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Iron Citrate Toxicity Causes aco1▲-induced mtDNA Loss in Saccharomyces cerevisiae

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Iron Citrate Toxicity Causes *aco1Δ*-induced mtDNA Loss in *Saccharomyces cerevisiae*

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Abstract

Aconitase is an enzyme of the Krebs cycle that catalyzes the isomerization of citrate to isocitrate. In addition to its enzymatic activity, Aco1 has been reported to bind to mitochondrial DNA (mtDNA) and mediate its maintenance in the budding yeast S. cerevisiae. In the absence of Aco1, cells rapidly lose mtDNA and become "petite" mutants. The purpose of this study is to uncover the mechanism behind mtDNA loss due to an aco1 deletion mutation. We found that an aco1 mutation activates the mitochondria-to-nucleus retrograde (RTG) signaling pathway, resulting in increased expression of citrate synthases (CIT) through the activation of two transcription factors Rtg1 and Rtg3. Increased activity of CIT leads to increased iron accumulation in cells, which is known to raise reactive oxygen species (ROS). By deleting *RTG1*, *RTG3*, genes encoding citrate synthases, or *MRS3* and *MRS4*, encoding two iron transporters in the mitochondrial inner membranes, mtDNA loss can be prevented in aco1 deletion mutant cells. We further show that the loss of SOD1, encoding the cytoplasmic isoform of superoxide dismutase, but not SOD2, encoding the mitochondrial isoform of superoxide dismutase, prevents mtDNA loss in aco1 mutant cells. Altogether, our data suggest that mtDNA loss in aco1 mutant cells is caused by the activation of the RTG pathway and subsequent iron accumulation and toxicity in the mitochondria.

Key Terms: Petite, RTG signaling pathway, Aco1, Reactive Oxygen Species, Superoxide dismutase

Introduction

Yeast: A Model Organism for Basic Research

Saccharomyces cerevisiae, also known as the budding yeast or Baker's yeast, has benefitted mankind for centuries. Besides its use in baking and brewing alcohol, yeast has also been employed as a powerful tool for understanding the cellular mechanism of eukaryotic cells. Approximately 31% of the yeast genes exhibit homology to mammalian genes (Botstein, Chervitz, and Cherry 1997), a number that is significant considering that yeast and humans have diverged from a common ancestor about one billion years ago.

Yeast cells can exist as diploids or haploids and have the ability to reproduce both sexually and asexually. Yeast reproduces asexually by budding. In the process of budding, nuclear DNA undergoes mitotic division, and the replicated DNA is transferred to the growing bud on the mother cell. Under unfavorable conditions a yeast cell can mate with another yeast cell of the opposite mating type, producing a diploid, which can then undergo the process of sporulation to generate haploid spores (Herskowitz 1988).The ability of yeast cells to be transformed with DNA, which can either be integrated into the yeast chromosome or exist as circular plasmids (Mumberg, Müller, and Funk 1995), along with a short generation time, has made yeast a great model organism to study basic biology at molecular levels.

Anaerobic and Aerobic Respiration in Yeast

Metabolism comprises catabolic and anabolic pathways that involve degradation and synthesis of biomolecules in cells, respectively. The metabolic pathways are highly interconnected. Energy generation due to breakdown of biomolecules is coupled with the

formation of high-energy molecules that can be utilized to carry out various cellular processes. Yeast is a facultative aerobe, which means that it can grow anaerobically through fermentation using D-glucose as a carbon source. If a fermentative carbon source is not available, it can switch to aerobic respiration through oxidative phosphorylation to make adenosine triphosphate (ATP) using oxygen as the terminal electron acceptor.

During fermentation, glucose is broken down through the glycolytic pathway in the cytosol of the cell. Glucose, a hexose, is broken down into two pyruvate molecules through ten sequential enzymatic reactions that also result in the formation of ATP and the reduced form of nicotinamide adenine dinucleotide (NADH). ATP is the energy currency that is utilized to drive endergonic biochemical reactions. NADH is produced by the reduction of NAD^+ by the action of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The net yield of glycolysis is two molecules each of pyruvate, ATP, and NADH for one molecule of glucose (Berg and Tymoczko 2002a).

NADH production from NAD^+ thwarts the redox balance of the cell. NAD^+ supply is limited, so for the continuation of glycolysis, NADH needs to be converted back NAD^+ (Berg and Tymoczko 2002a). This is achieved by alcoholic fermentation in yeast. The process occurs in two steps. The first step is the decarboxylation of pyruvate to acetaldehyde, which is catalyzed by pyruvate decarboxylase. The second step involves the reduction of acetaldehyde by NADH to ethanol through a reaction catalyzed by alcohol dehydrogenase, which results in regeneration of NAD^+ (Berg and Tymoczko 2002a).

Glycolysis results in partial oxidation of glucose to pyruvate. Pyruvate can be further oxidized to carbon dioxide through a cyclic pathway known by various names, including

tricarboxylic acid (TCA) cycle, citric acid cycle, or Krebs' cycle. Pyruvate is first converted to acetyl CoA through a reaction catalyzed by a multi-enzyme complex known as pyruvate dehydrogenase (PDH). Acetyl CoA then enters the TCA cycle (**Figure 1**), which occurs in the mitochondrial matrix.

