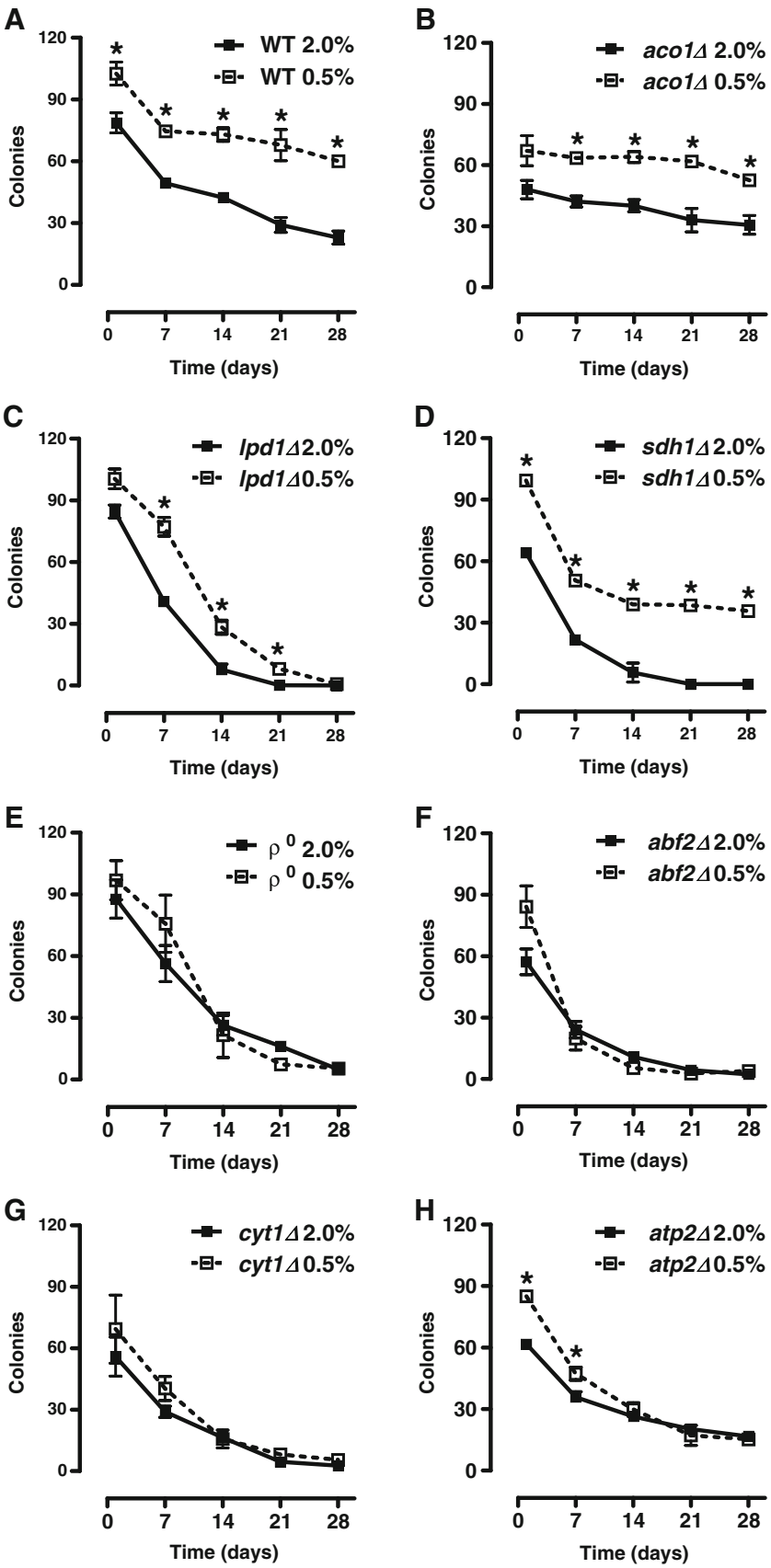
