



The efficiency of mitochondrial electron transport chain is increased in the long-lived *mrg19* *Saccharomyces cerevisiae*

Nitish Mittal,^{1,2} M. Madan Babu² and Nilanjan Roy¹

¹Department of Biotechnology, National Institute of Pharmaceutical Education and Research, Sector 67, S. A. S. Nagar, Punjab 160062, India

²MRC-Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, UK

Summary

Integrity of mitochondrial functionality is a key determinant of longevity in several organisms. In particular, reduced mitochondrial ROS (mtROS) production leading to decreased mtDNA damage is believed to be a crucial aspect of longevity. The generation of low mtROS was thought to be due to low mitochondrial oxygen consumption. However, recent studies have shown that higher mitochondrial oxygen consumption could still result in low mtROS and contribute to longevity. This increased mitochondrial efficiency (i.e. low mtROS generated despite high oxygen consumption) was explained as a result of mitochondrial biogenesis, which provides more entry points for the electrons to the electron transport chain (ETC), thereby resulting in low mtROS production. In this study, we provide evidence for the existence of an alternative pathway to explain the observed higher mitochondrial efficiency in the long-lived *mrg19* mutant of *Saccharomyces cerevisiae*. Although we observe similar amounts of mitochondria in *mrg19* and wild-type (wt) yeast, we find that *mrg19* mitochondria have higher expression of ETC components per mitochondria in comparison with the wt. These findings demonstrate that more efficient mitochondria because of increased ETC per mitochondria can also produce less mtROS. Taken together, our findings provide evidence for an alternative explanation for the involvement of higher mitochondrial activity in prolonging lifespan. We anticipate that similar mechanisms might also exist in eukaryotes including human.

Key words: adenosine tri-phosphate; caloric restriction; electron transport chain; *Mrg19*; reactive oxygen species.

Introduction

Over the past decades, aging has become an important area of research because of increase in age-associated diseases and elderly population in developed countries (Weinert & Timiras, 2003). It is difficult to define aging as it is a multifactorial physiological process, although it is generally marked as the progressive loss of function with the advancement of age (Kirkwood & Austad, 2000). The accumulation of nuclear and mitochondrial DNA (mtDNA) damage (i.e. genomic instability) is the characteristic feature of aging (McMurray & Gottschling, 2004). If genomic damage persists, it results in altered or impaired function of particular genes because of accumulation of mutated or non-functional proteins. According to the free radical theory of aging (Harman, 1956), it is believed that reactive oxygen species (ROS) causes oxidative damage to macromolecules such as DNA and promotes aging.

There are several sites of ROS production in a cell. For instance, Boveris *et al.* (1972) have showed that the microsomes are the major site of physiological ROS production followed by peroxisome and mitochondria in rat liver cells. The integrity of mtDNA is an important aspect for the aging process. For instance, it has been shown that the oxidative damage of mtDNA is several folds higher in comparison with nuclear DNA in aged mice (Richter *et al.*, 1988; Barja & Herrero, 2000). The proposed reason for increased mtDNA damage is the physical proximity of mtDNA to the site of ROS production and a lower DNA repair and protective systems compared with nuclear DNA (Barja & Herrero, 2000). Therefore, higher ROS production from mitochondria causes increased mtDNA damage and leads to mitochondrial dysfunction. Further, mitochondrial dysfunction has been linked to ROS generation (Choksi *et al.*, 2007) and premature aging in mice (Trifunovic *et al.*, 2004; Trifunovic & Larsson, 2008). In the view of the emerging role of mitochondrial ROS in aging, the free radical theory of aging has been modified to the mitochondrial theory of aging (Harman, 1972).

Many studies suggest that caloric restriction (CR) reduces ROS production, oxidative damage to DNA and maintains the genomic stability of cells (Sohal *et al.*, 1994; Lopez-Torres *et al.*, 2002; Sanz *et al.*, 2005). Caloric restriction has been a well-studied external manipulation that has been shown to increase the lifespan of a wide range of organisms from unicellular yeast to multicellular eukaryotes. The wide spectrum of organisms in which CR counters aging also points to its possibly conserved

Correspondence

Nilanjan Roy, Department of Biotechnology, National Institute of Pharmaceutical Education and Research, Sector 67, S. A. S. Nagar, Punjab 160062, India. Tel.: +91 172 2214 682-87 ext 2067; fax: +91 172 2214 692; e-mail: nilanjanroy@nipr.ac.in

Accepted for publication 12 August 2009

mechanism of action. Caloric restriction not only prolongs lifespan but also delays the onset of the age-associated disorders such as diabetes, cardiovascular and neurodegenerative diseases (Masoro, 2000). Because of its wide applicability, an improved understanding of the molecular mechanisms of CR is desirable. To understand the molecular mechanisms of CR-mediated longevity, several genetic mutants have been developed in yeast (*S. cerevisiae*). For instance, deletion of genes such as *TOR1*, *SCH9*, *MRG19* has been reported to increase the lifespan of yeast strain (Lin *et al.*, 2002; Kaeberlein *et al.*, 2005b; Kharade *et al.*, 2005; Bonawitz *et al.*, 2007; Lavoie & Whiteway, 2008). The lifespan of yeast is defined in two ways, replicative and chronological lifespan (Bitterman *et al.*, 2003). Replicative lifespan is defined as the number of divisions a mother cell undergoes before senescence. Chronological lifespan is measured in terms of the duration of time that yeast cells remain viable upon reaching the nondividing stationary phase.

As mitochondria have been established as a crucial site of ROS production and have been linked to aging, this organelle has become the focus of study for CR and genetic mutant mediated longevity in a wide range of organisms (Pamplona & Barja, 2007). Several evidences suggest that low ROS production might be one of the major reasons for longevity in model organism (Ramsey *et al.*, 2000; Barja, 2004). This low mitochondrial ROS production could be explained by two mechanisms: (i) low metabolic rate and (ii) increased mitochondrial biogenesis which provide more entry point for electrons and reduces electron stalling at the electron transport chain (ETC). Initially, it was shown that CR reduces the ROS as a result of lower metabolic rate in mice (Sohal & Weindruch, 1996). Other studies in *Caenorhabditis elegans* (Hansen *et al.*, 2005) and mice (Bevilacqua *et al.*, 2005) also support that low respiration rate is responsible for low ROS production and longevity. On the contrary, several studies have shown that CR increases lifespan of yeast by increasing the respiration rate (i.e. higher consumption of oxygen) rather than a decreased respiration rate (Lin *et al.*, 2002; Agarwal *et al.*, 2005; Bishop & Guarente, 2007a). Caloric restriction (Barros *et al.*, 2004) and genetic mutants such as deletion of *TOR1* (Bonawitz *et al.*, 2007) in yeast also produces low ROS with higher mitochondrial respiration. The same observation (low ROS and higher respiration rate) have also been made in multicellular eukaryotes such as *C. elegans* (Bishop & Guarente, 2007b) and mice (Nisoli *et al.*, 2005). The low ROS production despite higher respiration rate has been recently explained because of a higher mitochondrial biogenesis (Guarente, 2008). Taken together, while it is clear that low ROS generation is linked to longevity in several model organisms (Sohal & Weindruch, 1996; Barja, 2004), the link between ROS generation and mitochondrial functionality is still not fully resolved.

Furthermore, contradictory reports for the involvement of mitochondria in longevity exist in the literature. For instance, it has been shown that CR can increase lifespan in respiratory deficient (nonfunctional mitochondria) yeast (Kaeberlein *et al.*, 2005a). Similarly, systematic RNAi screening in *C. elegans* has

shown that impaired mitochondrial function is associated with longer lifespan (Lee *et al.*, 2003). On the contrary, several other reports suggest that disruption of ETC chain and use of ETC inhibitors minimize the lifespan of yeast (Barros *et al.*, 2004; Bonawitz *et al.*, 2006). These observations further raise the question on the mitochondrial involvement in longevity.

