

**Reduced cAMP-dependent protein kinase (PKA) signaling can increase
the fitness of *Saccharomyces cerevisiae* cells lacking mtDNA**

by

Emel Akdoğan

**A Thesis Submitted to the
Graduate School of Sciences and Engineering
in Partial Fulfillment of the Requirements for
the Degree of**

**Master of Science
in
Molecular Biology and Genetics**

Koç University

July 2015

Koç University
Graduate School of Sciences and Engineering
This is to certify that I have examined this copy of a master's thesis by

Emel Akdoğan

and have found that it is complete and satisfactory in all respects,
and that any and all revisions required by the final
examining committee have been made.

Committee Members:

Cory D. Dunn, Assist. Prof. (Advisor)

I. Halil Kavaklı, Assoc. Prof.

Arzu Celik, Assoc. Prof.

Date:

ABSTRACT

Damage to the mitochondrial genome (mtDNA) severely affects the cell and causes disease. Mutations to mtDNA also accumulate throughout the lifespan of many organisms and may be a proximal cause of aging. There is no effective treatment for ailments caused by mtDNA mutation. Since mitochondrial function and biogenesis are controlled by the nutrient environment of the cell, it is possible that perturbation of conserved, nutrient-sensing pathways may successfully treat mitochondrial disease.

Experiments using the tractable eukaryote *Saccharomyces cerevisiae* allow us to investigate the connection between nutrient-sensing and mitochondrial function. The main focus of my thesis is on the protein kinase A (PKA) pathway, which controls *S. cerevisiae* behavior according to glucose availability. We found that reduced PKA signaling leads to improved fitness after mtDNA loss. Our findings support the idea that glucose sensation is harmful for cells lacking the mitochondrial genome, however these effects are heavily dependent upon yeast genetic background. Interestingly, robust import of mitochondrial polytopic membrane proteins may be required in order for cells with no mtDNA to receive the full benefits of PKA reduction.

ÖZET

Mitokondriyal DNA (mtDNA) hasarları hücreyi olumsuz etkileyerek çeşitli hastalıklara yol açar. Mitokondriyal DNA’da meydana gelen bu hasarların, ilerleyen yaşla birlikte önemli miktarlara geldiği gösterilmiştir. Bu nedenle mitokondriyal DNA mutasyonlarının yaşlanma sürecinde önemli bir faktör olduğu belirtilmiş, hatta yaşlanmaya yol açan sebep olma olasılığını arttırmıştır. Ne yazık ki bu hasarların yol açtığı hastalıkları tedavi edebilecek yöntemler geliştirilememiştir. Mitokondriyal fonksiyon ve biyogenezi hücrenin besin ortamı tarafından kontrol edildiğinden, bu genellikle tüm canlılarda benzer yapıya sahip olan besin algılama yollarında yapılacak değişik düzenlemelerin, mitokondriyal hastalıkları başarıyla tedavi etmesi mümkündür.

Tek hücreli ökaryot *Saccharomyces cerevisiae* kullanarak yapılan deneyler, besin algılama ve mitokondriyal fonksiyon arasındaki bağlantıyı araştırmamızı mümkün kılar. Tezimin odak konusu ortamdaki glikoz durumuna göre *S. cerevisiae* davranışını kontrol eden protein kinaz A (PKA) sistemidir. Azalmış PKA aktivitesinin mtDNA kaybının sebep olduğu büyüme kusurlarını ortadan kaldırdığını gösterdik. Bulgularımız ortamdaki glikozun algılanmasının mitokondriyal genomu olmayan hücreler için zararlı olduğu görüşünü desteklemektedir, fakat bu sonuçların deneylerde kullanılan maya türüne özel olduğunu da belirtmeliyiz. İlginç bir şekilde sonuçlarımız, mitokondriyal protein girişini sağlayan politopik membran proteinlerinin, mtDNA’sı olmayan hücrelerde PKA aktivitesinin azalmasıyla sağlanan faydaları almak için gerekli olabileceğini göstermektedir.

This thesis is dedicated to the memory of my grandfather.

ACKNOWLEDGEMENTS

I am grateful to my advisor, Dr. Cory Dunn, for his professional guidance and intellectual support. His keen interest in my work, encouragement and support were central to my achievement. Although I will not leave very soon, his unwavering enthusiasm to the experiments and the lab environment that he provided are going to be terribly missed.

I wish to thank our lab members Abdurrahman Keskin and Arwa Bayraktar, and my previous fellow lab-mates Gökem Garipler and Göküz Baytek for their friendship and support. I want to thank Gökem for initiating my thesis project and giving me technical and moral support during my master studies.

I thank each member of my family starting with my grandmother for supporting me one way or the other.

I am thankful to my beloved parents who truly offered me unconditional love and support throughout my life and the master process. They encouraged me to be creative, to be a lifetime learner and to continually challenge myself. No word would be enough to express my appreciation.

I thank my brother, who is also one of my best friends. I know you will always be with me as I struggle to make my dreams come true. Your help and friendship are incredibly special for me.