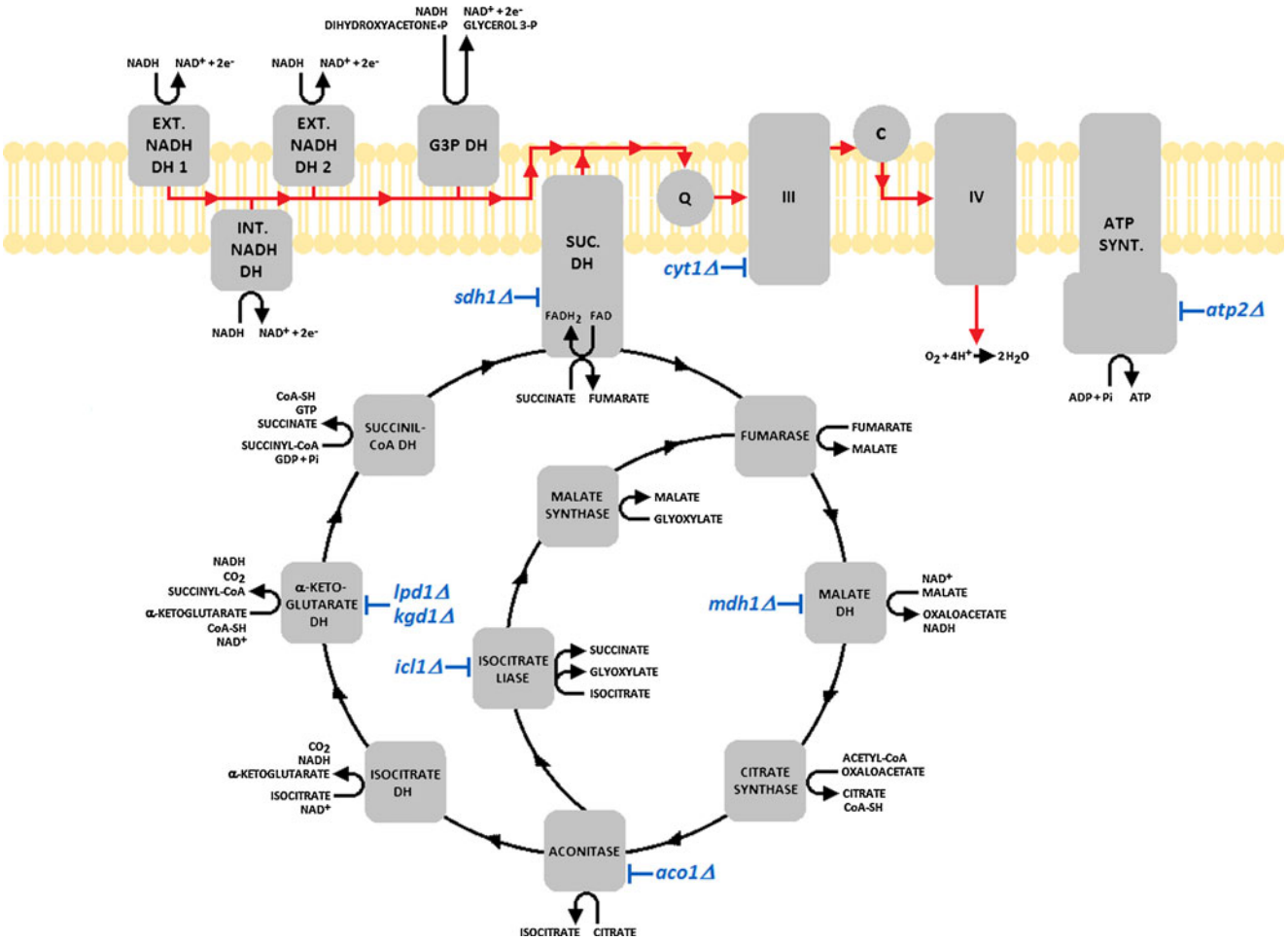