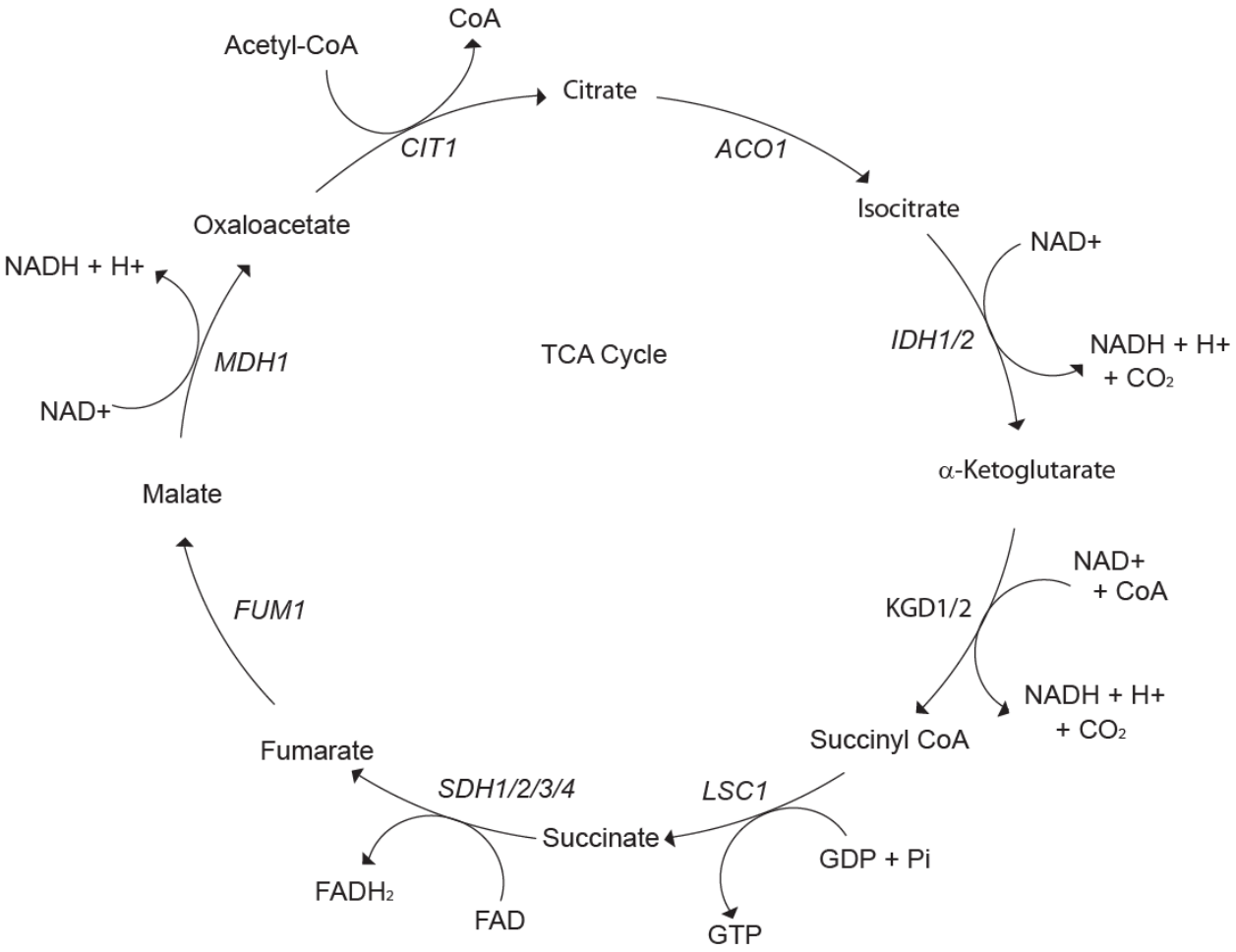


Figure 1: The Citric Acid Cycle

A diagram of the TCA cycle exhibiting the metabolic intermediates and enzymes involved in catalysis.

The primary function of the TCA cycle is the formation of high-energy electron carriers, namely NADH and $FADH_2$. One molecule of Guanosine triphosphate (GTP), an ATP-equivalent compound, is produced along with three molecules of NADH, one molecule of $FADH_2$, and two

molecules of carbon dioxide. NADH and $FADH_2$ can transfer electrons to the electron transport chain (ETC) that produces a proton gradient, which is then used to synthesize ATP (Berg and Tymoczko 2002b).

In addition to providing the reducing power, the TCA cycle is also involved in biosynthesis of other biomolecules such as glucose, amino acids, and fatty acids. Different intermediates of TCA are used in the synthesis of different biomolecules. For instance, oxaloacetate (through malate shuttle) is used in gluconeogenesis, and citrateis used for the synthesis of fatty acids, and the remaining intermediates are employed in the biosynthesis of amino acids such as glutamate synthesis from α-ketoglutarate; isoleucine, methionine, and valine from succinyl CoA; aspartate, phenylalanine, and tyrosine from fumarate (Voet, Voet, and Pratt 2008).

The electron transport chain consists of a series of integral membrane protein complexes located in the inner membrane of mitochondria. ETC arranges electron carriers in the order of increasing reduction potentials. When electrons are passed down the chain, the Gibbs free energy released by electrons is coupled to pump proton into the inter-membrane space of the mitochondria. The carrier proteins of ETC contain prosthetic groups such as heme, iron-sulfur clusters, FAD, FMN, and copper ions to facilitate the transfer of electrons. The first four complexes of ETC are involved in proton transfer from the mitochondrial matrix to the inter membrane space, whereas the fifth complex, also known as the F_1F_0 ATP synthase carries out ATP synthesis (Lodish, Berk, and Zipursky SL, et al. 2000).

NADH-CoQ reductase (complex I) accepts an electron pair from NADH, whereas succinate-CoQ reductase (Complex II) accepts electrons from $FADH_2$. Complex I and II transfer

their electron to $CoQH_2$-cytochrome c reductase (Complex III), which in turn transfers its electrons to Cytochrome c oxidase (Complex IV) via mobile electron carriers namely, ubiquinone and cytochrome c. Complex IV catalyzes the formation of water with the transfer of electrons to oxygen. ComplexesI and III translocate four protons each per electron pair, and Complex IV translocates only two protons. The proton gradient generated is then utilized by the F_1F_0 ATP synthase to synthesize ATP (Lodish, Berk, and Zipursky SL, et al. 2000).

Reactive Oxygen Species

Aerobic respiration is advantageous to cells energetically, but carries a disadvantage because of the production of reactive oxygen species (ROS). The most common source of ROS in cells is due to electron leakage during electron transfer in ETC. ROS that are produced in cells during aerobic respiration include: superoxide anion radical ($O_2\bullet^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\bullet$OH) (Farrugia and Balzan 2012). Besides mitochondria, NADPH oxidases contribute to the generation of ROS via electron transfer from NADPH to molecular oxygen (Griendling et al. 2000). The nitric oxide radical (NO$\bullet$) is another free radical that can react with $O_2\bullet^-$ to produce reactive nitrogen species that can lead to production of other radicals such as hydroxyl radical ($\bullet$OH) (Beckman et al. 1990).

Reactive oxygen species produced in minute concentrations in cells play important roles in cell physiology such as signal transduction, which mediates immune responses and apoptosis. ROS such as hydrogen peroxide have been reported to be involved in TNF-α induced cell apoptosis (Gotoh and Cooper 1998). At the site of infection nitric oxide and ROS are often secreted by macrophages to kill microbes in the vicinity. Reactive oxygen species in large quantity are detrimental to cells. Reactive oxygen species, as the name indicates, react with the biological molecules and degrade them, which ultimately results in ageing. ROS can also cause

DNA mutagenesis, leading to carcinogenesis. In addition to ageing, ROS has also been proposed to be the cause of various neurodegenerative diseases such as Friedreich's ataxia, Parkinson's disease, and Alzheimer's.

ROS Detoxification and Iron Citrate Toxicity

Defensive mechanisms exist in cells for the disposal of reactive oxygen species. Cells have enzymes that convert superoxide radicals to less potent peroxides, which are ultimately converted to water and molecular oxygen. Superoxide radicals are converted to hydrogen peroxide and molecular oxygen in the following reaction, which is catalyzed by superoxide dismutase.

$$2O_2^{\cdot -} + 2H^+ \rightarrow O_2 + H_2O_2$$

In eukaryotic cells two types of superoxide dismutase enzymes are present: cytosolic and mitochondrial superoxide dismutase, which are encoded by *SOD1* and *SOD2* respectively. Sod1 contains a Cu-Zn prosthetic group and localizes in the cytosol or the inter membrane space of the mitochondrion whereas Sod2 contains Mn prosthetic group and localizes to the mitochondrial matrix (Weisiger and Fridovich 1973). Cells contain enzymes called catalases that keep the intracellular concentration of hydrogen peroxide in check by converting hydrogen peroxide to water and molecular oxygen.

$$2H_2O_2 \rightarrow 2H_2O + O_2$$

When the homeostatic balance of iron in mitochondria is disturbed due to gene mutations, it can result in the accumulation of iron in mitochondria, which in turn can react with hydrogen peroxide to produce extremely potent hydroxyl radicals through Fenton reaction.

$$H_2O_2 + Fe^{2+} \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+}$$

Citrate is a known chelator of iron (Schmidt 1999) and a metabolic intermediate of the TCA cycle. An increase in citrate levels can disturb iron homeostasis, which can lead to an increase in iron levels in mitochondria. Yfh1 is a yeast homolog of human frataxin protein, which is encoded by*YFH1*. The function of frataxin and its yeast homolog Yfh1 is believed to be iron storage (Adamec et al. 2000). In yeast, *yfh1Δ* results in iron accumulation and a deficiency in respiration. The respiratory defect in *yfh1Δ* mutants likely results from iron-dependent production of hydroxyl radicals through the Fenton reaction and subsequent oxidative damages to the mitochondria (O. S. Chen, Hemenway, and Kaplan 2002).It has also been reported that the blocking of iron import into mitochondria prevents mtDNA loss in *yfh1Δ* mutants grown in media containing high concentration of iron, further emphasizing iron toxicity induced respiratory defect (Foury and Roganti 2002). Iron-citrate toxicity has been proposed to be the cause of Friedreich's ataxia, which is an autosomal recessive trait that results from mutations in the fratxin protein. Defects in this protein lead to neuronal degeneration and cardiomyopathy, scoliosis, and foot deformity (Delatycki, Williamson, and Forrest 2000). The major evidence for iron toxicity being the potential cause of Friedreich's ataxia comes from the studies of yeast homologue of human frataxin, Yfh1.