From our laboratory, it has been shown that deletion of *MRG19* increases both chronological and replicative lifespan of yeast (Kharade *et al.*, 2005). Mrg19p has been reported to be a putative transcription factor responsible for the utilization of carbon and nitrogen sources (Das & Bhat, 2005; Khanday *et al.*, 2002). However, recent studies (Lin *et al.*, 2008); (Nikko *et al.*, 2008) have shown that Mrg19 (*aka* Csr2) has been reported to function as an adaptor for the Rsp5p E3 ubiquitin ligase. Deletion of *MRG19* was also reported to increase cell density at stationary phase and oxygen consumption at the cellular level (Kabir *et al.*, 2000; Kharade *et al.*, 2005). The underlying mechanism of longevity in *mrg19* is not known so far. In this report, we have addressed the mitochondrial functionality of *mrg19* in relation to its wild-type (wt) counterpart. We demonstrate that deletion of *MRG19* results in higher mitochondrial efficiency and this is because of increased expression of ETC per mitochondria rather than mitochondrial biogenesis.

Results

In all experiments, succinate is used to activate isolated mitochondria until otherwise stated. The *P*-values have been calculated using student paired *t*-test available in Microsoft Excel.

Equal content of mitochondria in the *mrg19* and wt strain

Fluorescence-activated cell sorting (FACS) experiment has been carried out to quantify the mitochondrial content in *mrg19* and wt strains. The plasmid mitochondrial green fluorescent protein (mtGFP) was transformed in *mrg19* and wt w303 cells to compare mitochondrial content in both strains. The transformant was checked through fluorescence microscopy. Minor differences were observed in the morphology of the mitochondria in *mrg19* and wt cells (Fig. 1A,B phase contrast microscopy; Fig. 1C,D fluorescence microscopy). Furthermore, flow cytometry analysis was done for 100 000 events to measure the content of mitochondria per cell. The data (Fig. 1E) showed that there is no difference in the intensity of fluorescence. The average intensity of fluorescence was 134.39 and 126.1 for *mrg19* and wt cells, respectively. The difference was not significant and indicates that the mitochondrial content is same for the *mrg19* and wt cells. To further confirm that the amount of mitochondria is similar in both the strains, mtDNA (a biomarker for mitochondrial biogenesis) was isolated and quantified from equal total protein concentration of wt and *mrg19* strains as described in methods. The result showed no change 1.01 ± 0.05 (*P*-value = 0.4) in wt and *mrg19* mtDNA (Fig. 1F). Both FACS analysis and mtDNA estimation results together suggest that

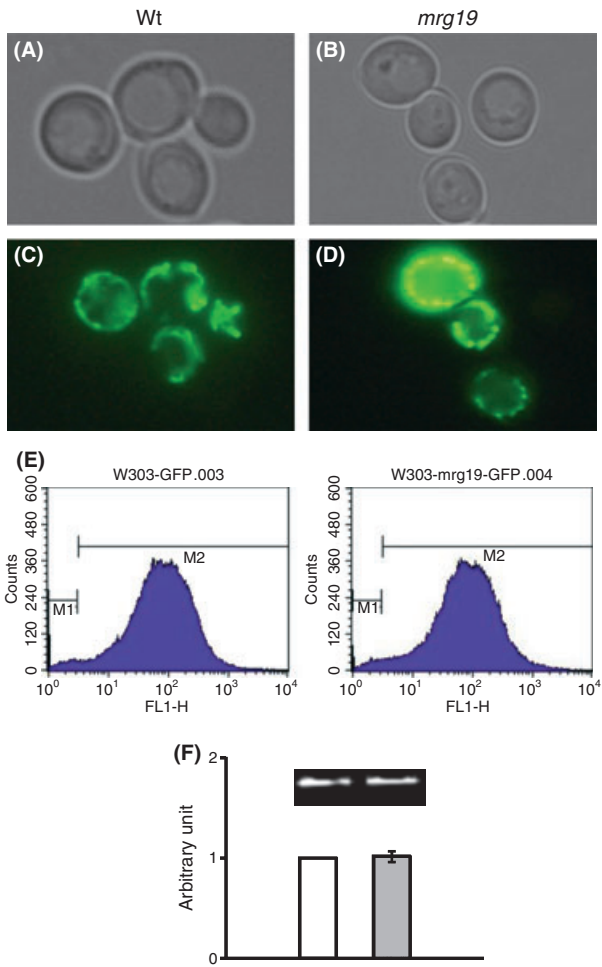


Fig. 1 No difference in mitochondrial content between *mrg19* and the wild-type cells. (A, B) show the bright field image of wt and *mrg19* cells corresponding to fluorescence microscopy images. (C, D) show the fluorescence microscopy images of wt and *mrg19* cells at 100x magnification in emersion oil after transformation of mtGFP plasmid. (E) Histogram of flow cytometry data for mtGFP transformed wt and *mrg19* cells. 100 000 events were measured for both wt and *mrg19* cells. (F) shows the bar graph of densitometric analysis for three independent estimate of wt (white bar) and *mrg19* (grey bar) mtDNA where wt were given the value of one and density of corresponding band in *mrg19* was measured in relation to the wt band. The difference between the density of both bands were found insignificant (P -value = 0.4). Inset of figure shows the representative gel of wt and *mrg19* mtDNA.

there is no difference in mitochondrial content of wt and *mrg19* cells. Moreover, mtDNA estimation suggests that equal protein in wt and *mrg19* strains are correlated to similar mitochondrial content. Hence, equal protein is used as a metric for equal mitochondria for further experiments.

Higher oxygen consumption in *mrg19* mitochondria

Oxygen consumption is an established metric to probe mitochondrial activity. Hence, the rate of oxygen consumption was measured in freshly isolated mitochondria of *mrg19* and wt cells as described in Materials and methods. The result (Fig. 2A)

showed that mitochondria of *mrg19* and wt consume 3.99 ± 0.11 and 1.27 ± 0.45 nmol of oxygen/min/mg of protein, respectively. This shows that *mrg19* mitochondria consume 3–4 times (P -value $< 4 \times 10^{-3}$) more oxygen than the wt.

Less ROS production in *mrg19* mitochondria

Total ROS produced by isolated mitochondria upon activation with substrates was evaluated by measuring H_2O_2 generation. As H_2O_2 is the most stable form of ROS which is also membrane diffusible, detection using Amplex Red dye has become a convenient and well accepted method for ROS measurement in isolated mitochondria (Chalmers & Nicholls, 2003; Barros et al., 2004; Votyakova & Reynolds, 2004). Amplex Red reacts with H_2O_2 in the presence of horseradish peroxidase (HRP) enzyme and is oxidized into the fluorescence compound resorufin. Therefore, fluorescence intensity of resorufin is directly proportional to H_2O_2 . As complex I and complex III are the major site of the ROS production in mitochondria, H_2O_2 production was measured with succinate (substrate for succinate dehydrogenase complex II) and NADH (substrate for NADH dehydrogenase, complex I). Our results show that *mrg19* mitochondria produce approximately twofold less H_2O_2 (P -value = 0.009) than wt (Fig. 2B) when succinate (substrate of complex II) is used to activate mitochondria and approximately 1.5-fold less H_2O_2 (P -value = 0.002) than wt cells (Fig. 2C) when NADH (substrate of complex I) is used to activate mitochondria. Altogether, these results suggest that mitochondria of *mrg19* produce less ROS despite consuming more oxygen.

