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PII: S0005-2728(18)30045-8
DOI: doi:10.1016/j.bbabio.2018.03.011
Reference: BBABIO 47891

To appear in:

Received date: 9 October 2017
Revised date: 13 March 2018
Accepted date: 19 March 2018

Please cite this article as: Alicia F. Dubinski, Raffaele Camasta, Tyler G.B. Soule, Bruce H. Reed, D. Moira Glerum , Consequences of cytochrome c oxidase assembly defects for the yeast stationary phase. The address for the corresponding author was captured as affiliation for all authors. Please check if appropriate. Bbabio(2018), doi:10.1016/j.bbabio.2018.03.011

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# Consequences of cytochrome *c* oxidase assembly defects for the yeast stationary phase

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**Keywords:** yeast, oxidative stress, mitochondria, cytochrome *c* oxidase (complex IV) assembly, stationary phase

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**ABSTRACT**

The assembly of cytochrome *c* oxidase (COX) is essential for a functional mitochondrial respiratory chain, although the consequences of a loss of assembled COX at yeast stationary phase, an excellent model for terminally differentiated cells in humans, remain largely unexamined. In this study, we show that a wild-type respiratory competent yeast strain at stationary phase is characterized by a decreased oxidative capacity, as seen by a reduction in the amount of assembled COX and by a decrease in protein levels of several COX assembly factors. In contrast, loss of assembled COX results in the decreased abundance of many mitochondrial proteins at stationary phase, which is likely due to decreased membrane potential and changes in mitophagy. In addition to an altered mitochondrial proteome, COX assembly mutants display unexpected changes in markers of cellular oxidative stress at stationary phase. Our results suggest that mitochondria may not be a major source of reactive oxygen species at stationary phase in cells lacking an intact respiratory chain.

## 1. INTRODUCTION

The yeast *Saccharomyces cerevisiae* is a facultative anaerobe and has proven to be a fruitful model organism for studying inherited mitochondrial defects in humans. However, until recently, most studies have examined mitochondria from yeast cells growing exponentially, where fermentation is the main metabolic pathway. Following the logarithmic phase of growth and the diauxic shift, yeast cells enter a phase of growth, known as the stationary phase, in which their metabolism is switched almost entirely to respiration. Stationary phase cultures have been defined as being saturated, with depleted carbon sources and cells that have become quiescent [1]. A yeast culture growing in typical rich glucose medium may take as long as seven days to reach 'true' stationary phase, at which point there is no further cell growth once the ethanol has been depleted [1, 2]. However, it is now clear that a stationary phase culture contains a mixed population of both quiescent and non-quiescent cells [3]. Nevertheless, there are

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characteristic features associated with stationary phase cultures, including the absence of replication and down-regulation of transcription and protein synthesis, elevated antioxidant defenses, and increased resistance to stress [1, 2, 4-7]. Mitochondrial function has been proposed to play a key role during stationary phase [8], which has obvious implications for organisms harboring respiratory chain defects, and the consequences of the loss of assembled cytochrome *c* oxidase (COX) during this critical period are poorly understood.

As the terminal electron acceptor in the respiratory chain, cytochrome oxidase is a large enzyme complex comprised of 11 subunits in the yeast, *Saccharomyces cerevisiae*. Having subunit polypeptides encoded in two genomes and multiple prosthetic groups complicates COX biogenesis. More than 40 proteins, referred to as assembly factors, are known to be required for generation of a functional COX holoenzyme [9]. In addition to the proteins that are involved in processing of the subunit polypeptides and insertion into the inner mitochondrial membrane, assembly factors are required for the biosynthesis of heme A, which is unique to COX, and for the delivery of copper to the nascent subunits 1 and 2 [10]. Copper transfer from the mitochondrial intermembrane space to the $Cu_A$ and $Cu_B$ sites of yeast COX is essential, and is thought to proceed in a "bucket brigade" fashion *via* three specific proteins – Cox17, Sco1 and Cox11 [11]. In general, yeast cells with COX assembly defects are unable to complete assembly of complex IV in the respiratory chain and are typified by a considerable reduction in steady-state levels of the mitochondrially-encoded subunits (Cox1/2/3) and a loss of the characteristic spectral peak at 605 nm [12].

The importance of COX assembly factors in human disease is underlined by the variety and severity of phenotypes resulting from their mutations. Many patients with Leigh syndrome, the most common mitochondrial disorder, have been found to have mutations in *SURF1* (*SHY1* in yeast) [13], which is believed to function in the provision of newly-synthesized heme A to Cox1 [14]. In addition, mutations in *SCO1* and *SCO2* have been found to cause encephalopathy [15, 16], and mutations in *COX10, COX15*, and *PET191* have been identified in cardiomyopathies [17-20]. Given the many commonalities between the signaling and metabolic pathways of yeast to a multitude of higher eukaryotes, a deeper understanding of COX assembly and function at stationary phase in yeast is likely to provide an improved understanding of the impact of inherited COX deficiencies in terminally differentiated cells in humans.

In this study, we sought to determine how COX function and assembly are affected at stationary phase in a respiratory competent yeast strain and how the loss of assembled COX would impact yeast during the stationary phase. We show that, surprisingly, there are reduced amounts of assembled and functional COX in respiratory competent yeast at the stationary phase, along with characteristics of increased levels of oxidative stress. In contrast, we find that typical markers of oxidative stress in COX assembly mutants are less abundant at the stationary phase, suggesting that a loss of respiration may not give rise to increased oxidative damage in mitochondria.

## 2. METHODS

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**2.1 Strains and Growth Curves** The strains used in this study, which have all been described previously, were: αW303ΔCOX4 (*α ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cox4::URA3*), αW303ΔCOX17 (*α ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cox17::TRP1*), aW303ΔSCO1 (*a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 sco1::URA3*), aW303ΔCOX11 (*a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cox11::HIS3*), aW303ΔCOX15 (*a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 cox15::HIS3*), and aW303ρ° (*a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 rho0*), while aW303 (*a ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1*) served as the respiratory competent parent strain [21, 22].

Overnight cultures of yeast were normalized to an $OD_{600}$ of 0.1 in YPD (2% glucose, 1% yeast extract, 2% peptone) and then grown in a shaking incubator for up to 8 days at $30^0$C and 230 rpm. At each time point, optical density at 600 nm was recorded to measure growth and reproductive capacity was tested by plating serial dilutions in triplicate on YPD agar plates, followed by quantification of colony forming units (CFUs) after 2 days of growth at $30^0$C.

**2.2 Western Blotting** Proteins in whole cell lysates, post-mitochondrial supernatant fractions, crude nuclei or mitochondria were separated on 12% polyacrylamide gels [23] or 16.5% polyacrylamide gels with 6 M urea [24]. Proteins were transferred to nitrocellulose membranes and immunoblotted with antibodies specific to Sdh2 (1:10,000 (gift from Dr. Bernard Lemire, University of Alberta)), Por1 (1:15,000, Sigma–Aldrich, Oakville, ON, Canada), Sod1 (1:500) and cytochrome *c* (1:400) (both from Antibodies-Online GmbH, Atlanta, GA, USA), Sco1 (1:1000, [25]), Cox17 (1:500, [26]), Act1 (1:10,000) and Atg3 (1:1000, both from Abcam, Cambridge, MA, USA), Lon1 (1:1000, gift from Dr. Caroline Suzuki, Rutgers-New Jersey Medical School), Aco1 (1:1000, gift from Dr. Anne-Laure Bulteau, Functional Genomics Institute, Lyon) or the COX holoenzyme originally developed in the Tzagoloff laboratory [27].

The Cox11 antibody was generated by expression of the soluble, intermembrane space portion (amino acids L106-N300) of the protein in *E. coli* BL21, which contained an N-terminal 6xHis tag. The protein was purified by affinity purification on a $Ni^{2+}$-NTA column and 0.1 mg purified protein injected per rabbit, using Freund's complete adjuvant for the initial injection and incomplete adjuvant for the subsequent booster shots (Pocono Rabbit Farms, Canadensis, PA, USA). The titre and specificity of the antisera were tested by comparing the cross-reacting material visualized in a wild-type strain (W303) to that of the *cox11* null strain (Fig. S1), and the antisera then used at a dilution of 1:500 for all subsequent experiments.

Membranes were imaged using a ChemiDoc MP (BioRad Laboratories, Mississauga, ON, Canada) after incubation with horseradish peroxidase-conjugated anti-rabbit (1:10,000; Abcam, Cambridge, MA, USA) or anti-mouse antibody (1:10,000; ThermoFisher, Burlington, ON, Canada). Blots were imaged using a BioRad ChemiDoc MP imager (BioRad Canada, Mississauga, ON, Canada) and analysis was carried out using the ImageLab software. For all Western blots, the Δcox4, Δcox15, and $\rho^0$ samples were run on separate gels from the Δcox17, Δcox11 and Δsco1 samples, with the same wild-type samples serving as an internal control for each blot.

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**2.3 Confocal Microscopy** Wild-type and mutant strains were grown in YPD for either 24 or 192 hrs and 2 x $10^7$ cells were harvested. Following washing in 100 µL PBS, cells were stained with 0.1 µg/mL 4',6-diamidino-2-phenylindole (DAPI) and 100 nM Mitotracker Red (Invitrogen, Burlington, ON, Canada) in the dark for 30 minutes. 1.5 µL of cells were mounted onto a 4% agar bed in minimal media (0.67% nitrogen base without amino acids, 2% glucose). Fluorescent images were obtained with a Zeiss LSM 700 laser scanning confocal microscope, using a 63x, 1.4 NA lens. At least 50 cells were examined and then a representative image was obtained. Mitochondrial morphology images were obtained by forming a Z-stack using the ZEN Blue software (Carl Zeiss, North York, ON, Canada).