Fig. 1 TCA cycle-deleted, but not respiratory-deficient *S. cerevisiae*, present increased CLS when grown under CR conditions. The colony-forming ability of WT (**a**), *aco1Δ* (**b**), *lpd1Δ* (**c**), *sdh1Δ* (**d**), ρ^0 (**e**), *abf2Δ* (**f**), *cyt1Δ* (**g**) and *atp2Δ* (**h**) mutants was assessed after 16 h and 7, 14, 21 and 28 days of culture either under standard (filled squares) or CR conditions (open squares). The number of colonies formed from 100 cells plated onto solid media over time was assessed as CLS, as described in "Materials and Methods". Panel **a**: *$p<0.05$ vs. 2.0% WT; Panel **b**: *$p<0.05$ vs. 2.0% *aco1Δ*; Panel **c**: *$p<0.05$ vs. 2.0% *lpd1Δ*; Panel **h**: *$p<0.05$ vs. 2.0% *atp2Δ*



Scheme 1 Aerobic metabolic pathways in *S. cerevisiae* mitochondria. ETC, TCA and glyoxylate cycle components are depicted in grey. Electron transfers are represented in red. Mutants used in this study are highlighted in blue. *C* cytochrome *c*; *DH* dehydrogenase; *G3P* glycerol 3 phosphate; *Q* coenzyme Q; *Suc* succinate

chrome *b* (Foury et al. 1998), mutants lacking mtDNA *per se* or harboring a defect in mtDNA maintenance exhibit substantial impairments in aerobic metabolism. *abf2Δ* cells (defective in the *ars* binding protein, a member of mitochondrial high mobility group of proteins important for mtDNA replication, recombination and stability; Diffley and Stillman 1991, 1992) do not grow in respiratory-selective medium when previously cultured in glucose (Zelenaya-Troitskaya et al. 1995).

We observed that loss of mitochondrial DNA, either in ρ^0 mutants or due to the *abf2Δ* mutation, which results in the respiration-deficient *petite* phenotype (Kao et al. 1993), completely suppressed the response to CR (Fig. 1, Panels e and f), as well long-term respiratory growth capacity (Fig. 2). These observations directly demonstrate that disruption of mitochondrial electron flow, and the consequences thereof, such as loss of oxidative phosphorylation, mitochondrial membrane potential and protein import impairment (Baker and Schatz 1991; Stuart et al. 1994), totally abrogate CR-mediated CLS extension in *S. cerevisiae*. In addition, the inability of ρ^0 and *abf2Δ* mutants to maintain long-term respiratory growth under the experimental conditions used here clearly associates mtDNA integrity and maintenance as key features for *S. cerevisiae* aerobic growth in rich media (Fig. 2).

Nuclearly-encoded respiratory chain components are necessary for CR effects and long-term respiratory growth capacity

Mitochondrial functionality requires a concerted interaction between both nuclear and mitochondrial genomes (Linnane et al. 1972; Falkenberg et al. 2007). Since the absence of mtDNA abolishes CR-mediated CLS extension (Fig. 1, Panels e and f) and aerobic growth in respiratory selective media (Fig. 2), we decided to investigate whether the lack of a specific subunit of the ETC encoded by nuclear DNA could promote the same phenotypes. CLS under control and