Equal membrane potential of *mrg19* and wt mitochondria

The mitochondrial membrane potential is a vital aspect of respiring mitochondria and has also been linked to mitochondrial function. It is known that mitochondrial membrane potential can affect ROS and ATP generation therefore mitochondrial membrane potential was also measured. This was measured by using JC-1 dye, the aggregation of which depends upon the membrane potential. Figure 2D shows that there is no difference in the ratio of fluorescence intensity at 595 and 535 nm for mitochondria of both strains. This implies that there is no difference in the membrane potential of both strains.

Higher ATP generation in *mrg19* mitochondria

The efficiency of ETC can also be evaluated by measuring the ATP generation in isolated mitochondria. In the same way, measurement of cellular ATP content tells about the physiological state (respiratory or fermentation) of the cell. ATP generation was estimated in isolated mitochondria of *mrg19* and wt cells. Figure 2E shows that 249 ± 9.6 and 220 ± 7.0 ng ATP/mg protein (P -value = 0.003) was generated by *mrg19* and wt mitochondria, respectively. The cellular content of ATP was also measured by using cell lysate of 10^8 cells. Cell lysate was

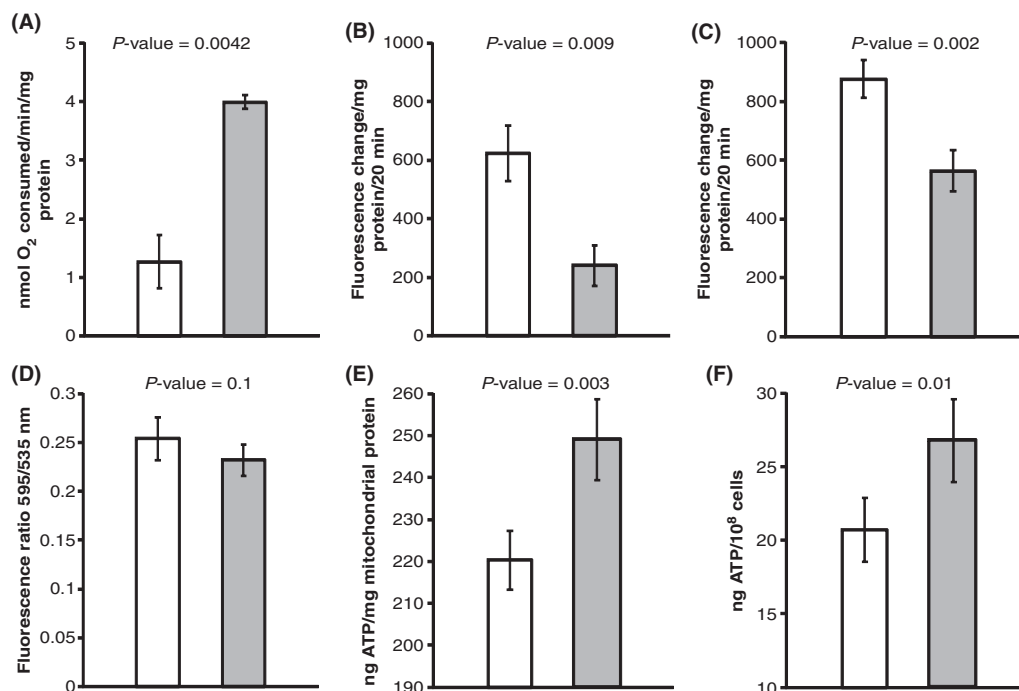


Fig. 2 Deletion of *MRG19* increases mitochondrial efficiency. Values shown here are the average of three independent experiments and error bars refer to the standard error of the mean. White and grey bars represent wt and *mrg19* mitochondria, respectively. (A) Oxygen consumption assay; mitochondria of *mrg19* and wt are energized using succinate. The result is measured in nmol oxygen consumed/min/mg protein for *mrg19* and wt mitochondria. (B, C) H_2O_2 measurement; total H_2O_2 produced by mitochondria was measured using amplex red dye when succinate and NADH were used as substrate, respectively. The results are shown as fluorescence change/mg protein/20 min. (D) Membrane potential of *mrg19* and wt mitochondria was measured by using JC-1 dye. The ratio of fluorescence at 595 and 535 corresponds to membrane potential. (E) ATP measurement; amount of ATP produced by isolated mitochondria is measured and the result is presented as ng ATP/mg protein. (F) Total ATP content of the cell was measured and the result is represented as ng ATP/ 10^8 cells.

prepared by an enzymatic method and was used immediately to measure cellular content of ATP in *mrg19* and wt cells. It was found that the cellular content of ATP is significantly higher (*mrg19*: 26.7 ± 2.8 ng ATP/ 10^8 cells; wt: 20.7 ± 2.1 ng ATP/ 10^8 cells; P -value = 0.01) in *mrg19* than wt cells (Fig. 2F). These results altogether provide evidence for a higher mitochondrial efficiency in *mrg19* cells than wt cells.

Higher expression of mitochondrial complex proteins

Two-dimensional Blue Native Page (2D BN-PAGE) is used to separate membrane proteins and has been well established to separate mitochondrial ETC proteins (Schagger & Pfeiffer, 2000; Lascaris *et al.*, 2003). 2D BN-PAGE was performed as described in Materials and methods to find differences in expression of proteins of the ETC. The images of the gels (Fig. 3A,B) showed visible differences between the proteins of complex V and complex II. Further quantitation of protein spots was performed using PDQuest software version 7.1 (Bio-Rad Laboratories India Pvt. Ltd., Gurgaon, India) as per the manufacturer's manual. The quantitation data also suggest higher expression of most of the proteins in *mrg19* than wt counterpart (Supporting information).

Saccharomyces cerevisiae has separate external and internal NADH dehydrogenases instead of complex-I, as found in mammalian mitochondria. The external NADH dehydrogenase

consists of Nde1 (62 kDa) and Nde2 (61 kDa), while the internal NADH dehydrogenase consists of the single protein Ndi1 (56 kDa). These three proteins could not be identified in 2D BN-PAGE because of their small molecular weight with respect to other complexes; Hence, the activity assay (see Materials and methods) was performed separately for these proteins (Fig. 3D). It was found that expression of the Nde2 and Ndi1 proteins was 5.8 ± 0.16 (p -value = 0.02) and 2.5 ± 0.28 (p -value = 0.03) fold higher in *mrg19* than in the wt cells, respectively (Supporting information). However, there is no change in the expression of Nde1. Quantitation of protein band upon performing the in-gel activity assay was done using Quantity-one software (BioRad). Results of 2D BN-PAGE and in-gel activity assay of NADH dehydrogenases suggest higher amounts of ETC proteins in the *mrg19* mitochondria.

Higher gene expression of mitochondrial ETC

Quantitative real time PCR was also performed for relative quantitation of ETC genes in wt and *mrg19* condition. Two genes from each complex was randomly selected and analyzed for their expression. Table 1 shows significant higher expression of ETC genes in *mrg19* than wt. Results of 2D BN-PAGE, in-gel activity assay of NADH dehydrogenases and qRT-PCR data strongly suggest the presence of higher amounts of ETC proteins in *mrg19* mitochondria than the wt.