**2.4 Quantitative PCR** Yeast total RNA was isolated from cells grown for either 24 hours or 192 hours with the Presto™ Mini RNA Yeast Kit (Geneaid, New Taipei City, Taiwan), followed by assessment of RNA integrity by agarose gel electrophoresis and measurement of RNA concentration with a Nanodrop 3300 (ThermoFisher, Massachusetts, USA). 1 µg of RNA was reverse-transcribed with the SensiFAST™ cDNA Synthesis Kit (FroggaBio, Toronto, Canada) and stored at -20$^0$C. Before qPCR was performed, primers were optimized for annealing temperature and tested for specificity in a PCR reaction with purified yeast genomic DNA. Primer sequences were as follows: Act1 forward: 5' CTGCCGGTATTGACCAAACT 3', Act1 reverse: 5' CGGTGATTTCCTTTTGCATT 3', Pim1 forward: 5' CATCCAGTCGATCGGATTCT 3', Pim1 reverse: 5'AATAAGGGCCGTCTGGCTAT3', Sod1 forward: 5' AACGTGGGTTCCACATTCAT 3', Sod1 reverse: 5' CACCATTTTCGTCCGTCTTT 3'.

qPCR was carried out using the SensiFAST™ SYBR No-ROX Kit, with SYBR Green as the intercalator. Each qPCR reaction was performed in triplicate and a negative control was included to ensure the absence of non-specific amplification. The thermocycling program consisted of 95$^0$C for 2 min, followed by 40 cycles of 5s at 95$^0$C, 10s at 55$^0$C, and 15s at 72$^0$C. After completion of the program, melt-curve data were collected to confirm the presence of the expected product. The ΔCq method was used to analyze the results for *ACT1* transcript, with samples being normalized to the WT at 24 hours. The *PIM1* and *SOD1* transcript levels at each phase of growth were normalized to *ACT1* transcript levels, using the ΔΔCq method [28]. Each sample was tested in triplicate and amplification was performed on three biological replicates.

**2.5 Miscellaneous Methods** Whole cell lysates were obtained from YPD cultures grown to either 24 (exponential phase, EP) or 192 hours (stationary phase, SP). Harvested and washed cells were digested with Zymolyase (BioShop, Burlington, ON, Canada), washed with 1.2 M sorbitol and homogenized using a Dounce homogenizer. Following centrifugation, the supernatant was collected for further analysis. For preparation of nuclei, a Dounce homogenizer was used after Zymolyase digestion, followed by spinning at 1,500xg for 10 minutes and washing once in sorbitol buffer (0.5 M sorbitol, 20 mM Tris pH 7.5, 0.5 mM EDTA). Mitochondria and post-mitochondrial supernatant fractions were prepared as described previously [29], based on a slight modification of the protocol originally described by Faye *et al.* [30]. All protein concentrations were determined using the Folin-Ciocalteu reagent [31].

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COX activity assays and cytochrome spectral analyses were both carried out using intact organelles as described by Tzagoloff *et al.* [32] and the zymographic analysis of Sod1 activity was performed according to the method of Beauchamp and Fridovich [33], with the gels being developed in the light for 1 hour and imaged using a BioRad ChemiDoc MP. Aconitase activity was assayed essentially as described by Bulteau *et al.* [34]. In brief, mitochondria were solubilized in 25 mM $KH_2PO_4$ pH 7.5 containing 0.05% Triton X-100 and activity was measured by measuring $NADP^+$ reduction at 340 nm for 2 minutes after addition of isocitrate dehydrogenase, using sodium citrate (1.0 mM) as the substrate.

## 3. RESULTS

### 3.1 Respiration deficient mutants exhibit decreased cell fitness at the stationary phase.

As part of a long-standing interest in identifying secondary functions for a number of copper-binding COX assembly factors, such as Sco1 and Cox11 [35, 36], we analyzed the promoters of the *COX17*, *SCO1* and *COX11* genes for peroxide-responsiveness in a LacZ assay. While we did not see any changes in β–galactosidase activity in response to changes in peroxide concentrations, we did observe that expression levels driven from the *COX17* promoter increased with increased time in culture (data not shown). This suggested that Cox17 might have a specific role in the yeast stationary phase, which is defined as the phase of growth after glucose depletion when the cell must rely on oxidative phosphorylation. In order to determine whether any observed changes were specific to the Δcox17 mutant, rather than being the result simply of a loss of assembled COX, we also examined five other mutant strains as a means to identify potential physiological variations when different parts of the COX assembly pathway are disrupted. In addition to the *cox17* null mutant, we included *sco1* and *cox11* null mutants (Δsco1, Δcox11), all of which are missing proteins involved in copper delivery for COX assembly. A *cox15* null mutant (Δcox15) also fails to assemble COX but through a defect in the heme A biosynthetic pathway, allowing us to differentiate between the effects of copper acquisition and heme A biosynthesis on the yeast stationary phase. The *cox4* null mutant (Δcox4) is missing the structural subunit 4 and therefore also lacks any detectable assembled COX, but is not thought to have any role in acquisition of prosthetic groups. Lastly, we included a $\rho^0$ strain in our analyses, which lacks mitochondrial DNA (mtDNA) and is therefore missing Complexes III, IV, and V of the respiratory chain; $\rho^0$ strains are frequently used as models for the total absence of respiratory chain activity. The inclusion of this strain allows us to differentiate phenotypic characteristics that are the result solely of the loss of COX as compared to the more generalized respiratory deficiency associated with $\rho^0$ cells.

The transition of yeast from the exponential phase through the diauxic shift and into the stationary phase has been well described in the literature. As reviewed by one of the pioneers of this field [37], studies should be carried out in a manner that is less likely to induce stress on the cells. By incubating yeast in YPD and monitoring their progress, rather than switching them to minimal media or an aqueous buffer after exhaustion of glucose, we chose a physiologically relevant experimental approach. We found that all of

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the mitochondrial mutants exhibit a minor growth defect (Figure 1A), as reflected in the lower optical densities after 24 hours of growth, when compared to the wild-type strain, which continues to increase in cell density from 24 to 48 hours and thereafter plateaus. The literature suggests that a true stationary phase is achieved at between five and eight days in culture [1, 7] and therefore all subsequent experiments with

Figure 1

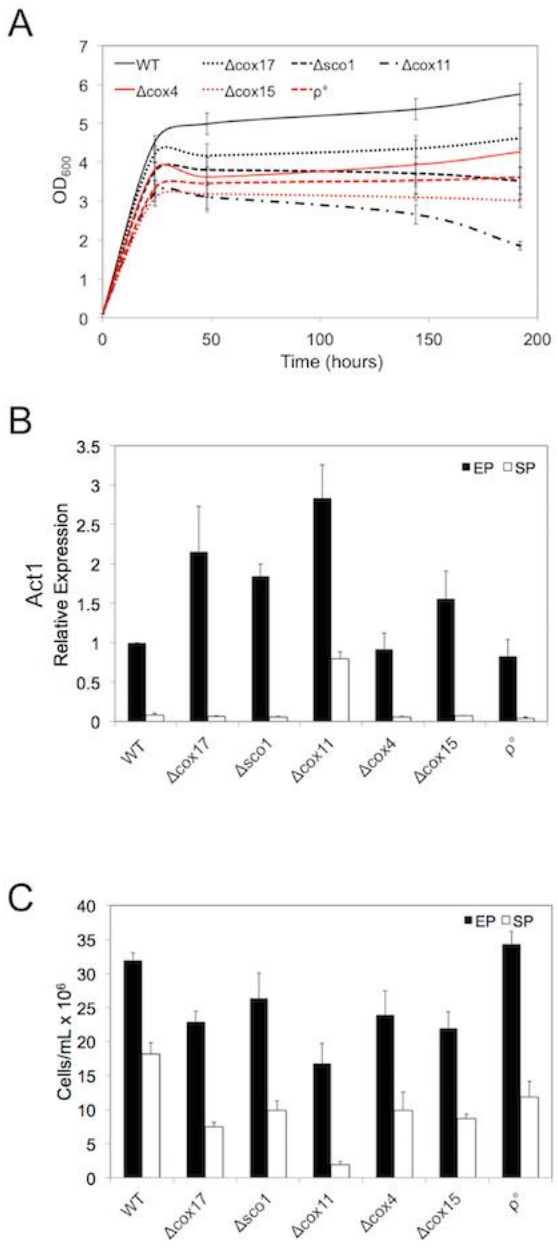


***Figure 1.*** **COX mutants are characterized by decreased reproductive fitness at stationary phase. (A)** The growth of mutants with defects in the copper delivery pathway (Δcox17, Δsco1, Δcox11), along with mutants with defects in non-copper related pathways (Δcox4, Δcox15, $\rho^0$), were assayed by measuring the $OD_{600}$ of the

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cultures at the time points shown. Error bars represent ±SEM of at least 3 independent experiments. **(B)** The relative abundance of the actin (Act1) transcript was assessed from total RNA in all strains at 24 hours (EP) and 192 hours (SP), using qPCR. Relative expression levels were normalized to that of the WT strain at exponential phase. Error bars indicate ±SEM from triplicates of three biological replicates. **(C)** The viability of the WT strain and mutants was assessed by counting colony-forming units (CFUs) after plating serial dilutions of cells from liquid culture. Error bars represent ± SEM from at least 3 independent experiments.

stationary phase cells in the present study used cultures grown for eight days. One of the characteristics that typifies stationary phase in yeast is a significant decrease in the RNA transcripts of many essential proteins, which are usually present at approximately 2-3% of exponential phase levels. In order to confirm that cells had entered stationary phase, total RNA was assessed for levels of actin transcript at both 24 hours and 8 days (Figure 1B) and we observed a considerable decrease in *ACT1* transcript levels in the wild-type strain and all mitochondrial mutants, as expected from the literature [6]. Interestingly, the quantity of actin transcript varied amongst the mutants at exponential phase, although the reason for this is currently unknown (Figure 1B). Nevertheless, significantly reduced levels of actin transcript at 8 days was consistent for all strains and gave us confidence that 8-day cultures were an acceptable representation of yeast stationary phase.