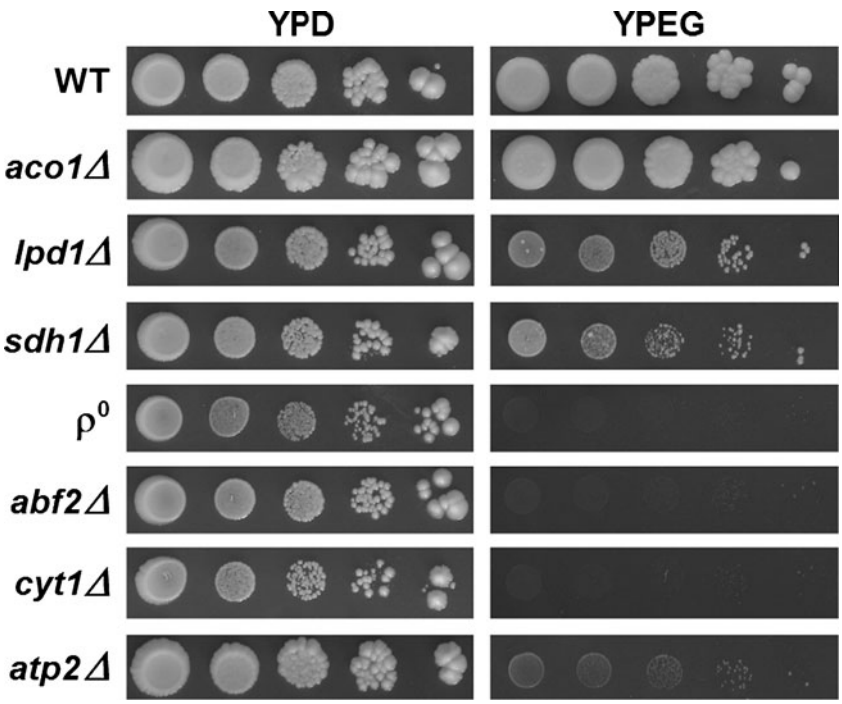


Fig. 2 Long-term respiratory growth capacity in *S. cerevisiae* mutants. The respiratory capacity of WT and mutant cells was assessed performing serial dilutions. Growth was recorded after 7 days in culture at 30 °C over solid YPD and YPEG media, as described in "Materials and Methods". The figure is a representative image of at least 4 equal repetitions

CR conditions, and long-term respiratory growth capacity were measured in *cyt1Δ* mutants, which lack cytochrome c_1, a component of the multicomplex ubiquinol-cytochrome *c* reductase, the first proton pump in the *S. cerevisiae* ETC (Sidhu and Beattie 1983). We verified that *cyt1Δ* cells do not respond to CR with CLS extension (Fig. 1, Panel g) nor present long-term respiratory growth capacity (Fig. 2). These findings further support the idea that respiratory integrity is essential for CR-mediated lifespan extension, and are in line with our previous results obtained in ρ^0 and *abf2Δ* mutants (Fig. 1).

ATP synthase defects partially abolish the response to CR and long-term respiratory growth ability in *S. cerevisiae*

Our results so far indicate that integrity of the ETC is required for CR effects in CLS extension (Fig. 1, Panels e, f and g; Fig. 2). However, ρ^0 cells also present defects in the ATP synthase (Foury et al. 1998). Thus, we tested whether *atp2Δ* mutants, which lack the β-subunit of F_1 in F_1F_O ATP synthase (Saltzgaber-Muller et al. 1983), respond to CR in a similar fashion. We observed that CR significantly increased cellular viability only up to the 7th day of culture in these mutants. From the 14th day on, CLS did not vary significantly between control and CR cells (Fig. 1, Panel h). Furthermore, *atp2Δ* mutants also presented the lowest long-term respiratory growth capacity between all mutants that exhibited positive growth in YPEG (Fig. 2).

mtDNA stability is dependent on initial glucose concentrations

While conducting CLS experiments using the mutants described above, we noticed that these strains presented different tendencies to form spontaneous *petite* colonies, almost exclusively related to mtDNA instability (Linnane et al. 1989; Ferguson and von Borstel 1992). We thus further determined the percentage of respiratory-competent (ρ^+) colonies in *aco1Δ* and *lpd1Δ* mutants cultured in standard and CR conditions over time, since both aconitase and dihydrolipoyl dehydrogenase have dual roles as TCA cycle enzymes and structural components of mitochondrial nucleiods in *S. cerevisiae* (Chen et al. 2005). These nucleoproteic structures, formed by the interaction between double-stranded DNA and packaging proteins (Rickwood et al. 1981), promote physical stability and functionality to the mitochondrial genome (Rickwood et al. 1981; Miyakawa et al. 1984; Newman et al. 1996; Brewer et al. 2003; Chen et al. 2005). The number of ρ^+ colonies was assessed by replicating the YPD solid plates from CLS determinations onto YPEG, as described in "Materials and Methods".

Although there is no significant difference in the percentage of ρ^+ colonies between standard and CR conditions in WT cells and in *aco1Δ* mutants, we observed an increased percentage of ρ^+ colonies as the culture time advances (Fig. 3, Panels a and b). This is probably due to the loss of ρ^0 cells replicating in the absence of fermentative substrates; since ρ^0 cells do not exhibit significantly

different mortality rates from WT cells until the 14th day of culture (Fig. 1), and WT cells present ethanol-supported growth but ρ^0 mutants do not, we infer that the number of these mutants in batch cultures becomes proportionally reduced as culture times advance.