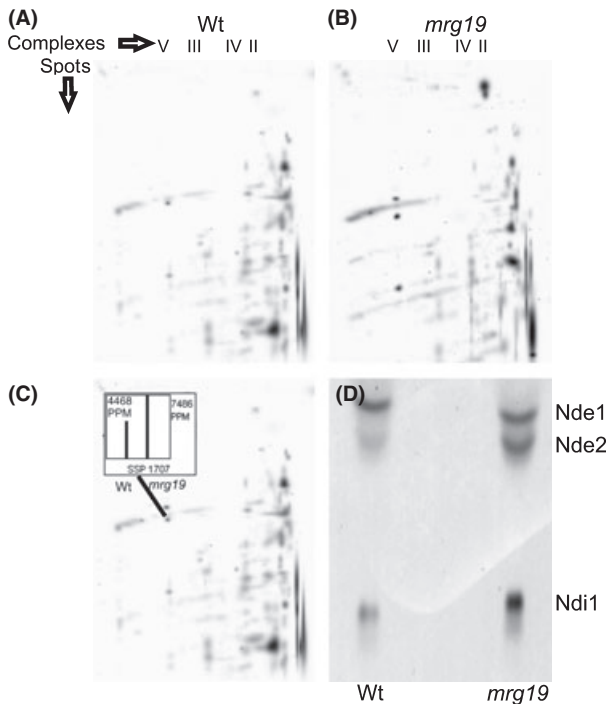


Fig. 3 Higher amounts of mitochondrial complex proteins in *mrg19* mitochondria. (A, B) represent 2D BN-PAGE for mitochondrial membrane protein of wt and *mrg19* mitochondria, respectively. (C) Gaussian master image that contains the spots of both the gels as generated by the PD Quest software during image analysis. The inset shows an example of spot quantitation. (D) In-gel assay for Nde1, Nde2 and Ndi1 protein; 100 µg of mitochondrial membrane protein was loaded for wt and *mrg19* mitochondria. Lane 1 and lane 2 show the activity bands of Nde1, Nde2 and Ndi1 of wt and *mrg19*, respectively.

Table 1 Table for qRT-PCR data. The first column shows the complex name, second column shows gene name, third column shows the fold change for gene expression in *mrg19* in comparison with wt, fourth column shows standard error of the mean and the fifth column shows statistical significance

Complexes	Genes	Fold change in <i>mrg19</i>	SEM	P-value
Complex I	<i>NDE2</i>	5.44	±1.40	1.7×10^{-2}
	<i>NDI1</i>	5.12	±0.33	1.25×10^{-4}
Complex II	<i>SDH1</i>	2.85	±0.20	4.5×10^{-4}
	<i>SDH4</i>	3.20	±0.68	1.5×10^{-2}
Complex III	<i>CYT1</i>	3.47	±1.07	4.1×10^{-2}
	<i>QCR8</i>	2.25	±0.59	5.1×10^{-2}
Complex IV	<i>COX4</i>	3.77	±0.32	5.4×10^{-4}
	<i>COX5A</i>	2.16	±0.33	1.2×10^{-2}
Complex V	<i>ATP4</i>	2.12	±0.63	7.5×10^{-2}
	<i>ATP1</i>	1.46	±0.20	4.0×10^{-2}

Status of antioxidant enzymes

To compare the status of antioxidant enzymes in *mrg19* and wt, qRT-PCR was performed for *SOD2*, *CTA1*, *GPX2* genes. The expression of *SOD2*, *CTA1*, *GPX2* were found 2.03 ± 0.16 (P -value = 0.001), 1.77 ± 0.27 (P -value = 0.025) and 1.32 ± 0.01 (P -value = 1.8×10^{-5}), respectively (Fig. 4B). In-gel activity assay was also performed for Sod2 as described in Materials and

methods section. It was found that the activity of Sod2 was 1.61 ± 0.07 (P -value = 5.4×10^{-4}) fold higher in *mrg19* than wt mitochondria (Fig. 4C,D). All together these results suggest higher antioxidant activity in the *mrg19* mitochondria when compared with the wt.

Discussion

Low mitochondrial ROS production as a result of mitochondrial biogenesis seems to be important for longevity in model organism ranging from yeast to mammals. For instance, over-expression of *HAP4* (transcriptional activator and global regulator of ETC genes) has been found to increase respiration and mitochondrial biogenesis in yeast (Lascaris *et al.*, 2003). Furthermore, mitochondrial biogenesis in mice (Nisoli *et al.*, 2005), human cell lines (Lopez-Lluch *et al.*, 2006) and human muscle (Civitarese *et al.*, 2007) have also been reported in calorie restriction condition. To investigate whether mitochondrial biogenesis in *mrg19* is responsible for the longevity, we checked the content of mitochondria present per cell in both the wt and *mrg19* strains. Surprisingly, flow cytometry analysis and mtDNA estimation showed comparable numbers of mitochondria in both the condition (Fig. 1E,F) suggesting that mitochondrial biogenesis is not the reason for the previously reported longevity of *mrg19*.

We have previously observed (Kharade *et al.*, 2005) that *mrg19* cells consume more oxygen than wt cells. To investigate whether this is caused by increased mitochondrial activity, oxygen consumption by isolated mitochondria of *mrg19* and its wt counterpart was measured. The results showed that *mrg19* mitochondria consume almost 3–4 times more oxygen (Fig. 2A). Increased oxygen consumption could be used either for ATP generation or lead to increased mtROS production or both. To evaluate the fate of higher oxygen consumption in *mrg19* mitochondria, mtROS production was checked and it was found that mtROS production in *mrg19* mitochondria was low (Fig. 2B,C). Higher oxygen consumption and less mtROS production suggest that the mitochondria of *mrg19* may work more efficiently. As membrane potential has been linked to mtROS production (Korshunov *et al.*, 1997; Nicholls, 2004), we investigated if the higher efficiency of *mrg19* mitochondria is caused by a difference in the membrane potential. Measurement of mitochondrial membrane potential surprisingly revealed no difference between the *mrg19* and wt mitochondria (Fig. 2D). Therefore, our observation that mtROS production is low suggests that the increased oxygen consumption is more likely to be used in ATP generation in *mrg19* mitochondria. As hypothesized, the ATP measurement in isolated mitochondria (Fig. 2E) showed higher ATP generation in *mrg19*. Altogether, these results point to an important conclusion that deletion of *MRG19* results in higher metabolic rate and higher efficiency of mitochondria in *mrg19* than wt. These results therefore suggest that higher metabolic rate and higher mitochondrial efficiency, which in turn is likely to cause less oxidative damage, contributes to previously observed longevity of *mrg19*.

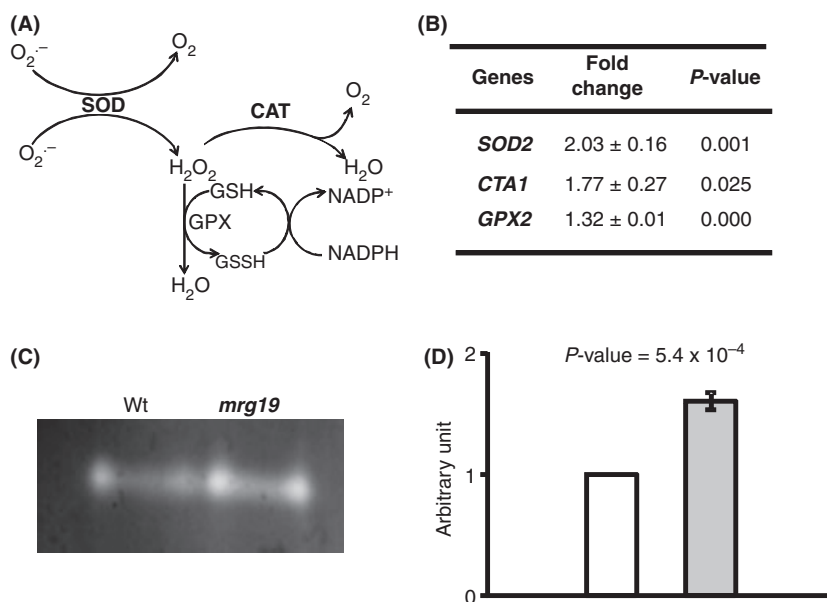


Fig. 4 Higher expression of mitochondrial antioxidant enzymes. (A) Schematic diagram of how antioxidant enzymes scavenge ROS. (B) qRT-PCR data for antioxidant enzymes present in mitochondria. (C) In-gel activity of Sod2 enzyme. (D) Bar graph for the quantitation of band intensity of Sod2. Densitometric analysis was performed for three independent assay by using Quantity-one software from Bio-Rad. Intensity of *mrg19* (grey bar) was normalized with respect to bands of the wt (white bar) as per the software manual.