Aside from the *cox11* null strain, all the remaining strains retained an apparently stable cell density by the 8$^{th}$ day in culture, which was surprising in the face of the supposition that a functional respiratory chain at the stationary phase is essential to cell survival. In order to assess the reproductive fitness of the cells, serial dilutions of cultures grown to either 24 hours or 8 days were plated onto YPD and CFUs were counted in triplicate. In cultures harvested at the exponential phase, we found that the COX assembly mutants displayed reduced fitness (16.8 - 26.3 x $10^6$ cells/mL) when compared to that of the wild-type strain (31.9 x $10^6$ cells/mL), which is in keeping with empirical observations regarding wet weight yields from mutant cultures (Figure 1C). Interestingly, the Δcox11 strain, which is specifically sensitive to exogenous hydrogen peroxide [36], has the lowest fitness of any of the COX assembly mutants (16.8 x $10^6$ cells/mL), whereas the $\rho^0$ strain displays CFUs similar to that of the wild-type during exponential phase (34.4 x $10^6$ cells/mL), as has been observed previously [38]. After 8 days in culture, the respiratory competent strain has a reproductive fitness that is reduced by about 50% (18.2 x $10^6$ cells/mL), which is in keeping with reports in the literature [7], while the COX assembly mutants display 20 – 40% of the CFUs of those exhibited at the exponential phase. The strain lacking mtDNA displayed a survival rate similar to that of the COX assembly mutants at stationary phase (11.8 x $10^6$ cells/mL; Figure 1C). Overall, our results show that *S. cerevisiae* strains lacking an intact mitochondrial electron transport chain can survive into the stationary phase, albeit with reduced viability.

**3.2 Cytochrome *c* oxidase is less abundant at stationary phase in wild-type yeast**

In order to provide a context for the loss of COX at stationary phase, we determined the characteristics of COX at the stationary phase in a respiratory competent strain. We

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observed that the specific COX activity of the wild-type strain in intact mitochondria from stationary phase cultures was approximately one-fifth of that measured in mitochondria isolated from exponential phase cells (Figure 2A). As expected, there were only background levels of COX activity in all of the mutant strains at both exponential and stationary phases (data not shown). To examine if the reduced COX activity was due to altered kinetics or a decrease in assembled enzyme, we performed a spectral analysis of reduced – oxidized mitochondrial cytochromes. This standard approach for quantifying the state of COX assembly revealed a marked reduction of the COX peak at 605 nm in mitochondria isolated from the stationary phase cultures (Figure 2B). There also appeared to be a concomitant reduction in the levels of cytochromes *b* and *c*, which is a common finding associated with reduced levels of assembled COX in a variety of yeast mutants [39], likely attributable to the involvement of COX in the respiratory supercomplexes [40]. These data demonstrate that the reduced COX activity is likely a reflection of a reduced amount of assembled COX in mitochondria from wild-type cells in stationary phase cultures.

Given the apparent decrease in assembled COX at stationary phase, we sought to determine whether this was accompanied by a decrease in steady-state levels of the polypeptide subunits. We separated mitochondrial proteins on gels specifically designed to separate low molecular weight proteins, such as the nuclear-encoded COX subunits, and immunoblotted using an antibody to the COX holoenzyme [27] (Figure 2C). In the wild-type strain, we observed similar levels of Cox2, a mtDNA-encoded subunit, as well as Cox4 and the small subunits (7, 7a, 8), which are all nuclear encoded, at exponential and stationary phases (Figure 2C). Interestingly, stationary phase wild-type cultures showed increases in the levels of subunits 5 and 6, which may be a reflection of the previously demonstrated association of these two proteins in COX assembly subcomplexes [39, 41], along with the observation that subunit 5b is expressed under anaerobic or stress conditions [42, 43]. In the mitochondrial mutants, nuclear-encoded subunits (Cox4, 5, 6, 7, 7a, 8) are present at the exponential phase but decrease in abundance at the stationary phase, whereas Cox2, the mitochondrially-encoded subunit, is not detectable in any mutant at either phase of growth, as has been shown previously for yeast strains lacking COX assembly [39]. Taken together, our results identify a detectable reduction in the amount of assembled COX at the stationary phase in respiratory competent yeast.

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Figure 2

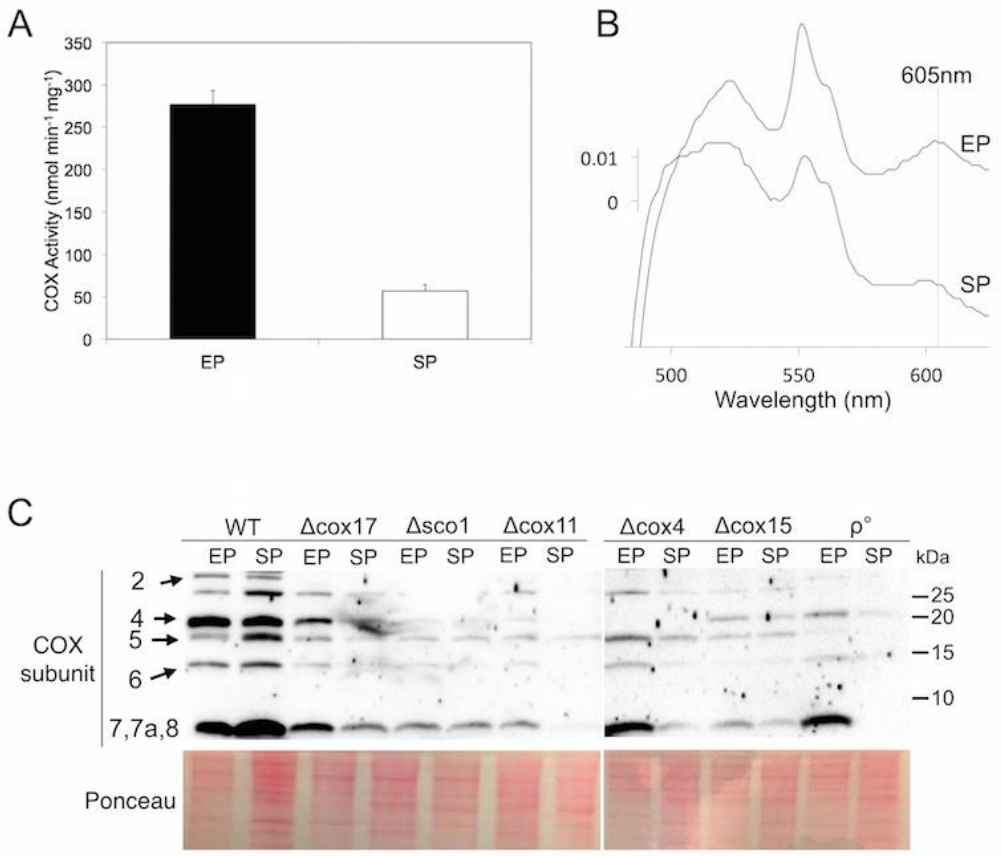


***Figure 2.*** **Assembled cytochrome *c* oxidase is less abundant at stationary phase in respiratory competent yeast. (A)** COX activity was assayed by measuring the oxidation of reduced cytochrome *c* at 550 nm. COX activity in the WT strain is presented at exponential (EP) and stationary phase (SP); error bars represent ±SEM from duplicates of two independent trials. **(B)** Cytochrome spectral analysis of the WT strain at EP and SP reveals a reduced peak at 605 nm, which represents the assembled COX. Spectra were analyzed from 4 independent mitochondrial isolations and a representative spectrum is shown; the absorbance scale is indicated on the left side of the spectrum. **(C)** Western blot analysis of the nuclear-encoded COX subunits was carried out on all strains, using an antibody to the COX holoenzyme [27]. The COX subunits are indicated on the left; the migration of molecular weight markers is indicated on the right; blots were repeated at least twice with different mitochondrial isolations to ensure reproducibility. The Ponceau-stained blot was used to verify equal protein loading and is shown beneath the immunoblot.

### 3.3 COX assembly mutants have altered mitochondrial morphology

The changes in cell viability and reduction of assembled COX led us to ask whether there might be broader mitochondrial changes associated with the stationary phase. We began

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by comparing the mitochondrial morphology of a wild-type strain, cultured to either exponential or stationary phase, by staining cells with the potential-sensitive fluorescent dye, MitoTracker Red. Confocal imaging revealed the well-documented reticular mitochondrial network in cells grown for 24 hours, with slightly heavier staining at the cell cortex (Figure 3). At stationary phase, the mitochondrial reticulum was still present, although less well-defined, and there were more intensely stained foci at the cell periphery (Figure 3), a finding that is in keeping with previous reports of respiratory competent yeast strains as they age [44]. This altered morphology in wild-type yeast from stationary phase cultures may, in fact, be related to the reduced COX content we observed.

Unlike the wild-type strain, the COX assembly mutants and the $\rho^0$ strain were characterized by a virtual absence of a mitochondrial reticulum in exponentially growing cultures, an effect that was magnified in cells taken from stationary phase cultures (Figure 3). Imaging of mutant cells was more difficult due to a relative lack of Mitotracker Red staining, suggesting a decreased membrane potential based on the mechanism of uptake of Mitotracker Red. Even so, the respiratory mutants exhibited considerably less staining compared to the wild-type strain, with the majority of the stain located at the cell cortex. Furthermore, the cortical staining was exaggerated at the cell periphery in the mutant strains at stationary phase. We also performed live cell imaging to investigate mitochondrial dynamics and found that while the wild-type strain had a dynamic mitochondrial reticulum, the respiratory mutants had a disrupted and slow moving network (data not shown). To our knowledge, this is the first time that the mitochondrial morphology of COX assembly mutants in the stationary phase has been reported and while there were no obvious morphological features in any mutant strain that made it unique, our data suggest that the disrupted mitochondrial morphology is likely an effect of a dysfunctional electron transport chain, a common trait in all of the respiration deficient mutants we examined.