Aconitase

Aconitase is an enzyme that catalyzes the isomerization of citrate to isocitrate. Aconitase contains a [4Fe-4S] iron-sulfur cluster at its active site(Berg and Tymoczko 2002b). Aconitase, like other citric acid cycle enzymes, is encoded by the nuclear genome. In yeast, Aco1 expression is down regulated in cells grown in media containing glucose (fermentative carbon source) and glutamate, and *aco1Δ* leads to glutamate auxotrophy due to a block in the metabolic pathway that leads to glutamate biosynthesis (Gangloff, Marguet, and Lauquin 1990).

Expression of *ACO1*, along with *CIT1, IDH1,* and *IDH*2, is under the control of the RTG pathway in yeast cells with reduced respiratory activity (Z Liu and Butow 1999). Rtg1, Rtg2, and Rtg3 are the positive regulators of the RTG pathway. Rtg2 has been reported to be a positive regulator of *ACO1* expression (Vélot, Haviernik, and Lauquin 1996), and mutations in *RTG* genes lead to glutamate auxotrophy, a trait which is also shared by *aco1* mutants (Liao and Butow 1993). Rtg1 and Rtg3 are the transcriptions factors that form a heterodimer. Rtg3 contains the transcriptional activation domain, and partial dephosphorylation of Rtg3 stimulates its translocation to the nucleus along with Rtg1 (Sekito, Thornton, and Butow 2000; Rothermel, Thornton, and Butow 1997). Rtg2 is a cytoplasmic protein containing an N-terminal ATP binding domain is required for partial dephosphorylation of Rtg3 and nuclear translocation of the Rtg1/3 heterodimer (Z. Liu et al. 2003). An overview of the regulation of the *RTG*-target gene is shown in **Figure 2.**

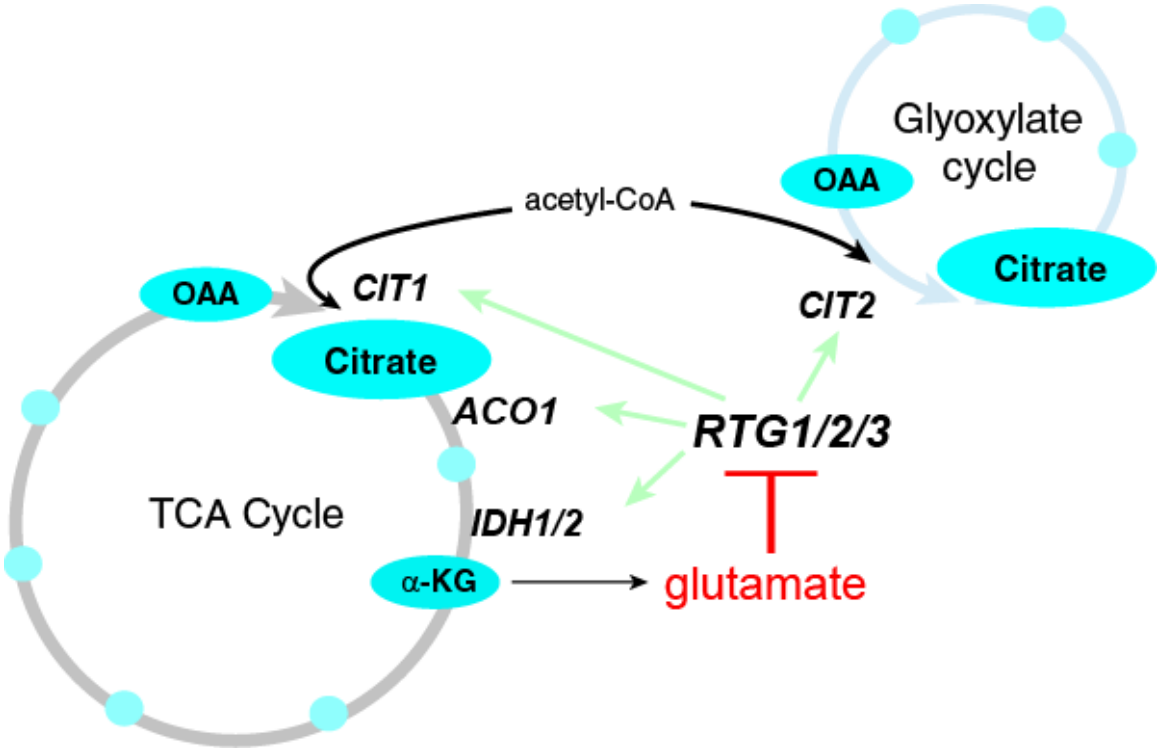


Figure 2: The RTG pathway is important for glutamate biosynthesis in *S. cerevisiae*

A model for the regulation of *CIT1/2, ACO1*, and *IDH1/2* by *RTG1/2/3*. The light green arrows indicate the target genes of RTG pathway in the TCA cycle and glyoxylate cycle. The pathway exhibits feedback inhibition by glutamate.

Cytosolic isoforms of aconitase can act as iron regulatory proteins (IRP) by disassociation of their iron sulfur cluster under low iron concentration (Hentze, Muckenthaler, and Andrews 2004). Aco1 is a multifunctional protein that has also been reported to associate with mtDNA (Kaufman et al. 2000). It has been proposed that Aco1 association with mtDNA is independent of its catalytic activity (X. J. Chen et al. 2005). It has also been reported that Aco1 is involved in mtDNA repair and stabilization in *pif1Δ* (mtDNA Helicase), and binding of Aco1 to mtDNA is essential for mtDNA maintenance (X. J. Chen, Wang, and Butow 2007).

Materials and Methods

Growth Conditions and Media

All yeast strains were grown at 30°C. Media used for growing yeast strains are as follows: minimal SD medium (0.67% yeast nitrogen base and 2% dextrose), YNBCasD (2% dextrose, 1% casamino amino acids, 0.67% yeast nitrogen base), and YPD (1% bacto yeast extract,2% bacto peptone, 2% dextrose). Agar (2%) was added to make plate media. When needed, the carbon source was replaced from dextrose to lactate (2%), glycerol (2%), or ethanol (2%). 30 mg/L of L-amino acids (L-leucine, L-lysine, L-histidine, L-glutamate, or L-methionine) and/or 20mg/ml uracil were added to minimal SD medium to cover the auxotrophic requirements (Amberg et al. 2005).

Yeast strains

All yeast strains used for this project were derived from two parent strains: BY4741 (*MATa/α ura3*Δ *leu2*Δ *his3*Δ*1 met15*Δ) or BY4743 (*MATa/α ura3*Δ*0/ura3*Δ*0his3*Δ*/his3*Δ*1 leu2*Δ*0/leu2*Δ*0 met15*Δ*0/MET15lys2*Δ*0/LYS2*).