Interestingly, culture condition was a determinant factor for mtDNA stability in *lpd1Δ* mutants, which exhibited a higher percentage of ρ^+ colonies during the early culture days, when in standard media (Fig. 3, Panel c). *lpd1Δ* mutants cultured under CR conditions also showed a significant decrease in mtDNA stability when compared to WT cells during the early culture days. Finally, *aco1Δ* cells cultured under standard conditions present lower ρ^+ counts relative to WT at the 1st day of culture (Fig. 3, Panel b).

Taken together, these results indicate that, under standard culture conditions, aconitase is a determinant player in *S. cerevisiae* mtDNA stability when glucose is still present in the media [glucose from both standard and CR culture media is exhausted within the first day (Goldberg et al. 2009)]. On the other hand, dihydrolipoyl dehydrogenase, under CR, is necessary to promote this same phenotype both before and after glucose exhaustion.

Discussion

Although respiratory metabolism plays a central role in *S. cerevisiae* lifespan and is involved in the beneficial effects of CR (MacLean et al. 2001; Fabrizio and Longo 2003; Samokhvalov et al. 2004), little is known about the role in aging of specific oxidative metabolism components. As a result, we evaluated the separate roles of the TCA cycle, electron transport chain and the ATP synthase in CLS and its response to CR.

We measured CLS in a series of TCA and ETC mutants, and verified if there is a correlation between long-term respiratory fitness and CLS extension due to CR. As expected, most of these metabolic mutants have lower CLS than WT cells (Fig. 1). We observed distinct responses relative to respiratory deficiency phenotypes: TCA cycle mutants (*aco1Δ*, *lpd1Δ* and *sdh1Δ*) were capable of growing in respiratory selective-media, while cells lacking mtDNA (ρ^0) or exhibiting marked mitochondrial genome instability (*abf2Δ*) were not (Fig. 2). In fact, mtDNA encodes several proteins required for respiratory fitness in *S. cerevisiae*, and its functional impairment completely abolishes respiratory growth (Tzagoloff et al. 1975). On the other hand, TCA cycle components are not crucial for growth in YPEG (Fig. 2), since this medium contains TCA intermediates, and reduced NAD and FAD can be reoxidized by the ETC in their absence. Interestingly, all TCA cycle mutants studied here also responded to CR with CLS extension (Fig 1), an effect not observed in ρ^0, *abf2Δ* mutants or *cyt1Δ* cells (Fig 1). Furthermore, although enzymes from the glyoxylate cycle are activated during CLS (Samokhvalov et al. 2004), this pathway is not required for a response to CR, as indicated by experiments using *icl1Δ* cells (deficient in isocitrate lyase), which also present enhanced CLS when cultured under CR conditions (results not shown). Another mutant in which these respiratory activity and CLS correlate is *atp2Δ*, which grows very poorly in respiratory media and responds marginally to CR. The lack of ATP synthase activity does not energetically impair cells in the logarithmic growth phase, when *S. cerevisiae* rely on glycolysis to generate the bulk of their ATP, but may be important for mitochondrial protein import and ATP generation in the stationary phase (Baker and Schatz 1991; Stuart et al. 1994). Thus, a strong correlation between CR responsiveness in CLS and ability to exhibit long-term respiratory growth is evident.

Interestingly, although our results indicate that the ability to metabolize respiratory substrates through a functional electron transport chain is required for the beneficial effects of CR in CLS, previous results show that respiratory mutants may exhibit enhanced replicative life span when cultured under CR conditions (Kaeberlein et al. 2005; Lin