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Figure 3

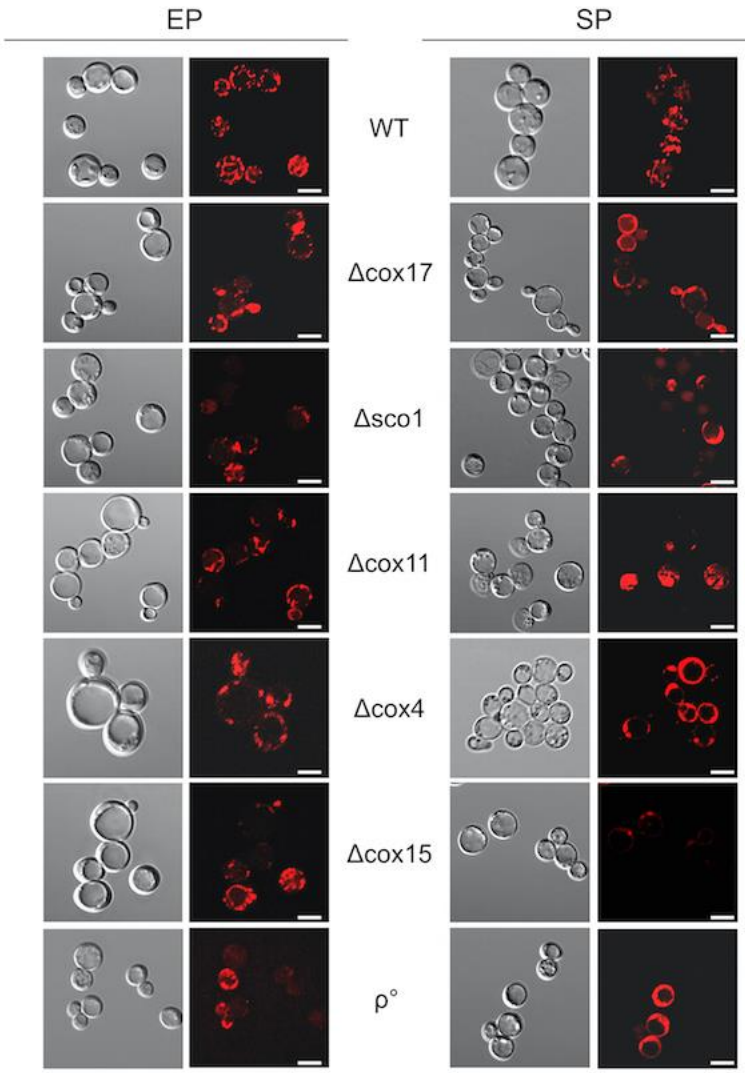


***Figure 3.*** **COX assembly mutants display a disordered mitochondrial reticulum.** Mitochondrial morphology was visualized by confocal microscopy from cells isolated from exponential (EP) and stationary phase (SP) cultures. Mitochondria were stained with MitoTracker Red and imaged by excitation with a 543 nm laser on a Zeiss LSM700 microscope. Representative images are shown for each strain from two independent experiments. Scale bars are 10 μm in all pictures except for Δcox4 and Δcox15 at EP, where they represent 5 μm.

### 3.4 Mitochondrial protein levels decrease in COX assembly mutants at stationary phase

In light of the presumptive decrease in mitochondrial membrane potential in the COX assembly mutants and the $\rho^0$ strain, we examined the steady-state levels of a variety of mitochondrial proteins located in several different compartments of the organelle.

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Initially, we examined Sco1, Cox11, and Cox17, which all work together to bring copper to subunits 1 and 2 of COX [11]. Although these three proteins are postulated to work in concert in acquiring copper for COX biogenesis, the effects of the loss of one of these proteins on the presumptive partners has not been reported. We found no significant differences in the levels of these three proteins in mitochondria isolated from exponential phase cultures taken from either the respiratory competent W303 strain or from the respiration-deficient mutants (Figure 4). The only exception was a slightly stronger signal for all three proteins in the strain lacking mtDNA. Likewise, we did not see a significant decrease in the level of any of these proteins in mitochondria isolated from wild-type yeast at the stationary phase (Figure 4). In contrast, all the mutant strains were characterized by a visible decrease in steady-state levels of Cox17, Sco1 and Cox11 at the stationary phase as compared to the exponential phase. Interestingly, there was almost no detectable Cox11 in the Δsco1 strain and, likewise, no detectable Sco1 in the Δcox11 strain in mitochondria taken from the stationary phase cultures. This result appears to support our earlier findings regarding a partial overlap in function between these two proteins [36], and suggests that this overlapping function may be more prominent at the stationary phase.

Along with examining the abundance of several COX assembly factors, we also assessed the levels of other mitochondrial proteins to understand how COX deficiency might affect the mitochondrial proteome on a broader scale. Western blotting showed that mitochondria isolated from stationary phase cultures of wild-type yeast had similar levels of Por1 and Sdh2 when compared to mitochondria isolated from exponential phase yeast (Figure 4). In contrast, all of the mutants showed reduced levels of porin (Por1) and an almost complete loss of detectable succinate dehydrogenase 2 (Sdh2) in stationary phase mitochondria, when compared to the exponential phase. The almost complete loss of Sdh2 was surprising to us, especially in the exponential phase in the *sco1* and *cox11* null mutants, as there have been no previous reports of loss of assembled COX adversely impacting Complex II. Since Por1 is commonly used as a loading control, this prompted us to use Ponceau-stained membranes in all subsequent experiments requiring a mitochondrial loading control (provided in Supplementary Figure S2). Examination of Cyc1, given its dual roles as a mobile electron carrier in the respiratory chain and an important trigger in apoptosis, revealed that unlike the relative stability of steady-state levels of Por1 and Sdh2 in wild-type yeast, the levels of Cyc1 at the stationary phase were reduced to about one-third compared to levels at the exponential phase (Figure 4), as revealed by densitometric analysis (data not shown). This reduction correlates with the reduced spectral peak shown above (Fig. 2B). Most of the mutants, except for the null *cox17* and $\rho^0$ strains, showed decreased levels of Cyc1 at exponential phase and all the mutants had barely detectable Cyc1 in mitochondria isolated from stationary phase cultures. The loss of the Cyc1 signal in mitochondria was not the result of loss of this protein to the cytoplasm, as there was no detectable Cyc1 protein in the cytosolic fractions in these cultures (data not shown). Since retro-translocation of intermembrane space (IMS) proteins to the cytosol has been shown to occur upon changes in the redox state of mitochondria [45], we also assessed the post-mitochondrial supernatant (i.e. cytosolic) fractions for steady-state levels of Cox17. As expected, the cytosolic levels of Cox17 in the stationary phase are increased. The decrease in the levels of

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mitochondrially-localized Cox17 is therefore likely the result of retro-translocation, while the reason(s) for the reductions seen with the other proteins remains unclear. To ensure that our experimental approach for isolation of mitochondria did not affect cells differently between the exponential and the stationary phases, due to potentially compromised mitochondrial integrity at the latter stage, we immunoblotted for Pma1, a marker of the plasma membrane. We found some contamination in the mitochondrial fractions that was more prominent at the exponential phase in all of the strains (data not shown), supporting the notion that the decrease in mitochondrial protein titers was not the result of experimental perturbations of the stationary phase cultures.

Figure 4

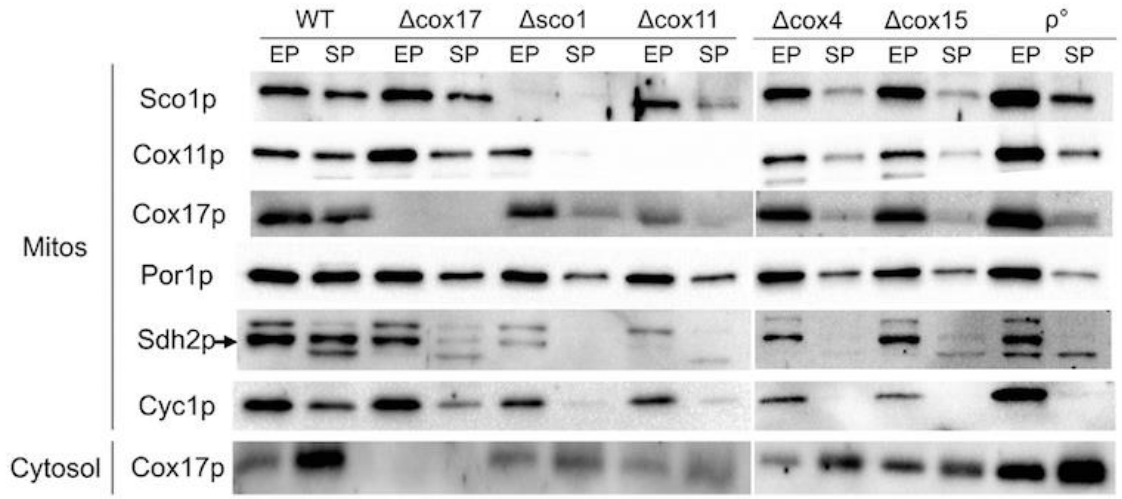


***Figure 4.*** **Mitochondrial protein levels decrease at stationary phase in respiration deficient mutants.** Mitochondrial protein samples (Mitos, 10 μg) from WT and mutants were subject to immunoblotting with antibodies to general mitochondrial proteins (porin, Por1; succinate dehydrogenase subunit 2, Sdh2; cytochrome *c*, Cyc1) or proteins involved in COX assembly (Sco1, Cox11, Cox17). All blots were stained with Ponceau S prior to immunblotting to ensure equal loading across all lanes of the gel. The band corresponding to Sdh2 is marked with an arrow. The band above Sdh2 is non-specific, as it was present in all samples taken from exponential phase cultures but not in samples from the stationary phase. The band below Sdh2 is likely a product of proteolysis and has been observed in a previous report using this antibody [46]. Cytosolic protein samples (Cytosol, 10 μg) were similarly examined to verify the retro-translocation of Cox17 at the stationary phase. Protein levels were analyzed at both the exponential (EP) and the stationary phase (SP); all blots were repeated in duplicate with two separate subcellular fractionations.