High-efficiency Yeast Transformation

Fresh cells were inoculated in 5.0mL YPD media using a sterile loop. The cell culture was then allowed to grow for 3 to 5 hours with constant swirling in a 30°C incubator. The cells were harvested by centrifuging 1.0mL cell culture in a microfuge tube at 5000xg for 1 min. The harvested cells were then washed with 1.0mL 0.1M LiOAC. Afterwards, the cells were mixed with DNA and transformation mix (67µL of water, 200.0µL of PEG (60%), 18.0 of µL LiOAc (2M), and 50.0µL of single stranded DNA), and incubated at 42°C for 1 hour. Cells were then plated on the selective media to allow selection for positive transformants.

β-galactosidase Activity Assay

Plasmids containing a *CIT2-lacZ* reporter gene were transformed into yeast strains. Strains were then grown for at least 6 generations in YNBcasD medium at 30°C to reach OD_{600}0.6. Afterwards cells were collected and their protein content is extracted using glass bead method as described by Amberg et al. (2005). *lacZ* activity was reported in nmol of o-nitrophenyl-β-D-galactopyranoside [ONPG] hydrolyzed per milligram of protein per minute at 30°C. The activities were recorded as average results of duplicate or triplicate assays. Using bovine serum albumin (BSA) as a standard, the total amount of protein in cell extracts was determined by the Bradford assay, which in turn was used to calculate the specific activity of the *lacZ* reporter gene (Amberg et al. 2005).

Generation of Yeast Double Mutants

Two single mutant strains were mated and the diploids were selected on appropriate media. The diploids were then allowed to undergo sporulation. Depending upon cell density, 50.0uL to 100.0uL sporulated cultures were aliquoted in a 1.5mL microfuge tube containing 950.0uL of water. The contents of the microfuge tube were then centrifuged at 3000xg for 3 minutes to obtain a cell pellet. Afterwards, the supernatant is pipetted out to leave 100uL solution in the microfuge tube. The contents of the tube were then treated with 1.0uL Zymolyase 20T (10 mg/ml) for 6 minutes. 10μL cell resuspensions were then spread on the plate to form a line of cells. Subsequently, the spores of tetrads were dissected on YPD or YNBcasD medium under a dissection microscope. The spores were then allowed to grow and the genotypes of haploids were determined.

DAPI Staining of mitochondrial DNA

4',6-diamidino-2-phenylindole (DAPI) is a fluorescent stain that binds strongly to DNA. Yeast cells were grown in 5.0mL YPD or YNBcasD medium at 30°C to OD_{600} 0.8. Cell pellets were obtained by centrifugation and resuspended in 500μL 95% ethanol. 0.5 μL of 1 g/ml DAPI stock was then added to the cell suspension for 30 min and cell pellets were washed with 1.0mL sterile water three times. DAPI-stained cells were observed using fluorescence microscopy on a Nikon Eclipse E800 microscope equipped with HBO 100W/2 mercury arc lamp, a Nikon Plan Fluor 100X objective lens, and epifluorescence with Nikon the UV-2E/C ultraviolet excitation filter set (excitation 340 – 380 nm and emission 435 – 485 nm).

Results

Goals of Research

The overall goal of this research is to characterize the cellular mechanisms of mtDNA loss in *aco1*Δ mutant cells in *Saccharomyces cerevisiae*.

Results

*aco1*Δ Activates RTG Pathway

Citrate buildup in *aco1Δ* mutant cells is due to the absence of the catalytic step in the TCA cycle that carries out the isomerization of citrate to isocitrate. This situation could be further aggravated if the pathway that regulates the expression of citrate synthase is activated due to *aco1Δ*. *ACO1* expression along with *CIT1*, *IDH1*, and *IDH2* is under the control of the RTG pathway as part of the glutamate homeostasis pathway (Figure 2) (Liu and Butow 1999). *aco1*Δ leads to glutamate auxotrophy (Gangloff, Marguet, and Lauquin 1990). Absence of feedback inhibition by glutamate leads to over activation of the RTG pathway, which leads to the increased expression of citrate synthases, consequently citrate. To determine the activity of RTG pathway, I performed a β-galactosidase assay on wild type rho^0 and *aco1*Δ mutant strains transformed with *CIT2-lacZ* reporter gene plasmid. Figure 3 shows that the expression level of *CIT2-lacZ* is almost doubled in *aco1*Δ in comparison to wild-type rho^0 cells, indicating that the RTG pathway is activated due to *aco1Δ*.