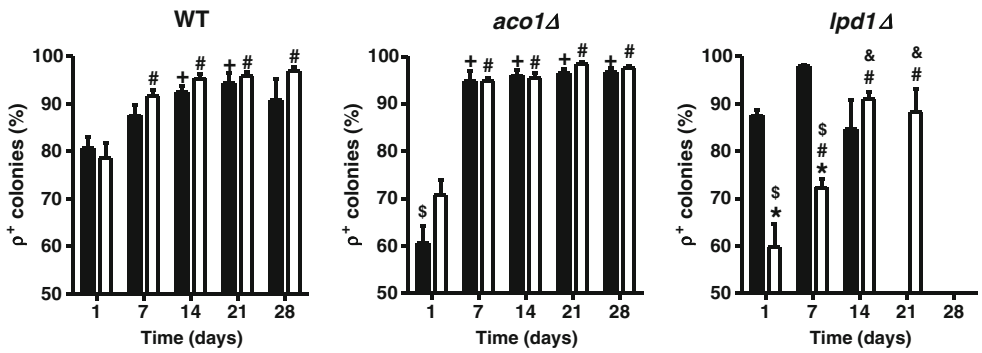


Fig. 3 mtDNA stability is modulated by aconitase and dihydrolipoyl dehydrogenase in a glucose-sensitive manner. The percentage of ρ^+ colonies from WT, *aco1Δ* and *lpd1Δ* cells was assessed over time, as described in "Materials and Methods". $^{\#}p<0.05$ vs. 16 h 0.5%; $^{+}p<0.05$ vs. 16 h 2.0%; $^{*}p<0.05$ vs. time-matched 2.0%; $^{\$}p<0.05$ vs. WT, $^{\&}p<0.05$ vs. 7 days

and Guarente 2006). This demonstrates that despite many similar properties of RLS and CLS, some conditions affect each aspect of yeast lifespan differently (Barea and Bonatto 2009; Barros et al. 2010). ETC integrity is strongly dependent on the stability of mtDNA, since it encodes for key components of the respiratory chain (Foury et al. 1998). mtDNA differs from nuclear DNA, as it (i) lacks protective histones, (ii) has different repair mechanisms (Lipinski et al. 2010) and (iii) presents mutagenesis rates of 10^{-1} to 10^{-3}, against 10^{-7} to 10^{-8}, for nuclear DNA mutation rates (Linnane et al. 1989; Ferguson and von Borstel 1992). Interestingly, mtDNA is physically associated with mitochondrial proteins, most of which have primary metabolic functions, forming a nucleoproteic complex known as nucleoid (Rickwood et al. 1981; Chen et al. 2005). Recent studies have focused on specific nucleoid proteins such as aconitase, suggesting these may participate in the maintenance of mtDNA stability, in addition to their well-established metabolic roles (Chen et al. 2005). Here, we further dissect the role of nucleoid proteins in mtDNA stability in *S. cerevisiae* by showing that the lack of aconitase and dihydrolipoyl dehydrogenase affect mtDNA stability differently depending on culture conditions (Fig. 3). In the early stages of the cultures, when glucose is still present, *aco1Δ* cells present fewer ρ^+ colonies, indicating that aconitase is important to maintain mtDNA stability in the presence of glucose. On the other hand, *lpd1Δ* cells cultured under CR conditions present high mtDNA instability, indicating that dihydrolipoyl dehydrogenase is important for mtDNA maintenance under low glucose culture conditions. Dihydrolipoyl dehydrogenase is also important for mtDNA stability in the early stages of the aging process, as indicated by low quantities of ρ^+ *lpd1Δ* cells at 7 days of culture under CR. Overall, we provide support for the finding that the metabolic state of *S. cerevisiae* may remodel the nucleoid, thus changing mtDNA stability (Kucej et al. 2008).

Although results using *S. cerevisiae* as a model organism cannot be immediately extended to more complex life forms, some parallels with aging studies in animals have already been demonstrated. For example, CR has been shown to increase maximal respiratory capacity in rodents by promoting mitochondrial biogenesis (Nisoli et al. 2005; López-Lluch et al. 2006; Cerqueira et al. 2011), an effect also observed in other animal models with enhanced lifespans such as fat-specific insulin knockout mice (Katic et al. 2007). Furthermore, the accumulation of lesions to mtDNA leads to premature aging (Trifunovic et al. 2004).

Overall, we demonstrate using *S. cerevisiae* as a model system that mtDNA and electron transport integrity are essential for CR-mediated CLS extension while, surprisingly, TCA and glyoxylate cycle activity are not. Interestingly, CR itself impacts mtDNA maintenance, since the levels of glucose in the media differentially affect the roles of two nucleoid proteins, aconitase and dihydrolipoyl dehydrogenase, in maintaining mtDNA stability. These results strengthen the idea that CR, respiratory activity and mtDNA integrity are key players in the aging process.