In order to explore possible causes for the decreased mitochondrial protein levels at stationary phase, we wondered whether Lon1, one of the major mitochondrial proteases, could be responsible for the observed decline in protein levels in the mutants. Lon1 is up-

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regulated under a number of different cellular stresses [47]. The higher steady-state levels of Lon1 in the mitochondrial mutants at exponential phase, as compared to the wild-type strain (Figure 5A and B), suggest higher levels of mitochondrial protein degradation in the exponential phase of growth for respiration deficient yeast. Because Lon1 plays a large role in degradation of mitochondrially-encoded COX subunits when COX assembly is blocked [48] [49], we observed the expected correlation between increased amounts of Lon1 (Figure 5A) and reduced levels of Cox2 in the mutants (Figure 2C). However, all strains, regardless of their ability to respire, revealed a decrease in steady-state levels of Lon1 during stationary phase (Figure 5A). In an effort to determine if the difference in protein titers was related to transcript levels, qPCR was performed on total RNA isolated from exponential and stationary phase cultures. When normalized to *ACT1* transcript levels, we found elevated levels of *PIM1* transcript, which encodes Lon1, in nearly all the mutants at both phases of growth (Figure 5C). The only exception was the Δcox11 strain, which had lower levels of *PIM1* at both phases of growth. Nevertheless, *PIM1* expression is down-regulated at the yeast stationary phase in both wild-type and respiration-deficient strains, regardless of whether there is a specific loss of COX or a more generalized loss of multiple respiratory chain enzymes. Taken together, our results suggest that Lon1 does not appear to play a significant role in the decreased mitochondrial protein levels seen in the COX assembly mutants at the stationary phase.

During our mitochondrial isolations, we noticed mitochondrial yields from stationary phase cultures were substantially decreased in mutant strains as compared to the wild-type strain, even though yields between all strains were similar at exponential phase. The decreased mitochondrial yield together with the altered mitochondrial morphology in the mutant strains (Figure 3) might be indicative of increased levels of mitophagy at stationary phase, which might also explain the lower abundance of the mitochondrial proteins we examined. Atg3, one of the many autophagy-specific proteins in yeast, is essential for mitochondrial homeostasis [50, 51]. We observed an increased abundance of Atg3 in most of the COX assembly mutants at the stationary phase as compared to the exponential phase (Figure 5C). Because we found virtually no change in Atg3 levels in the wild-type strain between the exponential and the stationary phases, the mutant-specific increase in Atg3 levels at stationary phase may be indicative of changes in autophagic quality control in strains that are defective in oxidative phosphorylation. Atg3 has been shown to help drive membrane biogenesis during autophagy in association with an Atg12-Atg5 conjugate [52] and is used as a marker for autophagy. The heightened levels of Atg3 may also explain the lack of a mitochondrial reticulum in the respiratory mutants at the stationary phase (Figure 3). Overall, our results suggest that the decreased abundance of many mitochondrial proteins in the mutants at stationary phase is likely due to a combination of retro-translocation to the cytosol of intermembrane space proteins, a substantially reduced mitochondrial membrane potential and changes in autophagy.

**3.5 COX assembly mutants display decreased mitochondrial oxidative stress at stationary phase** In light of the increased levels of Atg3 we observed in the mitochondrial mutants at the stationary phase, we were interested to see if there were any differences in key players in the antioxidant defenses between the wild-type and mutant

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stationary phase yeast cells, as the accumulation of reactive oxygen species (ROS) is known to induce autophagy [53]. We have previously shown that loss of Sco1 or Cox11 leads to a hypersensitivity to exogenous peroxide in yeast cells [22, 36] and Bode *et al* reported recovery of growth on ethanol by supplementation of growth media with either

Figure 5

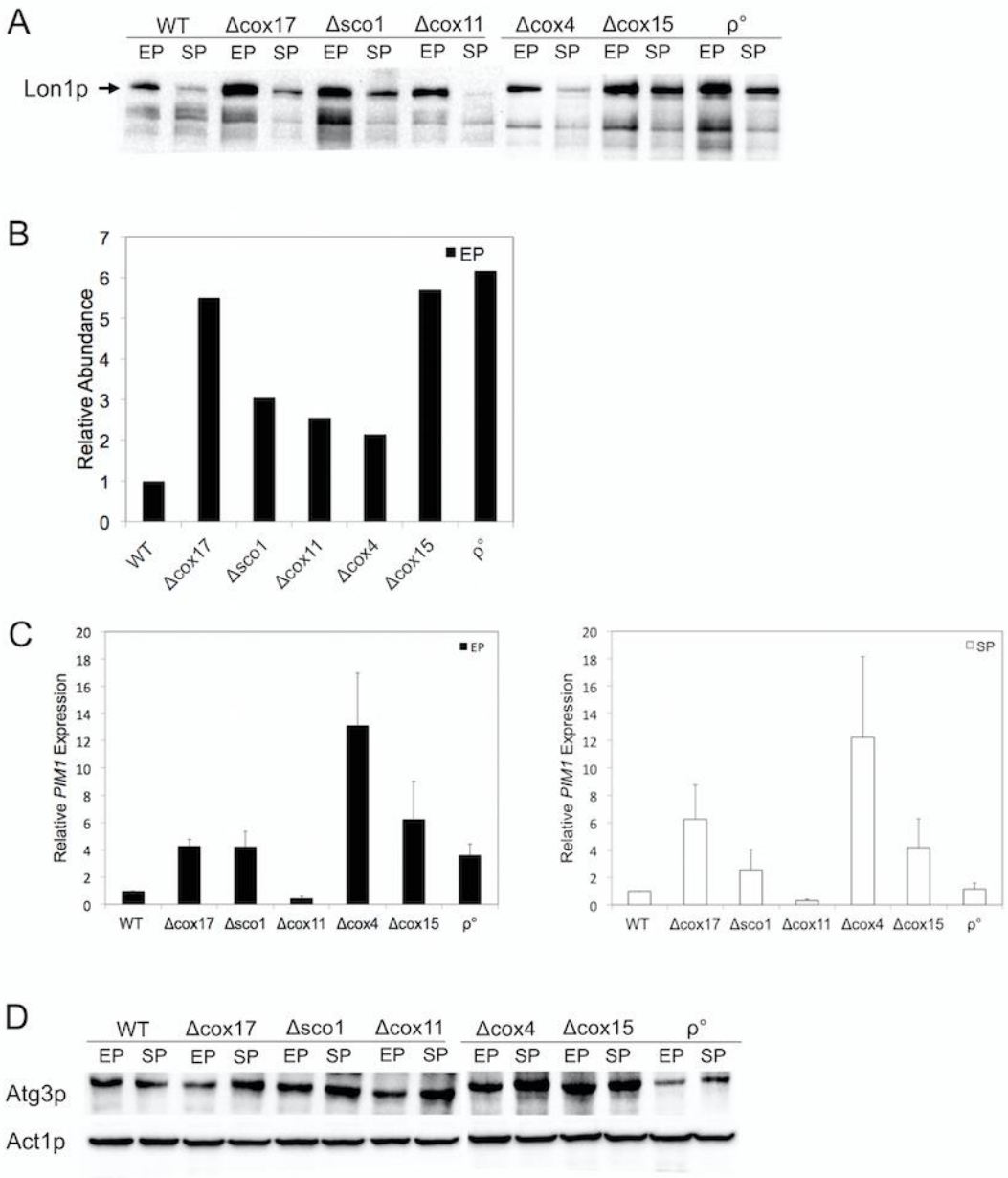


***Figure 5.*** **Analysis of mitochondrial quality control in COX assembly mutants. (A)** Western blot analysis of Lon1 protease (indicated by the arrow) was carried out using 25 μg of isolated mitochondria and an anti-yeast Lon1 antibody [54]. **(B)** Analysis of Western blotting using densitometry on exponential phase cultures. The WT strain at exponential phase was used as the reference. **(C)** Analysis of Pim1 transcript levels in all

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strains was carried out by qPCR. Using the ΔΔCq method, *PIM1* transcript levels were normalized to *ACT1* transcript levels to determine relative expression. Error bars represent ±SEM from triplicates of three independent experiments. **(D)** Western blot analysis of Atg3 with whole cell lysates (60 μg) was carried out on exponential (EP) and stationary phase (SP) cultures, using the actin blots as a loading control.

dithiothreitol (DTT) or glutathione (GSH) in a small subset of COX assembly mutants [55], suggesting that cellular growth arrest was due to oxidative stress. While a *cox17* null strain was described in the study by Bode *et al* [55] and was not rescued, we wondered whether any of the other strains in our study might experience a similar rescue in the presence of these known antioxidants. As shown in Figure 6A, the growth arrest exhibited by our mutants could not be rescued by the addition of either 5 mM DTT (Figure 6A) or 5 mM GSH (data not shown).