The observed higher oxygen consumption and low mtROS generation in *mrg19* cells could be due to one of the two possibilities: (i) increased mitochondrial biogenesis, thereby providing higher ETC proteins in the cell to consume the oxygen efficiently or (ii) similar mitochondrial content but with more ETC components per mitochondria, thereby increasing the total amounts of ETC proteins. As there was no difference in the mitochondrial number in *mrg19* and wt strains, we investigated the second possibility, i.e. increased ETC per mitochondria. Our qRT-PCR results (Table.1) showed higher expression of ETC transcript in *mrg19* mitochondria. The result of '2D BNPAGE' also showed a higher expression of ETC proteins in *mrg19* mitochondria when compared with the wt mitochondria (Fig. 3A–C). In the same way, complex-I (NADH dehydrogenase) activity assay also showed a higher activity for Nde2 and Ndi1 proteins in *mrg19* mitochondria (Fig. 3D). All these results indicate that *mrg19* cells maintain the same number of mitochondria and that the increased mitochondrial efficiency can be explained due to an increase in the amount of ETC per mitochondria. This might explain the observed higher oxygen consumption, ATP synthesis and low ROS production despite identical mitochondrial potential in *mrg19* and wt cells. In this context, it is interesting to note that Bonawitz *et al.* (2007) have shown that deletion of *TOR1* in yeast causes higher mitochondrial oxygen consumption and low ROS production. The authors have shown that higher expression of ETC components might be a reason for their observed low ROS production and increased oxygen consumption. Moreover, a recent study from same group (Pan & Shadel, 2009) has demonstrated higher number of ETC complexes per mitochondria in *TOR1* null mutant rather than more mitochondria/cell. Above two studies from same group together suggest that, similar to *mrg19* cells, higher oxygen consumption and low ROS production in *TOR1* null mutant is also achieved by higher ETC per mitochondria.

In our previous study (Kharade *et al.*, 2005), we have shown that cellular ROS is higher in *mrg19* cells when compared with wt cells. This raises an important question which is why cellular ROS is higher in *mrg19* cells? A trivial explanation could be that the ROS defense mechanism is compromised in *mrg19*, which may lead to higher ROS production at cellular level. However, in our previous study, we have shown that the in-gel activity of both cellular and mitochondrial antioxidant enzymes such as superoxide dismutases [Sod1 (cytoplasmic) and Sod2 (mitochondrial)], catalases [Ctt1 (cytoplasmic) and Cta1 (mitochondrial)] and glutathione peroxidases [Gpx1 (cytoplasmic) and Gpx2 (mitochondrial and cytoplasmic)] were significantly increased in *mrg19* cells in comparison with the wt. Thus, it is unlikely that the increased cellular ROS might be due to compromised ROS defense mechanism. Thus, the alternative explanation for higher cellular ROS generation could be generation of cellular ROS from organelles other than mitochondria in *mrg19* cells. Indeed, peroxisomes and microsomes have been shown to be potential sites of ROS generation other than mitochondria in a cell (Boveris *et al.*, 1972).

Our observation of increased cellular ROS (Kharade *et al.*, 2005) and low mitochondrial ROS (present study) also raises another fundamental question which is how such opposing behavior contribute to longevity? We believe that high cellular ROS increases the longevity according to the hormesis theory. Hormesis theory suggests that high ROS at the cellular level acts as mild stressor for the cells, which results in higher expression of cytosolic and mitochondrial antioxidant systems [as shown in the previous study, Kharade *et al.*, 2005]. We have reconfirmed the expression of mitochondrial antioxidant enzymes in the current study. Consistently, we find a higher expression of antioxidant enzymes *SOD2*, *GPX2*, *CTA1* transcript (Fig. 4B) and higher in-gel activity of Sod2 (Fig. 4C,D) suggesting that higher cellular ROS does indeed leads to increase antioxidant activity. In the current study, we have also

shown that mitochondrial ETC works more efficiently and produce less ROS in *mrg19* when compared with the wt. Thus, the higher mitochondrial antioxidant activity as explained by the hormesis theory in combination with higher ETC efficiency could minimize the mtDNA damage and may lead to longevity. We therefore propose that higher mitochondrial efficiency and hormesis work together to increase the lifespan of *mrg19* cells.

According to the mitochondrial theory of aging, it was initially believed that low metabolic rate is responsible for longevity. However, in a recent review, an alternative possibility was discussed wherein longevity is achieved by increased mitochondrial activity coupled with low mtROS production (Guarente, 2008). The possible explanation provided for the observed low mtROS despite the higher metabolic rate was increased mitochondrial biogenesis. This increased number of mitochondria provides more entry points for electrons and reduces the electron stalling at the ETC. Our results follow a similar pattern of low ROS despite high metabolic rate. However, a crucial difference in our case is that more electron entry points are provided as a result of an increase in the number of ETC per mitochondria rather than mitochondrial biogenesis (Fig. 5). Such a pathway is also economical to the cell as up-regulation of the ETC subunits will cost much less energy than channelizing resources to create new mitochondria. Thus, our data provides evidence for the existence of an alternative explanation as to how higher mitochondrial efficiency could result in longevity in yeast.

In a recent study, Powers *et al.* (2006) have determined the chronological lifespan of 4800 single-gene mutants in yeast and have ranked the mutants according to their survival scores. The authors showed a significant enrichment for

mitochondrial gene mutants in the shortest lived strains. This observation provides another independent evidence for the requirement of functional mitochondria for longevity. In this context, we believe that our study provides a conceptual framework to systematically investigate the mitochondrial functionality in other long lived genetic mutants to dissect the molecular mechanism behind mitochondrial functionality and longevity. We also anticipate that the mechanism proposed here may exist in higher eukaryotes including humans.

Materials and methods

Strains and growth condition

In all the experiments CCFY100 (*W303-1a Mat A ade2-1 ura3-1 trp1-289 leu2-3, 112 his3-11, 15 can1-100 HMRΔE::TRP1 rDNA::ADE2, CAN1 VR TEL::URA3*) and its *MRG19* deleted counterpart *mrg19* (*W303-1a Mat A ade2-1 ura3-1 trp1-289 leu2-3, 112 his3-11, 15 can1-100 HMRΔE::TRP1 rDNA::ADE2, CAN1 VR TEL::URA3 mrg19::LEU2*) of *S. cerevisiae* was used. For the FACS experiments, we used W303-1A (*Mat A ade2-1 ura3-1 trp1-289 leu2-3, 112 his3-11, 15 can1-100*) and its *MRG19* deleted counterpart *mrg19* (*Mat A ade2-1 ura3-1 trp1-289 leu2-3, 112 his3-11, 15 can1-100 MRG19::HIS3*) of *S. cerevisiae*. Cells were grown aerobically at 200 rpm at 30 °C in YPD (yeast extract 1%, peptone 2% and dextrose 2%). In all experiments, cells were harvested at 0.7–1.0 OD₆₀₀.

Fluorescence microscopy and flow cytometry to determine mitochondrial content

To determine if deletion of *MRG19* had any effect on the mitochondrial content in yeast cells, W303 and *MRG19* deleted W303 strain cells were transformed with the pVT100U-mtGFP plasmid. This plasmid contains a mitochondrial presequence, a short linker and the GFP coding region. The mitochondrial presequence is first 69 amino acids of subunit 9 of the F₀ ATPase of *Neurospora crassa* (Su9) which is a well characterized mitochondrial matrix targeting sequence. It encoded GFP that targeted specifically to mitochondria (Westermann & Neupert, 2000). The transformed cells were grown overnight in drop out media (for maintaining the selection pressure for the GFP plasmid), i.e. in YC-ura with 2% dextrose. Cells were harvested and vortexed for making single cell suspension before visualizing them under the fluorescence microscope to confirm the transformation and flow cytometry analysis.