Acknowledgements The authors would like to thank Dr. Luis Eduardo Soares Netto and Simone Vidigal Alves for the kind donation of *S. cerevisiae* mutants and Dr. José Ribamar dos Santos Ferreira Júnior for stimulating and highly helpful discussions. We are also indebted with Camille Cristine Caldeira Ortiz, Edson Alves Gomes and Doris Dias de Araújo for excellent technical assistance. This work was supported by grants from the *Fundação de Amparo à Pesquisa do Estado de São Paulo* (FAPESP), *Conselho Nacional de Desenvolvimento Científico e Tecnológico* (CNPq), *Instituto Nacional de Ciência e Tecnologia de Processos Redox em Biomedicina* (INCT Redoxoma) and The John Simon Guggenhein Memorial Foundation. EBT and KC are supported by FAPESP and CNPq fellowships, respectively.

References

Baker KP, Schatz G (1991) Nature 349:205–208
Barea F, Bonatto D (2009) Mech Ageing Dev 130:444–460
Barros MH, Bandy B, Tahara EB, Kowaltowski AJ (2004) J Biol Chem 279:49883–49888
Barros MH, da Cunha FM, Oliveira GA, Tahara EB, Kowaltowski AJ (2010) Mech Ageing Dev 131:494–502
Bitterman KJ, Medvedik O, Sinclair DA (2003) Microbiol Mol Biol Rev 67:376–399
Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD (1998) Yeast 14:115–132
Brewer LR, Friddle R, Noy A, Baldwin E, Martin SS, Corzett M, Balhorn R, Baskin RJ (2003) Biophys J 85:2519–2524
Cerqueira FM, Laurindo FR, Kowaltowski AJ (2011) PLoS One 31: e18433
Chapman KB, Solomon SD, Boeke JD (1992) Gene 118:131–136
Chen XJ, Wang X, Kaufman BA, Butow RA (2005) Science 307:714–717
DeRisi JL, Iyer VR, Brown PO (1997) Science 278:680–686
Dickinson JR, Roy DJ, Dawes IW (1986) Mol Gen Genet 204:103–107
Diffley JF, Stillman B (1991) Proc Natl Acad Sci USA 88:7864–7868
Diffley JF, Stillman B (1992) J Biol Chem 267:3368–3374
Fabrizio P, Longo VD (2003) Aging Cell 2:73–81
Fabrizio P, Li L, Longo VD (2005) Mech Ageing Dev 126:11–16
Falkenberg M, Larsson NG, Gustafsson CM (2007) Annu Rev Biochem 76:679–699
Ferguson LR, von Borstel RC (1992) Mutat Res 265:103–148
Fernández E, Moreno F, Rodicio R (1992) Eur J Biochem 204:983–990
Fontana L, Partridge L, Longo VD (2010) Science 328:321–326
Foury F, Tzagoloff A (1976) Eur J Biochem 68:113–119
Foury F, Roganti T, Lecrenier N, Purnelle B (1998) FEBS Lett 440:325–331
Frick O, Wittmann C (2005) Microb Cell Fact 4:30
Gancedo JM (1998) Yeast carbon catabolite repression. Microbiol Mol Biol Rev 62:334–361
Gangloff SP, Marguet D, Lauquin GJ (1990) Mol Cell Biol 10:3551–3561
Goldberg AA, Bourque SD, Kyryakov P, Gregg C, Boukh-Viner T, Beach A, Burstein MT, Machkalyan G, Richard V, Rampersad S, Cyr D, Milijevic S, Titorenko VI (2009) Exp Gerontol 44:555–571