In the absence of rescue by these two small molecule antioxidants, we next examined the Cu,Zn-superoxide dismutase, Sod1, given that elevated levels of this key component of cellular antioxidant defense are not only considered to be a hallmark of yeast stationary phase but also essential for long-term survival of yeast in the stationary phase [56, 57]. Western blotting of whole cell lysates of the wild-type strain revealed that the abundance of total Sod1 was only slightly higher in the stationary phase when compared to the levels observed in the exponential phase (Figure 6B). However, the signal intensity of the Sod1 band was noticeably increased in stationary phase cells from both the COX assembly mutants and the $\rho^0$ strain, both in comparison to the levels seen at the exponential phase and to the levels observed for the wild-type strain. Given that steady-state levels of Sod1 do not provide any information on the relative enzymatic activity of the enzyme in the cell extracts, we used an in-gel assay [58] to compare Sod1 activity in the wild-type and mutants at both the exponential and the stationary phase. In the wild-type culture we observed a decrease in Sod1 activity from exponential to stationary phase, even though protein abundance slightly increased in these cells (Figure 6B). In contrast, most of the COX assembly mutants displayed similar levels of Sod1 activity in the exponential and stationary phase cultures. The only exception appeared to be the Δcox11 strain, which displayed activity levels lower than that of the wild-type strain at exponential phase, but showed higher activity in the lysate taken from a stationary phase culture (Figure 6B). These results demonstrate that although the steady-state levels of total cellular Sod1 are increased in most of the respiration deficient yeast strains at stationary phase, there does not appear to be an accompanying increase in overall Sod1 activity. The strain lacking Cox11 is clearly an exception and will require further investigation in this regard. Because it is known that only a small portion of Sod1 (~2%) is required to dismutate superoxide [59], our finding of a substantial disconnect between steady-state Sod1 protein levels and Sod1 activity is not without precedent and further study may uncover the molecular mechanisms involved.

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Figure 6

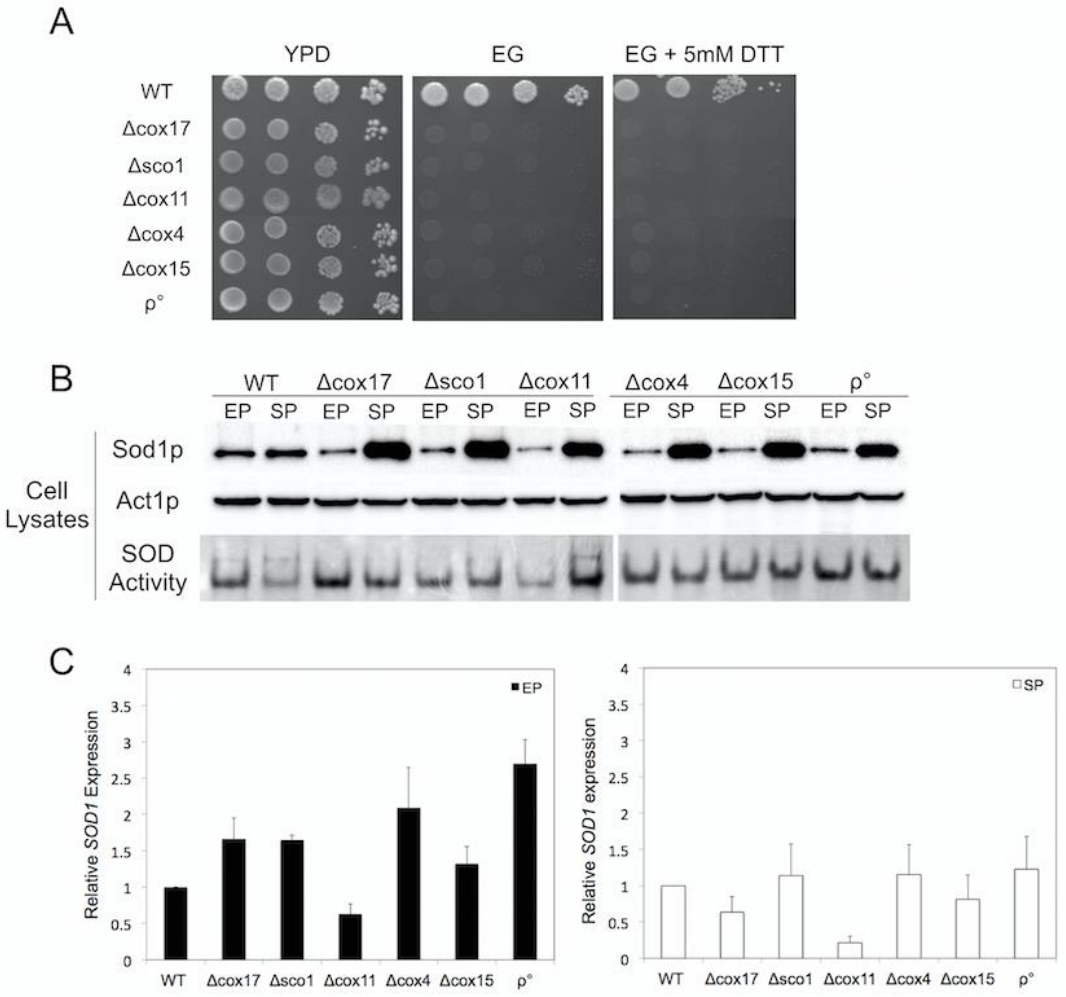


***Figure 6.*** **Examination of markers of cellular oxidative stress. (A)** Tenfold serial dilutions ($10^0$ – $10^{-3}$) of overnight cultures of the WT or respiration deficient mutant strains were normalized and spotted onto YPD (glucose), EG (ethanol/glycerol) or EG plates supplemented with 5 mM DTT. Plates were incubated at $30^0$C for 3 days. **(B)** Yeast strains were assessed for Sod1 protein and Sod activity in whole cell lysates (20 μg protein) at exponential (EP) and stationary (SP) phases. Sod1 was detected using a commercially available antibody; Act1 was used as a loading control. Sod1 activity was measured using a well-established in-gel assay [33]. **(C)** Sod1 transcript levels were assessed by qPCR, using total RNA from all strains. Using the ΔΔCq method, *SOD1* transcript levels were normalized to *ACT1* transcript levels to determine relative expression; error bars indicate ±SEM from triplicates of three biological replicates.

In order to better understand the substantial increase in steady-state levels of Sod1 in the

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mitochondrial mutants at the stationary phase, we used qPCR to determine whether increased Sod1 protein levels were reflected in increased *SOD1* transcript levels in mutants at both exponential and stationary phase (Figure 6C). When normalized to *ACT1* transcript levels, we observed a slight up-regulation of the *SOD1* transcript in almost all mutants at exponential phase. Strikingly, the *SOD1* transcript levels at stationary phase in most mutants are similar to those of the wild-type strain, suggesting that a complete loss of complex IV in the respiratory chain does not have a significant impact on *SOD1* transcript levels at this phase of growth. The decrease in *SOD1* transcript levels seen in the Δcox11 strain at both phases of growth may correspond to its decreased viability (Figure 1C). The decline in Sod1 transcript levels between the exponential and stationary phases is in clear contrast to the increased abundance of the Sod1 protein that we observed. Increased Sod1 protein abundance in the face of decreased transcript levels at the stationary phase is suggestive of changes in Sod1 protein stability during the different phases of growth, which may be caused by increased Sod1 aggregation [60].

It is widely recognized that Sod1 has a variety of forms and states of metallation, with varying stabilities, in the cell. While the bulk of Sod1 is found in the cytoplasm, with about 5-10% located in the intermembrane space of mitochondria [61], a recent study has shown that Sod1 can also translocate to the nucleus upon peroxide-induced oxidative stress, where it functions as a transcription factor [62]. The compartmentalization of Sod1 is associated with changes in both activity and stability of the protein and we therefore wanted to determine whether the subcellular distribution of Sod1 changes between the exponential and the stationary phases. We isolated mitochondrial, cytosolic, and nuclear fractions from yeast cultures, and observed a decrease in mitochondrially-localized Sod1 from exponential to stationary phase in the wild-type strain and all of the mitochondrial mutants (Figure 7A). Furthermore, Sod activity gels revealed not only a decrease in the mitochondrial Cu,Zn-Sod (Sod1) activity from exponential to stationary phase in all yeast strains, but also a considerable decrease in the activity of the Mn-Sod (Sod2), which resides in the mitochondrial matrix (Figure 7A). Together, these results show that a decrease in mitochondrial Sod1 protein levels is accompanied by a decrease in mitochondrial Sod activity in all strains during stationary phase. Western blotting of the cytosolic and nuclear fraction of cells revealed that the wild-type strain has lower cytosolic Sod1 levels but increased nuclear Sod1 levels at stationary phase when compared to the exponential phase (Figure 7). In contrast, all of the mutants displayed increased abundance of cytosolic Sod1 and decreased levels of nuclear-localized Sod1 at the stationary phase (Figure 7B). The activity of Sod1 in the cytosolic fractions varied considerably, with lower levels of activity in the stationary phase in most strains. There was no detectable Sod1 activity in the nuclear fractions from the respiration deficient mutants at stationary phase; in fact, we only detected Sod1 activity in nuclei isolated from the stationary phase cells of the wild-type strain.