The fluorescence microscopy images and corresponding phase contrast images of mtGFP transformed wt and *mrg19* cells were captured using Nikon epifluorescence microscope at 100× magnification in emersion oil. The flow cytometry data were acquired using FACScalibur™ flow cytometer (BD Biosciences, San Jose, CA, USA). The GFP was excited by an

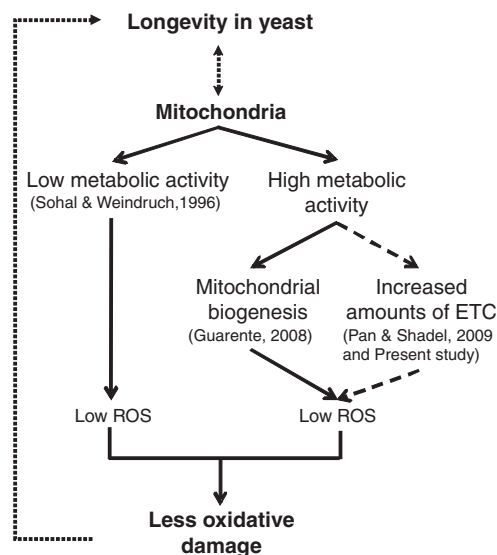


Fig. 5 The pathways describing how mitochondrial activity is linked to longevity in yeast. Dashed arrows represent the findings from this study. In this study, higher O₂ consumption, less ROS production and more ATP synthesis is not caused by increased mitochondrial content. Instead, it is likely to be due to an increased ETC per mitochondria.

argon laser and fluorescence was detected using a 530/30 nm bandpass filter in the FL1 channel. The Cell Quest software (BD Biosciences) was used for data acquisition and data on scatter parameters and histograms were acquired in log mode. Hundred thousand events were evaluated for each sample and the median peak channel obtained from the histograms of each standard was used to analyze their fluorescence intensity.

Isolation of mitochondria

Mitochondria were isolated according to protocol described in (Meisinger *et al.*, 2000) with following modification. Lyticase was used instead of zymolyase for converting cells into spheroplasts. Protein concentration was determined by modified Bradford (1976) method. Freshly isolated mitochondria were used in each experiment.

mtDNA isolation

Mitochondria were first isolated and protein concentration was quantified as described above then 1 mg mitochondrial protein of both wt and *mrg19* strains were used to isolate mtDNA according to method described in Defontaine *et al.* (1991). The mtDNA was then quantified according to Nisoli *et al.* (2005). In brief, equal volumes of mtDNA were run on 0.8% agarose gel. Gels were scanned using Bio-Rad GS 800 densitometer and quantified by Bio-Rad Quantity One (version 4.4) software as described in the software manual.

Oxygen consumption

The O₂ consumption assay was performed with isolated yeast mitochondria at 30 °C as described in Barros *et al.* (2004). Mitochondria were incubated in respiration buffer (0.6 M sorbitol, 20 mM Tris-HCl, 0.5 mM EDTA) in the presence of 5 mM succinate. O₂ consumption was measured by using oxygraph (Hansatech Instruments, Norfolk, England) with a computer-interfaced Clark electrode operating in air tight chamber with continuous stirring.

Hydrogen peroxide measurement

H₂O₂ was measured in isolated mitochondrial suspension in SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS pH 7.2) in the presence and absence of the substrate as described in Barros *et al.*, 2003, except that 5 mM succinate and 10 μM NADH were used as a substrate. In brief, the rate of oxidation of 50 μM Amplex Red to highly fluorescent product resorufin in the presence of 1.0 U/mL HRP was measured for 20 min at 30 °C by using Perkin Elmer LS 50B fluorescence spectrophotometer with continuous stirring. The instrument was operating at excitation and emission wavelength of 563 and 587 nm, respectively. The rate of oxidation of Amplex Red, in the absence of mitochondria from reaction mixture, was negligible.

Membrane potential measurement

Mitochondrial membrane potential was measured by using JC-1 dye as described in Reers *et al.* (Reers *et al.*, 1995) with some minor modifications. In brief, 250 μg of crude mitochondria were activated by incubating with 5 mM succinate, SEM buffer (250 mM sucrose, 1 mM EDTA, 10 mM MOPS pH 7.2) and 5 μg of JC-1 dye for 15 min. The reaction mixture was centrifuged at 12,000 rpm for 15 min at 4 °C to wash out unincorporated dye. The mitochondria were then resuspended into 500 μL of SEM buffer. The amount of monomers and aggregate of JC-1 dye was measured by using Perkin Elmer LS 50B fluorescence spectrophotometer with continuous stirring. The instrument was operated at excitation wavelength of 490 nm and the emitted light was measured at 535 and 595 nm for monomers and aggregated forms of JC-1 dye, respectively.

ATP measurement

The total ATP content of cells was measured by using adenosine 5'-triphosphate (ATP) bioluminescent somatic cell assay kit from Sigma-Aldrich chemicals Pvt. Ltd., Bangalore, India. 10⁸ cells were converted into spheroplasts by treating with lyticase enzyme. Spheroplasts were homogenized at 4 °C to prepare cell lysate. This cell lysate was used immediately to determine ATP content as per the manufacturer's protocol. *In vitro* mitochondrial ATP synthesis was also measured by a bioluminescent assay. In brief, 250 μg of crude mitochondria was energized by incubating with 5 mM succinate, 2 ng ADP and respiration buffer (0.6 M sorbitol, 25 mM potassium phosphate buffer pH = 7.0, 1 mM EDTA, 1 mM MgCl₂) for 15 min at 37 °C. ATP generated was measured by using ATP bioluminescent somatic cell assay kit (Sigma) as indicated in the manufacturer's protocol. Bioluminescent intensity was converted into ng ATP amount by preparing standard ATP curve.

2D BN PAGE

Two dimensional Blue native electrophoresis was performed using the method described in Brookes *et al.* (2002). Briefly, mitochondrial proteins were extracted by using 10% w/v n-dodecyl-β-D-maltoside. A 5–12% gradient gel with 4% stacker was used for the first dimension of the Blue Native Page. One hundred micrograms of membrane proteins was loaded for wt and *mrg19*. The lane of separated protein was then cut and slid on to 12% SDS PAGE for separation of the subunits in the complexes on second dimension. The gel was stained with silver staining method and scanned by using Bio-Rad GS 800 densitometer. The spots were analyzed with PDQuest software of Bio-Rad according to the software manual.

Nde1, Nde2 and Ndi1 in-gel activity

The mitochondrial membrane was prepared according to Brookes *et al.*, 2002. Membrane proteins were separated by

CN-PAGE with some modification and in-gel activity was performed. In brief, 15% gel was used to separate the proteins along with 4% stacking gel. The anode buffer comprised of imidazole (25 mM) pH 7.0. The cathode buffer comprised tricine (50 mM) and imidazole (7.5 mM). The gel, buffers and electrophoresis apparatus were chilled at 4 °C before samples (100 µg protein for *mrg19* and wt strain each) were loaded. Electrophoresis was run at 40 V, 4 °C for overnight till the dye front reached almost the end of the gel. NADH dehydrogenase activity was analyzed by incubating the gel for 1 h in a solution containing Tris 100 mM (pH 7.4), glycine 768 mM (pH 7.4), nitroblue tetrazolium 0.04% w/v, NADH 0.1 mM. Furthermore, the gel was incubated in 50% methanol and 10% acetic acid for 15 min to fix the color of NADH dehydrogenase reacting bands and then preserved in 10% acetic acid (Molnar et al., 2004). Gels were scanned using Bio-Rad GS 800 densitometer and quantified by Bio-Rad Quantity One version 4.4 software, as described by Agarwal et al. (2005).