Gombert AK, Moreira dos Santos M, Christensen B, Nielsen J (2001) J Bacteriol 183:1441–1451
Jazwinski SM (2002a) Exp Gerontol 37:1141–1146
Jazwinski SM (2002b) Annu Rev Microbiol 56:769–792
Jiang JC, Jaruga E, Repnevskaya MV, Jazwinski SM (2000) FASEB J 14:2135–2147
Kaeberlein M, Hu D, Kerr EO, Tsuchiya M, Westman EA, Dang N, Fields S, Kennedy BK (2005) PloS Genet 1:e69
Kao LR, Megraw TL, Chae CB (1993) Proc Natl Acad Sci USA 90:5598–5602
Katic M, Kennedy AR, Leykin I, Norris A, McGettrick A, Gesta S, Russell SJ, Bluher M, Maratos-Flier E, Kahn CR (2007) Aging Cell 6:827–839
Kenyon C (2001) Cell 105:165–168
Klein CJ, Olsson L, Nielsen J (1998) Microbiology 144:13–24
Kucej M, Kucejova B, Subramanian R, Chen XJ, Butow RA (2008) J Cell Sci 121:1861–1868
Lin SJ, Guarente L (2006) PLoS Genet 2:e33
Lin SJ, Defossez PA, Guarente L (2000) Science 289:2126–2128
Linnane AW, Haslam JM, Lukins HB, Nagley P (1972) Annu Rev Microbiol 26:163–198
Linnane AW, Marzuki S, Ozawa T, Tanaka M (1989) Lancet 25:642–645
Lipinski KA, Kaniak-Golik A, Golik P (2010) Biochim Biophys Acta 1797:1086–1098
López-Lluch G, Hunt N, Jones B, Zhu M, Jamieson H, Hilmer S, Cascajo MV, Allard J, Ingram DK, Navas P, de Cabo R (2006) Proc Natl Acad Sci USA 103:1768–1773
MacLean M, Harris N, Piper PW (2001) Yeast 18:499–509
McAlister-Henn L, Thompson LM (1987) J Bacteriol 169:5157–5166
Miyakawa I, Aoi H, Sando N, Kuroiwa T (1984) J Cell Sci 66:21–38
Müller I, Zimmermann M, Becker D, Flömer M (1980) Mech Ageing Dev 12:47–52
Newman SM, Zelenaya-Troitskaya O, Perlman PS, Butow RA (1996) Nucleic Acids Res 24:386–393
Nisoli E, Tonello C, Cardile A, Cozzi V, Bracale R, Tedesco L, Falcone S, Valerio A, Cantoni O, Clementi E, Moncada S, Carruba MO (2005) Science 310:314–317
Oliveira GA, Tahara EB, Gombert AK, Barros MH, Kowaltowski AJ (2008) J Bioenerg Biomembr 40:381–388
Piper PW (2006) Yeast 23:215–226
Repetto B, Tzagoloff A (1989) Mol Cell Biol 9:2695–2705
Reverter-Branchat G, Cabiscol E, Tamarit J, Ros J (2004) J Biol Chem 279:31983–31989
Rickwood D, Chambers JA, Barat M (1981) Exp Cell Res 133:1–13
Sadler I, Suda K, Schatz G, Kaudewitz F, Haid A (1984) EMBO J 3:2137–2143
Saltzgaber-Muller J, Kunapuli SP, Douglas MG (1983) J Biol Chem 258:11465–11470
Samokhvalov V, Ignatov V, Kondrashova M (2004) Biochimie 86:39–46
Schaffrath R, Breunig KD (2000) Fungal Genet Biol 30:173–190
Sidhu A, Beattie DS (1983) J Biol Chem 258:10649–10656
Sinclair D, Mills K, Guarente L (1998) Annu Rev Microbiol 52:533–560
Slonimski PP, Tzagoloff A (1976) Eur J Biochem 61:27–41
Smith DL Jr, McClure JM, Matecic M, Smith JS (2007) Aging Cell 6:649–662
Stuart RA, Gruhler A, van der Klei I, Guiard B, Koll H, Neupert W (1994) Eur J Biochem 220:9–18
Tahara EB, Barros MH, Oliveira GA, Netto LE, Kowaltowski AJ (2007) FASEB J 21:274–283
Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE, Bohlooly-Y M, Gidlöf S, Oldfors A, Wibom R, Törnell J, Jacobs HT, Larsson NG (2004) Nature 429:417–423
Tzagoloff A, Dieckmann CL (1990) Microbiol Rev 54:211–225
Tzagoloff A, Akai A, Needleman RB (1975) J Biol Chem 250:8236–8242
Zelenaya-Troitskaya O, Perlman PS, Butow RA (1995) EMBO J 14:3268–3276