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Figure 7

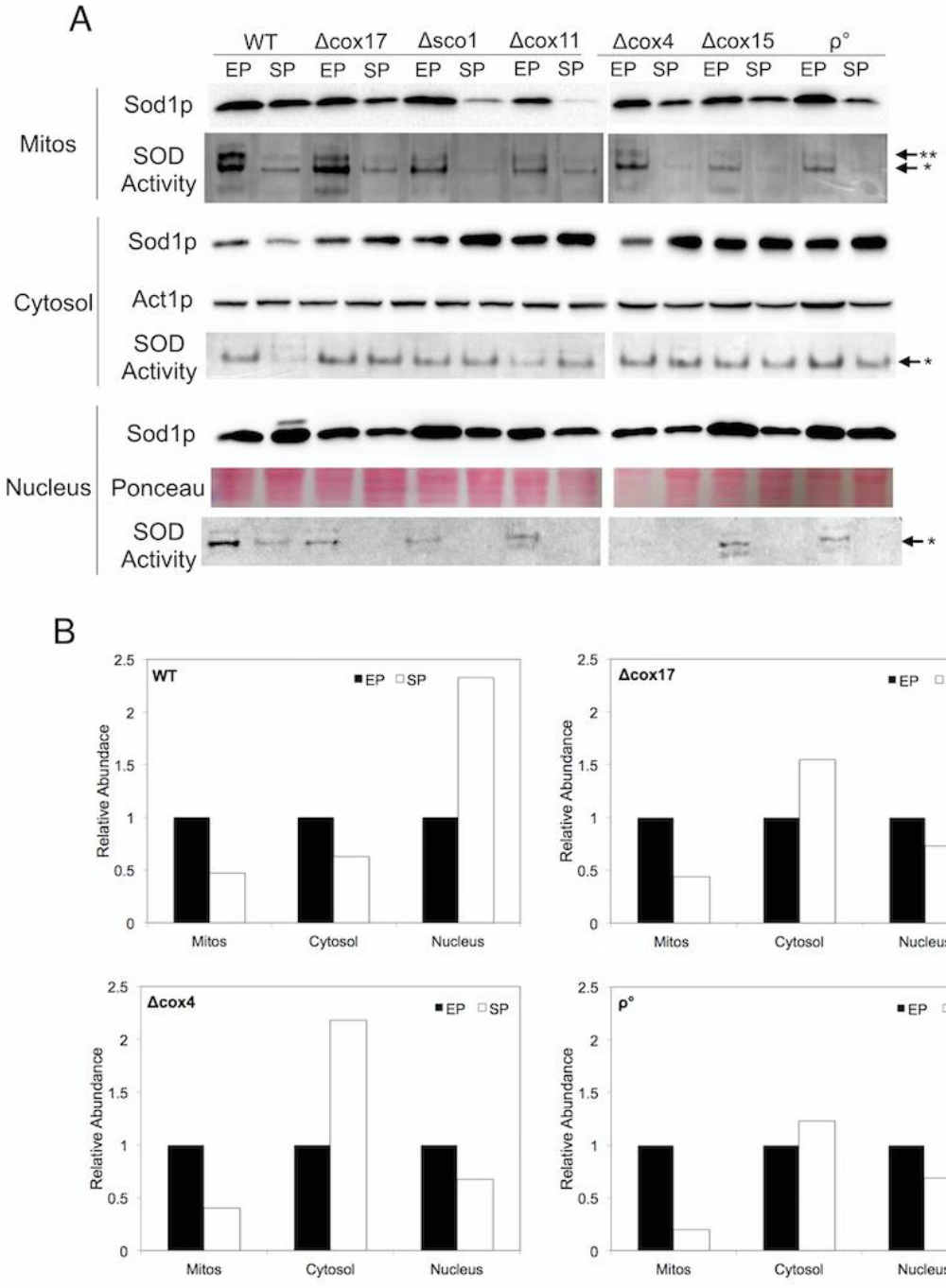


***Figure 7.*** **Sod1 compartmentalization changes from exponential to stationary phase. (A)** Sod1 immunoblotting was performed with mitochondrial, cytosolic, and nuclear fractions for all strains. Sod activity was assessed in the mitochondrial, cytosolic and nuclear fractions using the same in-gel assay as in Figure 6B. The arrow pointing to the band with one asterisk represents Sod1, while the arrow with two asterisks represents

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Sod2. Act1 and Ponceau staining act as the loading controls for the cytosolic and nuclear fractions, respectively. **(B)** Analysis of Western blots from Panel A using densitometry for strains with the least obvious differences in protein levels. For each strain in each compartment, the exponential phase band was used as the reference.

Interestingly, several of the mutants appear to have higher steady-state levels of nuclear Sod1 protein than the wild-type strain at exponential phase (Figure 7), suggesting the presence of cellular peroxide stress during the exponential phase [62]. The redistribution of Sod1 to the nucleus in a wild-type strain stationary phase culture suggests increased oxidative stress in these cells. The fact that nuclear Sod1 abundance decreases at stationary phase in the mitochondrial mutants, along with the observed increase in the cytosolic Sod1 pool, suggests that respiration-deficient strains may not experience increased peroxide-related oxidative stress at stationary phase.

The redistribution of Sod1 at stationary phase and the unexpected changes in overall Sod activity led us to further investigate mitochondrial oxidative stress by assessing the activity of aconitase, a citric acid cycle enzyme found in the mitochondrial matrix. Aconitase is considered to be a sensitive marker of oxidative stress, as the 4Fe-4S cluster at its active site is susceptible to oxidative damage [63, 64]. To differentiate from the cytosolic aconitase, which participates in the glyoxylate cycle and functions in iron regulation, we used isolated mitochondria to ensure we were only considering the mitochondrial aconitase. We found that mitochondria isolated from a wild-type strain at stationary phase had slightly reduced aconitase activity when compared to those from an exponential phase culture (Figure 8A). All of the respiratory mutants displayed levels of aconitase activity significantly lower than that of the wild-type strain when assayed in mitochondria isolated from exponential phase cultures. Although this is the first report of mitochondrial aconitase activity in COX assembly mutants, our findings are concordant with those of Bode et al [55] and support increased levels of oxidative stress in all mutants during exponential growth. Strikingly, all the COX assembly mutants exhibited an increase in aconitase activity at stationary phase when compared to exponential phase. Notably, the Δcox11 strain showed a 4.2 times increase in aconitase activity and the Δcox15 strain displayed a 6-fold increase in activity. The $\rho^0$ strain was the only respiration deficient strain that displayed similar levels of aconitase activity at both phases of growth. We also assessed steady-state levels of mitochondrial Aco1 using Western blotting (Figure 8B) and found that in the wild-type strain, the abundance of mitochondrial Aco1 protein was slightly decreased at stationary phase. The concordance between Aco1 protein and activity levels in the wild-type strain suggests similar levels of mitochondrial oxidative damage between the exponential and stationary phases. Unexpectedly, all mitochondrial mutants displayed reduced steady-state levels of the mitochondrial aconitase at stationary phase when compared to exponential phase (Figure 8B). One potential explanation for the observed increase in the mitochondrial aconitase activity levels in COX assembly mutants in the face of reduced steady-state levels of Aco1 is the possibility that, as with Sod1, only a small amount of the protein is required for activity, although we have not found any reports in the literature to this effect. Together with the reduction in the Sod1 mitochondrial and nuclear pools at stationary phase (Figure 7), the elevated aconitase activity observed in the COX assembly mutants

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studied here strongly suggests that the stationary phase in these strains is associated with lower levels of mitochondrial oxidative stress than seen in exponential phase cells.

Figure 8

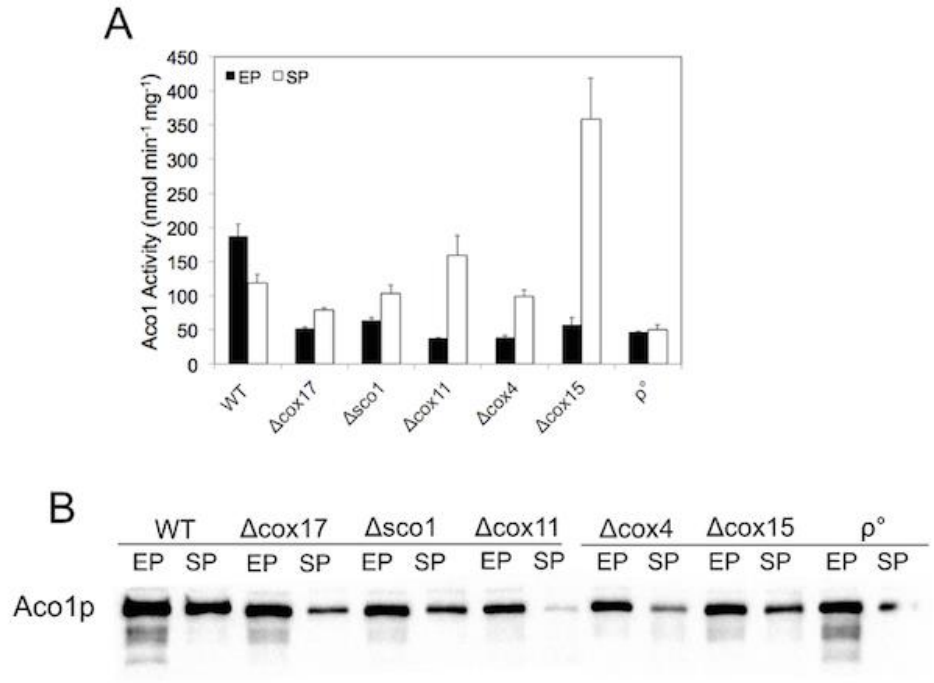


***Figure 8.*** **Assessment of aconitase as a marker of mitochondrial oxidative stress**. **(A)** Aco1 activity was assayed in mitochondria isolated from the WT and the mutant strains at both phases of growth. A spectrophotometric assay using citrate as the substrate measured the production of NADPH at 340 nm [65]. The activities represent the average of 4 separate assays; error bars = ±SEM. **(B)** Immunoblotting of mitochondria (25 µg protein) was carried out to detect the abundance of Aco1 at both exponential (EP) and stationary (SP) phases.