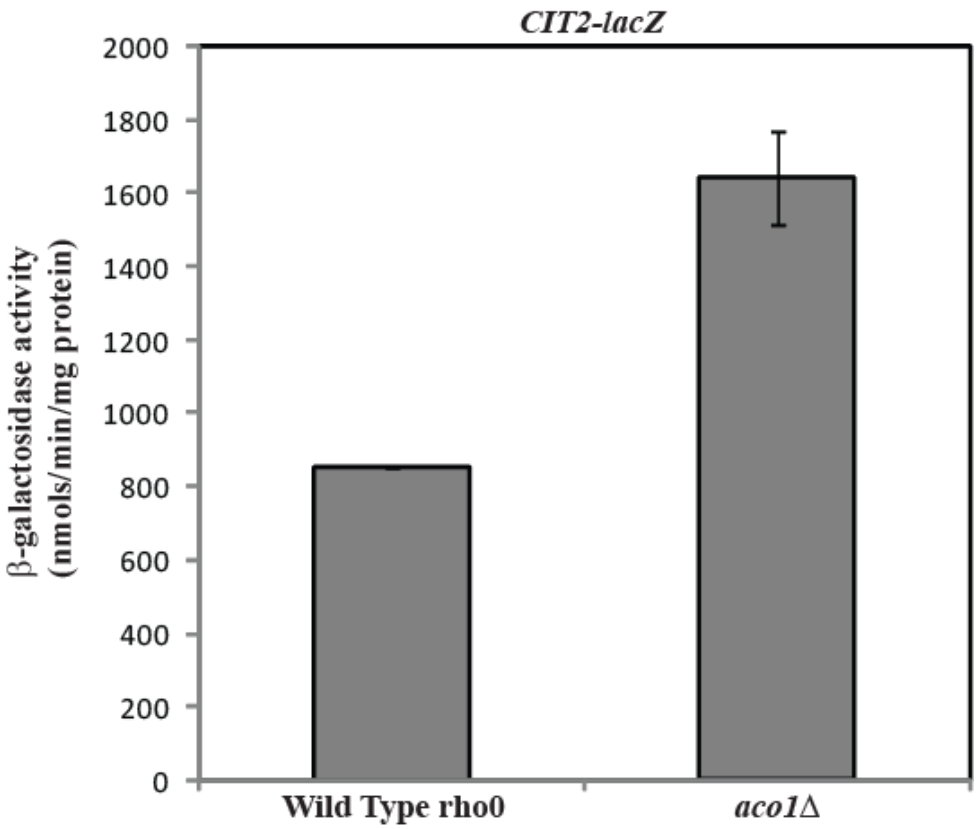


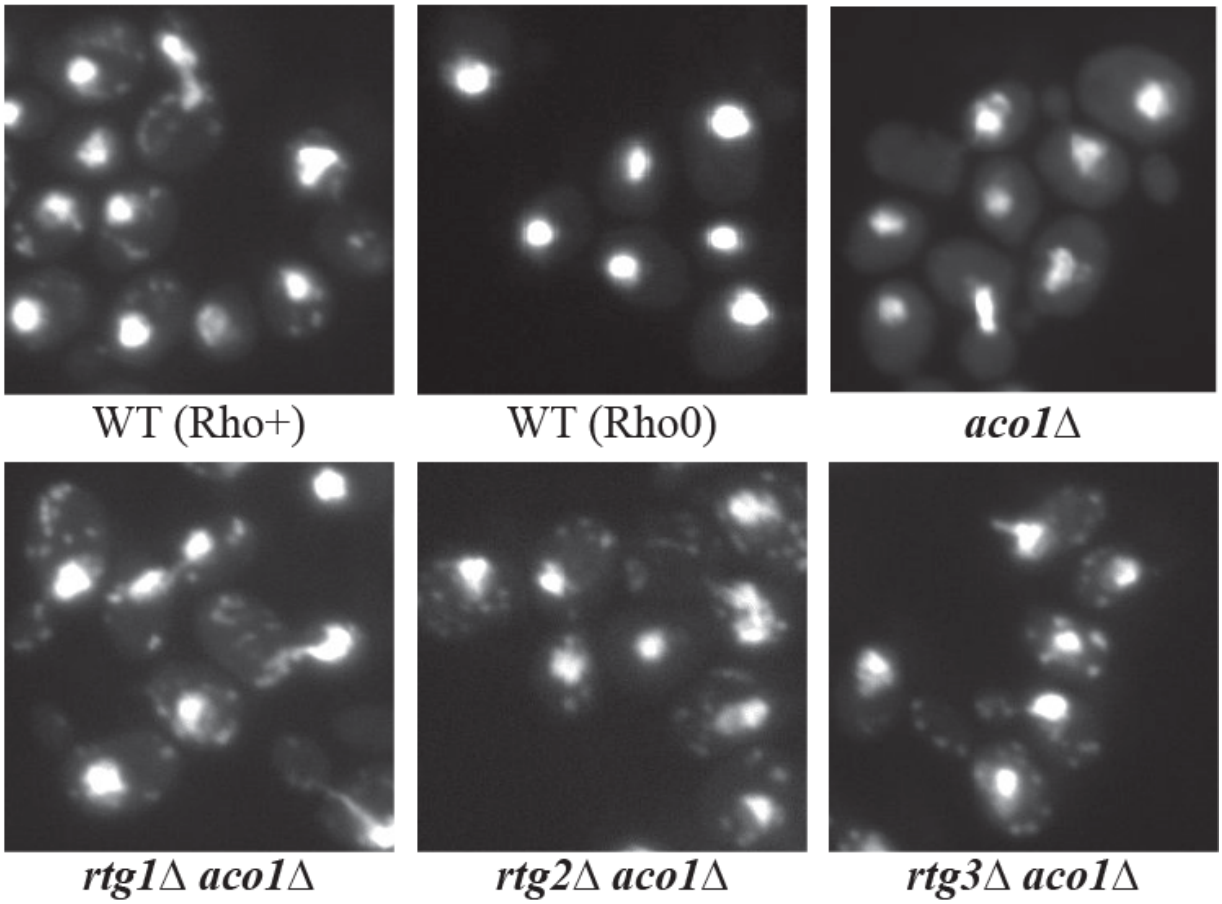
Figure 3: The RTG pathway is activated in *aco1Δ*mutant cells.
Wild Type rho^0 and *aco1Δ* strains carrying plasmids encoding a *CIT2-lacZ* reporter gene were grown in YNBCasD media and β-galactosidase activities were determined as described in Materials and Methods.

Mutations in *RTG* genes prevent mtDNA loss

Next I examined whether activation of the RTG pathway in *aco1Δ* mutant cells contributes to mtDNA loss. Accordingly, *rtg1Δ aco1Δ, rtg2Δ aco1Δ,* and *rtg3Δ aco1Δ* double deletion mutants were constructed and grown in YPD medium to mid-logarithmic phase. The presence or absence of mtDNA was determined by staining ethanol-fixed cells with 4',6-diamidino-2-phenylindole (DAPI). Fluorescent microscopy was used to observe DAPI-stained DNA. Figure 4A shows that although mtDNA was lost in *aco1Δ* mutant cells as reported previously, *rtg1*Δ *aco1*Δ*, rtg2*Δ *aco1*Δ*,* and *rtg3*Δ *aco1*Δ double mutants maintained mtDNA. This data indicate that the RTG pathway mediates mtDNA loss in *aco1*Δ mutant cells. I

quantified the percentage of rho^0 cells in wild-type rho$^+$ cells with intact mtDNA, wild-type rho^0 cells that have lost mtDNA, an *aco1*Δ single mutant, and *rtgΔ aco1*Δ double deletion mutants. The percentage of rho^0 cells was greatly reduced in *rtgΔ aco1*Δ double mutants compared to the *aco1Δ* single mutant (**Figure 4B**).

A



B

Strains	% Rho^0 cells	# Cells Analyzed
WT (rho+)	2.5%	551
WT (rho0)	100.0%	433
rtg1 Δ aco1 Δ	4.7%	406
rtg2 Δ aco1 Δ	18.3%	454
rtg3 Δ aco1 Δ	6.0%	399
aco1 Δ	100.0%	440

Figure 4: mtDNA Maintenance in *rtg1Δ aco1, rtg2Δ aco1Δ, and rtg3Δ aco1Δ* Mutants
(A) *aco1Δ* mutant strains also containing mutation in one of RTG genes were grown in YPD media and stained with DAPI as mentioned in materials and methods. mtDNA observed as small white dots in cells. (B) Percentage of rho^0cells was determined by counting approximately 400 cells.

Although mtDNA is maintained in *rtgΔ aco1*Δdouble mutant cells, the question that remains is whether mtDNA is functional in such mutants. To this end, the double mutants were mated with rho^0 cells of the opposite mating type to generate diploids, which were subsequently streaked onto plates with ethanol or glucose as the sole carbon source. Figure 5 shows that diploids generated from the crossing of rho0 cells with *rtgΔ aco1*Δ double mutants were all able to grow on ethanol media, indicating that mtDNA in *rtgΔ aco1*Δ haploid cells were functional.