Quantitative real time PCR

Fifty micrograms of total RNA, isolated from the wt and *mrg19* strains using hot-phenol method, was reverse transcribed to cDNA according to method described in Vyas et al. (2005). Primers were designed using online Primer3 software (Rozen & Skaletsky, 2000). Triplicate PCR reactions were carried out for genes of interest with the intercalating dye SybrGreen. Each sample (25 µL) contained 12.5 µL SYBR® Premix Ex Taq™ (Perfect Real Time) mix (Takara Bio. Inc., Shiga, Japan), 0.2 µM each primer, 8.2 ng cDNA. PCR cycles were programmed on Smart Cycler® (Cepheid, Sunnyvale, CA, USA). The data of qRT-PCR was analyzed by comparative C_T method (Schmittgen & Livak, 2008). *URA3* gene was used as a reference gene for analysis.

SOD2 in-gel activity

Isolated mitochondria of wt and *mrg19* were used for Sod2 activity. One hundred and fifty micrograms of wt and *mrg19* mitochondrial proteins were separated on 12% denaturing polyacrylamide gel with 5% stacking gel. Electrophoresis was performed at 60 V at 4 °C till the dye front reached almost the end of the gel. Sod2 gel activity was performed according to Kharade et al. (2005). Gels were scanned using Bio-Rad GS 800 densitometer and quantified by Bio-Rad Quantity One version 4.4 software according to the software manual.

Acknowledgments

The authors thank Dr Paiké Jayadeva Bhat, Indian Institute of Technology, Mumbai, India for providing the plasmid to delete *MRG19*. The authors also thank Westermann B at the Institut für Physiologische Chemie, Germany for providing pVT100U-mtGFP, Praveen Sharma at National Institute of Pharmaceutical

Education & Research, Mohali, India, Sukhwinder Kaur and Dr. Girish Varshney at Institute of Microbial Technology, Chandigarh, India for helping in flow-cytometry experiment, Sarath Chandra Janga and Arthur Wuster, MRC Laboratory of Molecular Biology, England for thoughtful comments on the manuscript. NR thanks ICMR for financial support. NM, MMB and NR thank the Commonwealth split-site fellowship program for their support.

References

- Agarwal S, Sharma S, Agrawal V, Roy N (2005) Caloric restriction augments ROS defense in *S. cerevisiae*, by a Sir2p independent mechanism. *Free Radic. Res.* **39**, 55–62.
- Barja G (2004) Free radicals and aging. *Trends Neurosci.* **27**, 595–600.
- Barja G, Herrero A (2000) Oxidative damage to mitochondrial DNA is inversely related to maximum life span in the heart and brain of mammals. *FASEB J.* **14**, 312–318.
- Barros MH, Netto LE, Kowaltowski AJ (2003) H(2)O(2) generation in *Saccharomyces cerevisiae* respiratory pet mutants: effect of cytochrome c. *Free Radic. Biol. Med.* **35**, 179–188.
- Barros MH, Bandy B, Tahara EB, Kowaltowski AJ (2004) Higher respiratory activity decreases mitochondrial reactive oxygen release and increases life span in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **279**, 49883–49888.
- Bevilacqua L, Ramsey JJ, Hagopian K, Weindruch R, Harper ME (2005) Long-term caloric restriction increases UCP3 content but decreases proton leak and reactive oxygen species production in rat skeletal muscle mitochondria. *Am. J. Physiol. Endocrinol. Metab.* **289**, E429–E438.
- Bishop NA, Guarente L (2007a) Genetic links between diet and life span: shared mechanisms from yeast to humans. *Nat. Rev. Genet.* **8**, 835–844.
- Bishop NA, Guarente L (2007b) Two neurons mediate diet-restriction-induced longevity in *C. elegans*. *Nature* **447**, 545–549.
- Bitterman KJ, Medvedik O, Sinclair DA (2003) Longevity regulation in *Saccharomyces cerevisiae*: linking metabolism, genome stability, and heterochromatin. *Microbiol. Mol. Biol. Rev.* **67**, 376–399 (table of contents).
- Bonawitz ND, Rodeheffer MS, Shadel GS (2006) Defective mitochondrial gene expression results in reactive oxygen species-mediated inhibition of respiration and reduction of yeast life span. *Mol. Cell. Biol.* **26**, 4818–4829.
- Bonawitz ND, Chatenay-Lapointe M, Pan Y, Shadel GS (2007) Reduced TOR signaling extends chronological life span via increased respiration and upregulation of mitochondrial gene expression. *Cell Metab.* **5**, 265–277.
- Boveris A, Oshino N, Chance B (1972) The cellular production of hydrogen peroxide. *Biochem. J.* **128**, 617–630.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.
- Brookes PS, Pinner A, Ramachandran A, Coward L, Barnes S, Kim H, Darley-Usmar VM (2002) High throughput two-dimensional blue-native electrophoresis: a tool for functional proteomics of mitochondria and signaling complexes. *Proteomics* **2**, 969–977.
- Chalmers S, Nicholls DG (2003) The relationship between free and total calcium concentrations in the matrix of liver and brain mitochondria. *J. Biol. Chem.* **278**, 19062–19070.
- Choksi KB, Nuss JE, Boylston WH, Rabek JP, Papaconstantinou J (2007) Age-related increases in oxidatively damaged proteins of