## 4. DISCUSSION

The delineation of the COX assembly pathway in yeast has occurred primarily through studies using cells grown to late exponential phase, leaving the implications of a loss of assembled COX for yeast homeostasis during the stationary phase largely unexplored. Discoveries in yeast COX assembly have had an undeniable impact on the study of human disease, initially facilitating the identification of mutations and later contributing to the understanding of the molecular mechanisms underpinning inherited COX deficiencies in humans. A better understanding of the impacts of a COX deficiency in the stationary phase is relevant not only to yeast, which exist primarily in this state in the wild, but also to humans, since yeast in the stationary phase can serve as an excellent model for terminally differentiated cells. Homeostasis in such tissues clearly has

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implications for the pathophysiological consequences of inherited COX defects. In this study, we used strains that all lack assembled COX but differ in the lesion that leads to this phenotype, allowing us to identify differences in characteristics that serve as the starting point for experiments aimed at delineating secondary functions for any one of these assembly factors. We also included a $\rho^0$ strain, which lacks Complexes III – V, as this allowed us to differentiate between a loss of COX and a more generalized oxidative phosphorylation defect.

In order to ensure that our cells exhibited characteristics of stationary phase cultures, all strains were cultured for 8 days and exhibited plateaued growth curves and reduced levels of actin transcript. In addition, our approach of maintaining cultures in the same glucose-exhausted milieu, is considered to be more physiologically relevant [37]. We have also chosen to describe the state of cells in stationary phase cultures in terms of their reproductive capacity, based on quantification of CFUs, as recommended by Werner-Washburne *et al.* [37]. As has been described in the literature, our stationary phase cultures contain a mixture of quiescent and non-quiescent cells [3], but we have nevertheless discovered some novel features of respiration deficient mutants in the stationary phase. While the finding of reduced fitness of all strains is not surprising after 8 days in culture, the presence of CFUs in the stationary phase cultures from COX assembly mutants suggests a smaller role for COX at stationary phase than previously thought. The reduced COX activity in a respiratory competent strain, which is mirrored by a decrease in the characteristic $aa_3$ peak at 605 nm, further supports the presence of decreased oxidative capacity in yeast stationary phase. In this regard, it has been reported that respiration rates of respiratory competent yeast in the stationary phase were only 20% of those measured in the exponential phase [66] and the results from our study would suggest that the decreased amount of COX may be largely responsible for this change. Interestingly, this decreased relative abundance of COX is similar to the observation that human skin fibroblasts, a primarily glycolytic cell type, contain less assembled COX compared to more aerobic cells types, such as skeletal muscle [67]. Taken together, our results provide a partial explanation for why mitochondrial mutants are still able to give rise to viable colonies at stationary phase and future experiments will be geared towards identifying the mechanisms that underlie this change in oxidative capacity.

Our finding of a perturbed mitochondrial network in COX assembly mutants at the stationary phase is novel, but not unexpected, given that respiratory competent yeast also exhibit more cortical mitochondrial localization under these conditions. The reduced staining with Mitotracker Red that we observed in the mutant strains, which may be suggestive of a decrease in membrane potential, led us to examine the abundance of several mitochondrial proteins at stationary phase. Both COX assembly factor proteins (Sco1, Cox11, Cox17) and mitochondrial proteins involved in non-COX related pathways, except Cyc1, remained at similar levels in the wild-type strain, regardless of growth phase, but were reduced in abundance at stationary phase in all of the mitochondrial mutants. We also identified a surprising loss of Sdh2 in our COX assembly mutants, even in exponential phase. We are not aware of any previous reports in this regard, but this intriguing result is worth further investigation. Our novel results argue

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that the decreased mitochondrial protein titers at stationary phase in the mutants may be due to decreased membrane potential and increased autophagy, rather than elevated levels of Lon1 protease. Mitochondria have many other proteases, such as Yme1, and future experiments will be aimed at defining the mechanism underlying the degradation of mitochondrial gene products. Additionally, Atg3 is just one of many proteins involved in autophagy and more experiments are needed in order to understand the potential change in autophagic response of mitochondrial mutants at stationary phase. The loss of proteins from the intermembrane space during stationary phase could be due to retro-translocation to the cytosol [45], although this phenomenon would only apply to Cox17 and Cyc1. The decrease in the levels of the mitochondrial proteins we investigated at stationary phase suggests that other proteins in the mitochondrial fractions are proportionately increased in abundance, given that all our analyses used the same amount of total mitochondrial protein. A proteomic analysis using mass spectrometry is currently underway and should allow for a broader understanding of the changes in the yeast mitochondrial proteome at stationary phase, both in the presence and absence of a functional respiratory chain.

The respiration deficient strains analyzed in this study displayed increased Sod1 protein levels at stationary phase that were not matched by up-regulated Sod1 transcript levels, suggesting alterations to Sod1 protein stability in the stationary phase. Sod1 has recently been shown to participate in a multitude of other cellular pathways that are unrelated to its role in scavenging superoxide radicals [68] and our results, showing a discordance between Sod1 activity and steady-state protein levels during both exponential and stationary phase in COX assembly mutants, suggest that further investigation of potential roles for Sod1 in respiration-driven cell signaling are warranted. In addition, striking differences in Sod1 distribution between sub-cellular compartments has proven to be an important principle, particularly as mitochondrially-localized Sod1 bearing the G93A mutation was found to correlate with features of amyotrophic lateral sclerosis in a mouse model of the disease [69]. Respiratory competent stationary phase yeast cells are typified by decreased Sod1 protein levels in the mitochondria and the cytosol, with the abundance of the protein elevated in the nucleus. Together with decreased mitochondrial aconitase at stationary phase, our results suggest the possibility of increased mitochondrial oxidative stress, which is likely peroxide driven, in respiratory competent yeast cells at stationary phase. The decreased Sod1 activity in mitochondria during stationary phase that we observed in the wild-type strain may, in fact, be one of the contributing factors to this increased mitochondrial oxidative stress in cells with a functional respiratory chain. The use of high-resolution respirometry will allow direct detection of superoxide and hydrogen peroxide, the two most common ROS and allow us to better assess the subcellular distribution of oxidative stress in respiratory competent yeast. In contrast to the wild-type strain, all respiration deficient mutants display increased levels of Sod1 in the cytosol at stationary phase, with a concomitant reduction in amounts of Sod1 in both the nucleus and the mitochondria. Interestingly, while the COX assembly mutants displayed increased aconitase activity at stationary phase, the $\rho^0$ strain did not, suggesting that the loss of complexes of III and V abrogates the apparent benefit of a loss of COX that we observed in the other mutants studied here. Our results therefore suggest that COX assembly mutants experience a decrease in mitochondrial oxidative damage and that the mitochondria in strains lacking COX may not be a major producer of ROS at

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stationary phase.

The considerable increase in overall abundance of Sod1 from exponential to stationary phase in the mutant strains suggests a number of potential roles for Sod1 during stationary phase as discussed above. Given that the endoplasmic reticulum (ER) is now believed to play a critical role in ROS homeostasis [70, 71], the increased levels of cytosolic Sod1 we observed in mitochondrial mutants at stationary phase may reflect a greater need for antioxidant defenses in closer proximity to the ER. Alternatively, increased cytosolic Sod1 in the mitochondrial mutants could indicate enhanced interaction with Yck1 during the stationary phase [72], thereby suggesting that the mutants are using aerobic fermentation as the main pathway for energy production. In addition to changes in subcellular distribution, Sod1 has also been shown to undergo increased aggregation in stationary phase yeast [60], which may also explain the increase in apparent stability of the protein. Our results also underscore the fact that increased Sod1 protein levels are not sufficient on their own to be used as an indicator of increased oxidative stress. The differential compartmentalization of Sod1 in the wild-type strain and the mitochondrial mutants may reflect the multitude of Sod1 functions, which are likely dependent on cellular oxidative damage, and further investigations will shed light on metabolic adaptations during the stationary phase that accompany the loss of an intact mitochondrial respiratory chain.

To our knowledge, ours is the first systematic characterization of a series of mutants with defects in different aspects of the COX assembly pathway at stationary phase. Our data shed new light on mitochondrial function at the stationary phase, revealing reduced levels of COX activity and assembly, disrupted mitochondrial morphology, and changes in the subcellular distribution of Sod1 in a respiratory competent wild-type strain. While our respiration-deficient yeast strains display abnormal mitochondrial morphology regardless of growth phase, we identified reduced steady-state levels of a number of key mitochondrial proteins and changes in the subcellular distribution of Sod1 in the COX assembly mutants as compared to the respiratory competent strain in stationary phase cells. Finally, the recovery of aconitase activity in the stationary phase in our mutants is suggestive of a decrease in mitochondrial oxidative stress. Taken together, we have identified important physiological changes in the titer of COX in replicating and terminally differentiated cells that has a profound effect on aerobic metabolism in stationary phase yeast. In addition, we have uncovered a difference in markers of mitochondrial oxidative stress between the exponential and the stationary phases in respiration deficient yeast strains. Defining the broader consequences for the loss of assembled COX in saturated yeast cultures has implications for the understanding of how an inherited COX deficiency would impact terminally differentiated cell types in patients and future studies will be aimed at delineating the mechanism that underlies the significant differences in the mitochondrial proteome and ROS in the presence and absence of assembled COX.

**ACKNOWLEDGEMENTS**

This work was supported by a Discovery Grant (RGPIN-227415-2012) from the Natural

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Sciences and Engineering Research Council of Canada (NSERC) to DMG and the Undergraduate Research Internship (URI) Program at the University of Waterloo to AD. We gratefully acknowledge the contributions of Seville Scarcello, Chanele Polenz, Polina Myrox and Tobi Alegbe (University of Waterloo) in data acquisition for the growth curves and strain viabilities, as well as the contributions of Graham Banting and Simona Veniamin (University of Alberta) in generating and characterizing the Cox11 antibodies, and thank Alex Tzagoloff for helpful comments on the manuscript. We also thank the reviewers for helpful comments and criticisms.

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**Highlights:**

- Respiratory competent yeast have lower amounts of assembled COX at stationary phase
- Loss of assembled COX results in altered mitochondrial morphology
- Nuclear Sod1 abundance decreases in mutants at stationary phase
- Mitochondrial aconitase activity increases in mutants at stationary phase

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