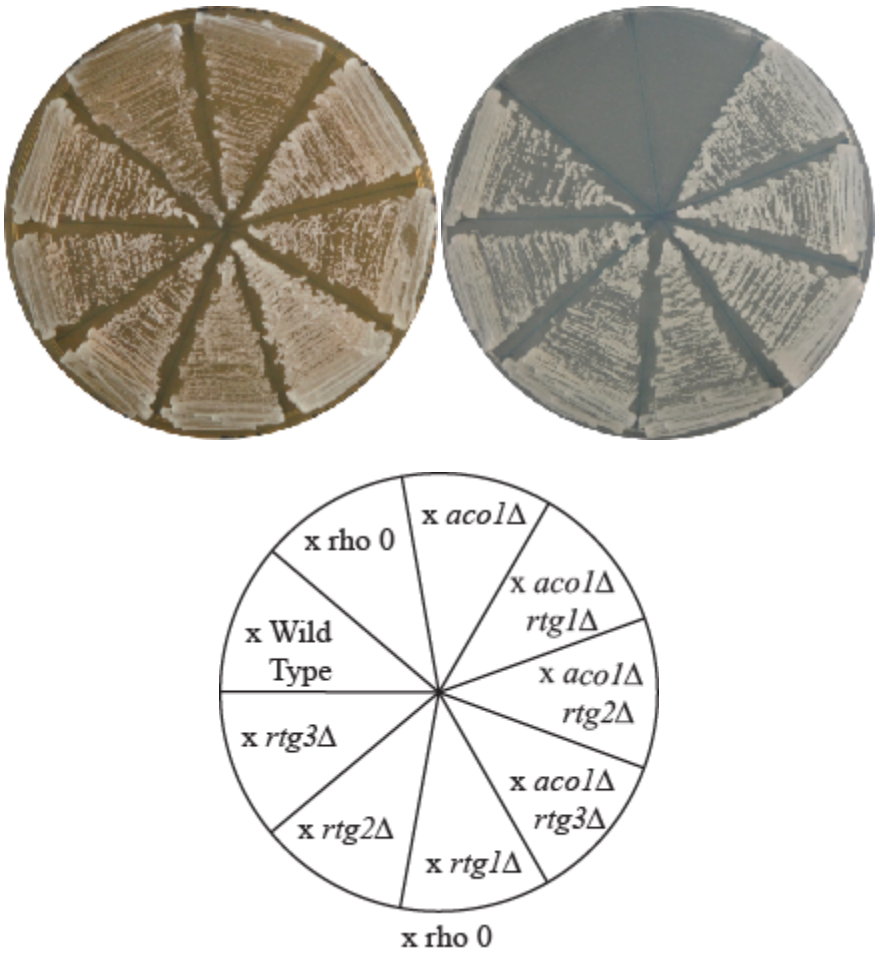


Figure 5: mtDNA is Functional in *aco1Δ rtgΔ* Double Deletion Mutants

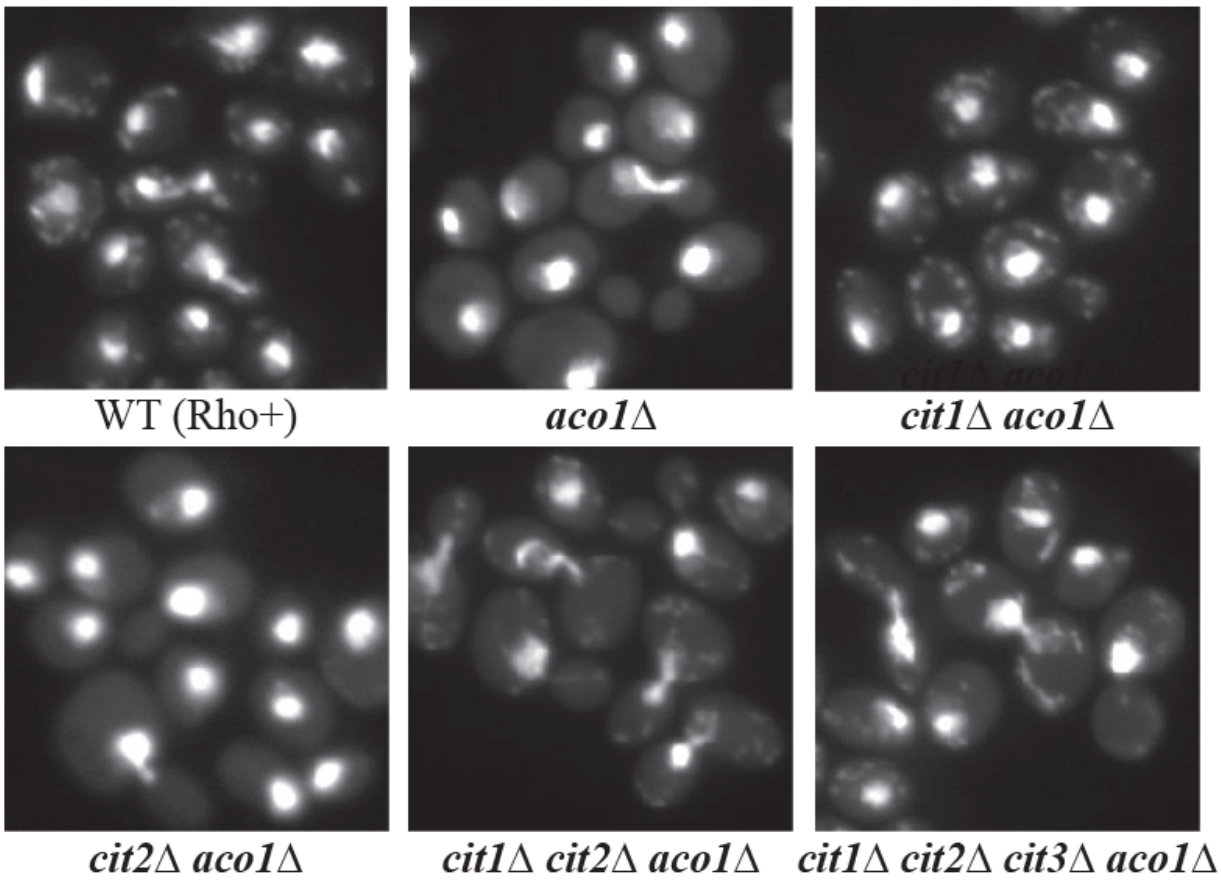
Wild-type rho^0 cells lacking mtDNA were crossed to wild-type rho$^+$, wild-type rho^0, *aco1Δ, rtg1Δ aco1Δ, rtg2Δ aco1Δ, rtg3Δ aco1Δ, rtg1Δ, rtg2Δ,* and *rtg3Δ*strains. The resultant diploid strains were streaked onto YPD and YP-ethanol media to determine the functionality of mtDNA. Cells were grown at 30℃ for two to three days. Top left, YPD plate; top right, YP-ethanol plate.

Blocking Citrate Synthesis Prevents mtDNA loss

Citrate synthesis is catalyzed by three citrate synthases encoded by *CIT1, CIT2,* and *CIT3.*It has been shown previously that a *cit1Δ* mutation partially suppresses mtDNA loss in *aco1Δ* mutant cells (Lin et al. 2008). Expression of *CIT1*, *CIT2* and possibly *CIT3*, is under the control of the RTG pathway. Suppression of mtDNA loss in *rtgΔ aco1Δ* double mutant cells may be due to reduced expression of genes encoding citrate synthase. To test this possibility, we introduced *aco1Δ* into a *cit1Δ cit2Δ cit3Δ* triple mutant and the resulting quadruple mutant

maintained mtDNA. This data suggest that reduced expression of genes encoding citrate synthase is responsible for *rtgΔ*-mediated suppression of mtDNA loss in *aco1Δ* cells. To determine which citrate synthase encoding gene is responsible for mtDNA loss in aco1D mutant, we generated *aco1Δ cit1Δ*, *aco1Δ cit2Δ*, *aco1Δ cit3Δ* double mutants, and *aco1Δ cit1Δ cit2Δ* triple mutant by crossing the respective haploid mutant strains and tetrad dissection. We found that 6 out of 8 *aco1Δ cit1Δ* double mutant strains, 0/6 *aco1Δ cit2Δ* double mutant strains, 0/6 *aco1Δ cit3Δ*, and 6/6 *aco1Δ cit1Δ cit2Δ* triple mutant strains maintained mtDNA (Fig. 6A and data not shown). We also calculated the percentage of rho^0 cells in a *cit1Δ aco1Δ* double, a *cit1Δ cit2Δ aco1Δ* triple and a *cit1Δ cit2Δ cit3Δ aco1Δ* quadruple mutant and found that they had less than 10% rho0 cells. Taken together, these data suggest that *CIT1* is mainly responsible for mtDNA loss in *aco1Δ* mutant cells.