- mouse kidney mitochondrial electron transport chain complexes. *Free Radic. Biol. Med.* **43**, 1423–1438.
- Civitaresse AE, Carling S, Heilbronn LK, Hulver MH, Ukropcova B, Deutsch WA, Smith SR, Ravussin E (2007) Calorie restriction increases muscle mitochondrial biogenesis in healthy humans. *PLoS Med.* **4**, e76.
- Das M, Bhat PJ (2005) Disruption of *MRG19* results in altered nitrogen metabolic status and defective pseudohyphal development in *Saccharomyces cerevisiae*. *Microbiology* **151**, 91–98.
- Defontaine A, Lecocq FM, Hallet JN (1991) A rapid miniprep method for the preparation of yeast mitochondrial DNA. *Nucleic Acids Res.* **19**, 185.
- Guarente L (2008) Mitochondria – a nexus for aging, calorie restriction, and sirtuins? *Cell* **132**, 171–176.
- Hansen M, Hsu AL, Dillin A, Kenyon C (2005) New genes tied to endocrine, metabolic, and dietary regulation of lifespan from a *Caenorhabditis elegans* genomic RNAi screen. *PLoS Genet.* **1**, 119–128.
- Harman D (1956) Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* **11**, 298–300.
- Harman D (1972) The biologic clock: the mitochondria? *J. Am. Geriatr. Soc.* **20**, 145–147.
- Kabir MA, Khanday FA, Mehta DV, Bhat PJ (2000) Multiple copies of *MRG19* suppress transcription of the *GAL1* promoter in a *GAL80*-dependent manner in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **262**, 1113–1122.
- Kaeberlein M, Hu D, Kerr EO, Tsuchiya M, Westman EA, Dang N, Fields S, Kennedy BK (2005a) Increased life span due to calorie restriction in respiratory-deficient yeast. *PLoS Genet.* **1**, e69.
- Kaeberlein M, Powers RW, III, Steffen KK, Westman EA, Hu D, Dang N, Kerr EO, Kirkland KT, Fields S, Kennedy BK (2005b) Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. *Science* **310**, 1193–1196.
- Khanday FA, Saha M, Bhat PJ (2002) Molecular characterization of *MRG19* of *Saccharomyces cerevisiae*. Implication in the regulation of galactose and nonfermentable carbon source utilization. *Eur J Biochem.* **269**, 5840–5850.
- Kharade SV, Mittal N, Das SP, Sinha P, Roy N (2005) *Mrg19* depletion increases *S. cerevisiae* lifespan by augmenting ROS defence. *FEBS Lett.* **579**, 6809–6813.
- Kirkwood TB, Austad SN (2000) Why do we age? *Nature* **408**, 233–238.
- Korshunov SS, Skulachev VP, Starkov AA (1997) High protonic potential actuates a mechanism of production of reactive oxygen species in mitochondria. *FEBS Lett.* **416**, 15–18.
- Lascaris R, Bussemaker HJ, Boorsma A, Piper M, van der Spek H, Grivell L, Blom J (2003) Hap4p overexpression in glucose-grown *Saccharomyces cerevisiae* induces cells to enter a novel metabolic state. *Genome Biol.* **4**, R3.
- Lavoie H, Whiteway M (2008) Increased respiration in the *sch9Delta* mutant is required for increasing chronological life span but not replicative life span. *Eukaryot. Cell* **7**, 1127–1135.
- Lee SS, Lee RY, Fraser AG, Kamath RS, Ahringer J, Ruvkun G (2003) A systematic RNAi screen identifies a critical role for mitochondria in *C. elegans* longevity. *Nat. Genet.* **33**, 40–48.
- Lin SJ, Kaeberlein M, Andalis AA, Sturtz LA, Defossez PA, Culotta VC, Fink GR, Guarente L (2002) Calorie restriction extends *Saccharomyces cerevisiae* lifespan by increasing respiration. *Nature* **418**, 344–348.
- Lin CH, MacGurn JA, Chu T, Stefan CJ, Emr SD (2008) Arrestin-related ubiquitin-ligase adaptors regulate endocytosis and protein turnover at the cell surface. *Cell* **135**, 714–725.
- Lopez-Lluch G, Hunt N, Jones B, Zhu M, Jamieson H, Hilmer S, Cascajo MV, Allard J, Ingram DK, Navas P, de Cabo R (2006) Calorie restriction induces mitochondrial biogenesis and bioenergetic efficiency. *Proc. Natl. Acad. Sci. USA.* **103**, 1768–1773.
- Lopez-Torres M, Gredilla R, Sanz A, Barja G (2002) Influence of aging and long-term caloric restriction on oxygen radical generation and oxidative DNA damage in rat liver mitochondria. *Free Radic. Biol. Med.* **32**, 882–889.
- Masoro EJ (2000) Caloric restriction and aging: an update. *Exp. Gerontol.* **35**, 299–305.
- McMurray MA, Gottschling DE (2004) Aging and genetic instability in yeast. *Curr. Opin. Microbiol.* **7**, 673–679.
- Meisinger C, Sommer T, Pfanner N (2000) Purification of *Saccharomyces cerevisiae* mitochondria devoid of microsomal and cytosolic contaminants. *Anal. Biochem.* **287**, 339–342.
- Molnar AM, Alves AA, Pereira-da-Silva L, Macedo DV, Dabbeni-Sala F (2004) Evaluation by blue native polyacrylamide electrophoresis colorimetric staining of the effects of physical exercise on the activities of mitochondrial complexes in rat muscle. *Braz. J. Med. Biol. Res.* **37**, 939–947.
- Nicholls DG (2004) Mitochondrial membrane potential and aging. *Aging Cell* **3**, 35–40.
- Nikko E, Sullivan JA, Pelham HR (2008) Arrestin-like proteins mediate ubiquitination and endocytosis of the yeast metal transporter *Smf1*. *EMBO Rep.* **9**, 1216–1221.
- 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) Calorie restriction promotes mitochondrial biogenesis by inducing the expression of eNOS. *Science* **310**, 314–317.
- Pamplona R, Barja G (2007) Highly resistant macromolecular components and low rate of generation of endogenous damage: two key traits of longevity. *Ageing Res. Rev.* **6**, 189–210.
- Pan Y, Shadel GS (2009) Extension of chronological life span by reduced TOR signaling requires down-regulation of Sch9p and involves increased mitochondrial OXPHOS complex density. *Aging* **1**, 131–145.
- Powers RW, III, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S (2006) Extension of chronological life span in yeast by decreased TOR pathway signaling. *Genes Dev.* **20**, 174–184.
- Ramsey JJ, Harper ME, Weindruch R (2000) Restriction of energy intake, energy expenditure, and aging. *Free Radic. Biol. Med.* **29**, 946–968.
- Reers M, Smiley ST, Mottola-Hartshorn C, Chen A, Lin M, Chen LB (1995) Mitochondrial membrane potential monitored by JC-1 dye. *Methods Enzymol.* **260**, 406–417.
- Richter C, Park JW, Ames BN (1988) Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc. Natl. Acad. Sci. USA.* **85**, 6465–6467.
- Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol.* **132**, 365–386.
- Sanz A, Caro P, Ibanez J, Gomez J, Gredilla R, Barja G (2005) Dietary restriction at old age lowers mitochondrial oxygen radical production and leak at complex I and oxidative DNA damage in rat brain. *J. Bioenerg. Biomembr.* **37**, 83–90.
- Schagger H, Pfeiffer K (2000) Supercomplexes in the respiratory chains of yeast and mammalian mitochondria. *EMBO J.* **19**, 1777–1783.
- Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. *Nat. Protoc.* **3**, 1101–1108.
- Sohal RS, Weindruch R (1996) Oxidative stress, caloric restriction, and aging. *Science* **273**, 59–63.
- Sohal RS, Agarwal S, Candas M, Forster MJ, Lal H (1994) Effect of age and caloric restriction on DNA oxidative damage in different tissues of C57BL/6 mice. *Mech. Ageing Dev.* **76**, 215–224.
- Trifunovic A, Larsson NG (2008) Mitochondrial dysfunction as a cause of ageing. *J. Intern. Med.* **263**, 167–178.

- Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE, Bohlooly YM, Gidlof S, Oldfors A, Wibom R, Tornell J, Jacobs HT, Larsson NG (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* **429**, 417–423.
- Votyakova TV, Reynolds IJ (2004) Detection of hydrogen peroxide with Amplex Red: interference by NADH and reduced glutathione auto-oxidation. *Arch. Biochem. Biophys.* **431**, 138–144.
- Vyas VK, Berkey CD, Miyao T, Carlson M (2005) Repressors Nrg1 and Nrg2 regulate a set of stress-responsive genes in *Saccharomyces cerevisiae*. *Eukaryot. Cell* **4**, 1882–1891.
- Weinert BT, Timiras PS (2003) Invited review: theories of aging. *J. Appl. Physiol.* **95**, 1706–1716.
- Westermann B, Neupert W (2000) Mitochondria-targeted green fluorescent proteins: convenient tools for the study of organelle biogenesis in *Saccharomyces cerevisiae*. *Yeast* **16**, 1421–1427.

Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 Bar graph for the quantitation of band intensity of Nde1, Nde2 and Ndi1. Densitometric analysis was performed by using

Quantity-one software from Bio-Rad. Bands of the wt were given the value of one and density of corresponding band in *mrg19* was measured in relation to the wt band.

Fig. S2 Densitometric analysis of 2D BN-PAGE for subunits of wt and *mrg19* mitochondrial respiratory complexes. PD Quest provides an identifier for every spot on the gel. The red circles on the gel show the location and identity of the spots.

Table S1 Table for densitometric quantity of spots present in both wt and *mrg19* gels. The first column shows the complex name, second column shows identifier of spot, third and fourth column show normalized quantity of spot density and fifth column shows ratio of quantity of *mrg19* spot to wt spot. The complexes have been identified in accordance to Lascaris *et al.* (2003).

As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.