A



B

Strains	% Rho^0 cells	# Cells Analyzed
WT (rho+)	2.5%	551
WT (rho0)	100.0%	433
aco1 Δ	100.0%	440
cit1 Δ aco1 Δ	6.5%	432
cit2 Δ aco1 Δ	100.0%	510
cit1 Δ cit2 Δ aco1 Δ	9.9%	444
cit1 Δ cit2 Δ cit3 Δ aco1 Δ	8.2%	454

Figure 6: Mutations in genes encoding citrate synthases prevent mtDNA loss due to *aco1Δ*. (A) mtDNA in indicated strains grown in YPD medium were examined by DAPI staining. (B) The percentage of rho^{0}cells in indicated strains was determined from approximately 400~550 DAPI-stained cells.

Deficiency of Iron Import into the Mitochondrial Matrix Prevents mtDNA Loss due to *aco1Δ*

Citrate levels raise iron levels through iron chelation (O. S. Chen, Hemenway, and Kaplan 2002). It has been proposed that iron citrate toxicity contribute to oxidative damage and mtDNA loss in *yfh1* mutant cells. Mitochondrial iron transport is mediated by iron transporters Mrs3 and Mrs4 in the mitochondrial inner membrane. Mutations in *MRS3* and *MRS4* lead to reduced iron levels in the mitochondrial matrix. To determine whether iron import into the mitochondria contributes to mtDNA loss in *aco1Δ* mutant cells, we generated an *mrs3Δ mrs4Δ aco1Δ* triple mutant. DAPI staining showed that mtDNA was maintained in the triple mutant (Figure 7). Quantitative analysis showed that the percentage of rho^0 cells in the triple mutant was similar to that in wild-type rho^+ cells (data not shown). These data support the notion that mtDNA loss in *aco1Δ* mutant cells is due to iron citrate toxicity. The *mrs3Δ mrs4Δ aco1Δ* triple mutants were also mated to rho^0 tester strain and the resulting diploids were streaked onto plate with ethanol as the sole carbon source. We found that the diploids could grow on ethanol medium, indicating that mtDNA in the *mrs3Δ mrs4Δ aco1Δ* triple mutant is functional (data not shown).

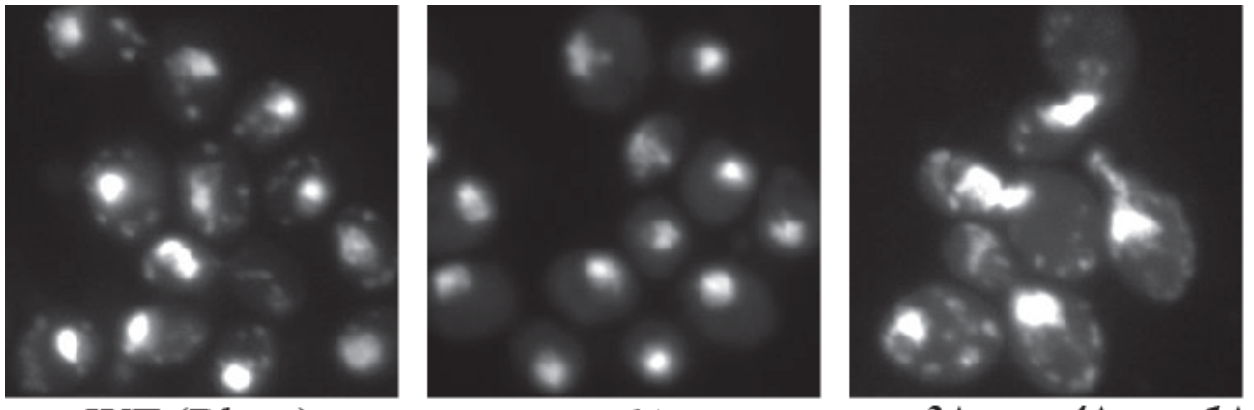


Figure 7: mtDNA is maintained in a *mrs3Δ mrs4Δ aco1Δ* triple mutant.
mtDNA in indicated strains grown in YPD medium were examined by DAPI staining.

A mutation in *SOD1*, but not *SOD2*, prevents mtDNA loss due to *aco1Δ*

Ferrous iron reacts with hydrogen peroxide through the Fenton reaction to produce potent hydroxyl radicals, which are believed to cause oxidative damage to mitochondria. Hydrogen peroxide is partly produced by the superoxide dismutases. In yeast, yeast cells contain two superoxide dismutases, Sod1 and Sod2, localized in the cytosol and the mitochondrial matrix, respectively. We hypothesized that a reduced production of hydrogen peroxide due to mutations in *SOD1* or *SOD2* might suppresses mtDNA loss in *aco1Δ* mutant cells by reducing the amount of hydroxyl radicals through the Fenton reaction. To this end, we generated *sod1Δ aco1Δ* double mutants by crossing *aco1Δ* and *sod1Δ* single mutant strain and tetrad dissection. Similarly, we also generated *sod2Δ aco1Δ* double mutant strains. Using DAPI staining, we found that 33 out of 34 *sod1Δ aco1Δ* double mutant strains generated maintained mtDNA while all six *sod2Δ aco1Δ* double mutants obtained lost mtDNA (Figure 8 and data not shown). These data suggest that hydrogen peroxide generated from reactions catalyzed by Sod1 contribute to mtDNA loss in *aco1Δ* mutant cells. Mutations in *SOD1*cause oxidative damage due to accumulation of superoxide radicals. Since a *sod1Δ* mutation suppressed mtDNA loss in *aco1Δ* mutant cells, we propose that hydroxyl radicals are more damaging to mtDNA than superoxide radicals.

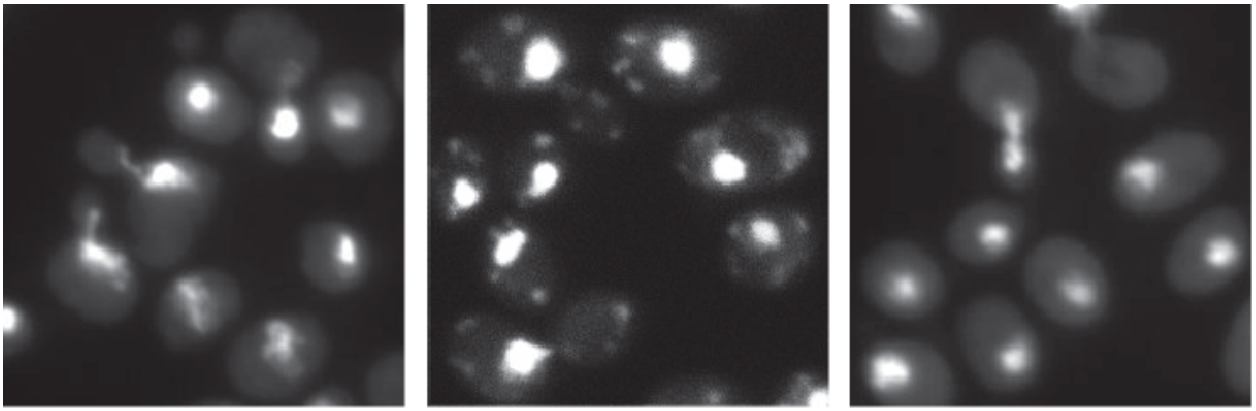


Figure 8: A mutation in *SOD1*, but not *SOD2*, suppresses mtDNA loss due to *aco1Δ*. Indicated strains were stained with DAPI and pictures were taken under fluorescence microscope.

Growth media affect mtDNA loss in aco1D mutant cells

It has been proposed that mtDNA loss in *aco1Δ* mutant cells is due to lack of physical protection of mtDNA in the absence of Aco1 protein. One key evidence to support the conclusion is the observation that the expression of two catalytically inactive Aco1 mutants, $Aco1^{C382S}$ and $Aco1^{C445S}$, under the control of the *ADH1* promoter from the pRS416 centromeric plasmid, prevented mtDNA loss in *aco1Δ* mutant cells. To maintain the plasmids, transformants were grown in YNBcasD medium. In light of discovery that iron citrate toxicity is responsible for mtDNA loss; one alternative explanation for mtDNA retention in cells expressing $ACO1^{C382S}$ and $ACO1^{C445S}$ mutant alleles is lower levels of iron in YNBcasD medium in comparison to YPD medium. To this end, we transformed *aco1Δ/ACO1* heterozygous diploid mutant cells with empty pRS416 vector and transformants were sporulated and dissected on YPD or YNBcasD medium. Eleven *aco1Δ* haploid mutants from YPD dissection plate were grown in YPD liquid medium and mtDNA was determined by DAPI staining, and we found that all of the 11 *aco1Δ* mutant dissectants lost mitochondrial DNA (Figure 9 and data not shown). In contrast, nine

aco1Δ mutant strains from YNBcasD dissection plate were grown in YNBcasD medium and DAPI staining showed that 7 out of the 9 *aco1Δ* mutant strains carrying the empty pRS416 vector maintained mtDNA (Figure 9 and data not shown). When these seven *aco1Δ* mutant dissectants that kept mtDNA were passed on YPD plate twice and grown in YPD medium, all of them lost mtDNA. Together, these data suggest that mtDNA loss in *aco1Δ* mutant cells is growth medium-dependent, which is likely due to differences in iron contents and/or types (ferrous or ferric) on YPD versus YNBcasD.

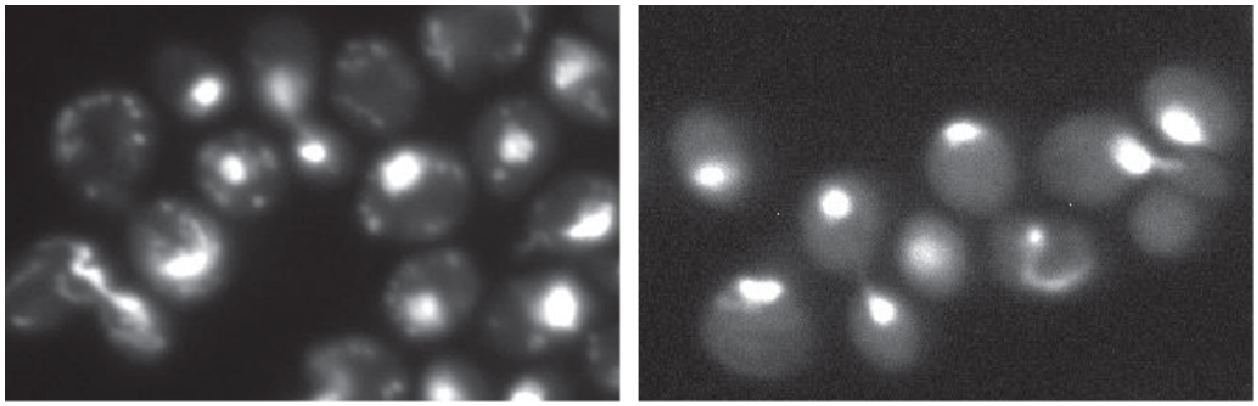


Figure 9: *aco1Δ*mutants carrying empty pRS416 vector maintain mtDNA when grown in YNBcasD medium.

aco1Δ mutant cells carrying pRS416 empty vector were obtained from tetrad dissection and grown on YNBcasD or YPD medium. mtDNA was nalyzed by DAPI staining and fluorescence microscopy.

Discussion

This study on budding yeast *aco1Δ* mutants elucidates a new hypothesis, which is that iron-citrate toxicity causes mtDNA loss in *aco1Δ* mutants. Citrate accumulation due to aconitase deletion, which is further elevated in *aco1Δ* by over activation of RTG pathway, corresponds to the conditions that lead to mtDNA loss. Reduced iron level due to regulation of citrate synthesis and elimination of potential generators of hydroxyl radical such as iron and hydrogen peroxide

through Fenton reaction, which is the cause of iron toxicity, results in mtDNA maintenance. mtDNA can, sometimes, accumulate mutations and become petite, rendering it non-functional. The *rtg1Δ aco1Δ, rtg2Δ aco1Δ, rtg3Δ aco1Δ, mrsΔ mrs4Δ aco1Δ*, when tested for mtDNA functionality, showed positive growth on non-fermentative media.

Yeast strains containing mutations in isocitrate dehydrogenase (*idh*) strains show increase in citrate levels and petite colony formation, phenotypes that are also shared by *aco1Δ* mutants. These phenotypes are repressed in *idhΔ cit1Δ* double deletion mutants due to decrease in endogenous citrate levels, whereas the increase in exogenous citrate results in increased petite frequency among these mutants (Lin et al. 2008). Similarly, our study proposes that the maintenance of mtDNA in *aco1Δ cit1Δ* double deletion mutants is due to a decrease in endogenous citrate levels.

Iron-citrate toxicity induced mtDNA loss in *yfh1Δ* has also been proposed (O. S. Chen, Hemenway, and Kaplan 2002). mtDNA loss due to *yfh1Δ*and *aco1Δ* is similar, such that in both cases decreasing cell citrate concentration can prevent mtDNA loss. Similarly, impeding iron import into the mitochondria also suppresses mtDNA loss in *aco1Δ*, which is consistent with the *yfh1Δ* (Foury and Roganti 2002). These similarities suggest that suggest that the iron homeostasis is crucial to the maintenance of mtDNA.

Chen et al. proposed that the mtDNA protective role of Aco1p is independent of its catalytic activity by transforming strains lacking Abf2, a DNA packaging protein, with Aco1 stripped of its catalytic activity. They obtained catalytically inactive aconitase by mutating cysteine residues to serine located at enzyme's active site. They constructed 416-ACO1 centromeric plasmid lacking enzymatic activity, and transformed it into *abf2Δ* mutants and

observed increase in frequency of rho+ cells (2005). We observed similar results in *aco1Δ* mutants containing pRS-416 plasmid. When those mutants containing empty vector were grown in YNBCasD media, mtDNA was maintained as compared to YPD media. This is an unusual behavior because *aco1Δ* has always led to mtDNA loss. This data implies that media constituents contribute towards maintenance of mtDNA in cells grown in YnBCasD as compared to YPD. If Chen et al. had transformed *abf2Δ* with pRS416 empty vector; they would have realized that maintenance of mtDNA is not due to expression of Aco1 cysteine mutants.

This study provides a possible mechanism for mtDNA loss in *aco1Δ* mutants, which can be employed to study the disease mechanism of Friedreich's ataxia. The similarity between the causes of respiratory defects in *yfh1Δ* and *aco1Δ* mutants may lead to the discovery of novel methods that can help understand various ROS related mitochondrial defects.

Conclusion

The results of this research establish a possible mechanism for *aco1Δ* induced mtDNA loss in budding yeast. This research establishes that mtDNA loss in *aco1Δ* mutants is due to the activation of the RTG pathway and subsequent iron citrate toxicity. This research can be employed to study the etiology of diseases, in particular Friedreich ataxia that results from mitochondrial defects due to iron citrate toxicity.

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APPROVAL SHEET

This is to certify that Muhammad Ali Farooq has successfully completed his Senior Honors Thesis, entitled:

Iron Citrate Toxicity Causes aco1 Δ-induced mtDNA Loss in Saccharomyces cerevisiae

Director of Thesis
Zhengchang Liu

for the Department
Mary J. Clancy

for the University
Abu Kabir Mostofa Sarwar
Honors Program

May 3, 2013
Date