Lastly, I thank my friends whom I won't name one by one, but immeasurable thanks for being such great people full of love and generosity, and utterly devoid of lies and insecurities. You taught me early on the only thing of value in life is to have true friends that share any achievement or failure with you. No matter how far we are, it is a relief to know that you are always there to support my endeavors.

TABLE OF CONTENTS

Contents

ABSTRACT	iii
ÖZET	iv
ACKNOWLEDGEMENTS	vi
LIST OF TABLES	x
LIST OF FIGURES	xi
NOMENCLATURE	xiii
Chapter 1	1
INTRODUCTION	1
Chapter 2	4
LITERATURE REVIEW	4
2.1 Overview of mitochondria and mitochondrial DNA (mtDNA).....	4
2.2 Defects in mtDNA are associated with human disease.....	6
2.3 There is no effective treatment for mtDNA-associated diseases.....	9
2.4 <i>Saccharomyces cerevisiae</i> can survive after mtDNA loss.....	10
2.5 <i>Saccharomyces cerevisiae</i> is a good model organism in which to study mitochondrial dysfunction.....	15
2.6 Nutrient signaling pathways have roles in the response to mtDNA damage.....	17
2.6.1 The cAMP-Protein Kinase A (PKA) Network.....	17
2.7 Literature Review Figures.....	21
Chapter 3.....	23
REDUCED cAMP-DEPENDENT PROTEIN KINASE (PKA) SIGNALING CAN INCREASE THE FITNESS OF <i>SACCHAROMYCES CEREVISIAE</i> CELLS LACKING MTDNA.....	23
3.1 Introduction.....	23
3.2 Results.....	29

3.2.1 Reducing cAMP/PKA pathway activity increases proliferation rate of yeast cells lacking mtDNA.....	29
3.2.2 Reduction of PKA activity reverses the nuclear transcriptional response associated with mtDNA damage.....	31
3.2.3 Reduction of PKA activity does not rescue the protein import deficiency of mitochondria with no DNA.....	32
3.2.4 A robust import pathway component for mitochondrial polytopic membrane proteins, Tom70p, is required for the beneficial effects of PKA reduction in cells without mtDNA.....	33
3.2.5 Many transcriptional regulators involved in PKA pathway are not required for the beneficial effects of <i>PDE2</i> overexpression in cells lacking mtDNA.....	36
3.2.6 Cells with deletion of TOR pathway regulatory components respond to <i>PDE2</i> overexpression when mtDNA is damaged.....	39
3.2.7 Increased P-body formation by the down-regulation of PKA activity is not the mechanism how <i>PDE2</i> overexpression benefits the cells with damaged mtDNA.....	40
3.3 Discussion.....	41
3.4 Conclusion.....	51
3.5 Materials and Methods.....	53
3.5.1 Yeast Strains and Culture Conditions.....	53
3.5.2 Protein Extraction and Western Blotting.....	53
3.5.3 Preparation of Cultures for RNA Extraction.....	55
3.5.4 RNA Isolation, cDNA Library Preparation and RNA-Sequencing (performed by Assoc. Prof. I. Halil Kavaklı and Mehmet Tardu).....	55
3.5.5 Transcriptome Analysis.....	56
3.5.6 Microscopy.....	56
3.6 Figure legends.....	57
3.7 Figures.....	62
3.8 Tables.....	70
Chapter 4.....	85
ACTIVATION OF THE PLEIOTROPIC DRUG RESISTANCE PATHWAY CAN PROMOTE MITOCHONDRIAL DNA RETENTION BY FUSION-DEFECTIVE MITOCHONDRIA IN <i>SACCHAROMYCES CEREVISIAE</i>	85
4.1 Introduction.....	85
4.2 Results and Discussion.....	86

4.2.1 Defects in the Mitochondrial-Endoplasmic Reticulum-Cortex Anchor (MECA) do not rescue the growth phenotype of petite-negative cells disrupted in mitochondrial fusion.....	87
4.2.2 Severe proliferation defects of <i>aac2Δ fzo1Δ</i> cells perturbed in sphingolipid biosynthesis do not favor growth assays to ask if <i>PDR</i> activation leads to increased sphingolipid biosynthesis.....	88
Chapter 5.....	90
MUTATION OF THE MITOCHONDRIAL LARGE RIBOSOMAL RNA CAN PROVIDE PENTAMIDINE RESISTANCE TO <i>SACCHAROMYCES CEREVISIAE</i>	90
5.1 Introduction.....	90
5.2 Results and Discussion.....	91
5.3 Figure Legend.....	92
5.4 Figure.....	93
Chapter 6.....	95
UNDERSTANDING THE MECHANISMS OF MITOCHONDRIAL ARCHITECTURE AND FUNCTION.....	95
6.1 Introduction.....	95
6.2 Results.....	96
6.2.1 Separate deletions of the fusion components in <i>mos1Δ dnm1Δ</i> cells resulted in varying effects on the growth rate.....	96
6.3 Discussion.....	96
6.3.1 Ugo1p loss results in severe proliferation defect in <i>dnm1Δ mos1Δ</i> cells.....	96
6.3.2 <i>FZO1</i> deletion partially rescue the proliferation defect in <i>dnm1Δ mos1Δ</i> cells..	98
6.4 Figure Legends.....	99
6.5 Figures.....	100
BIBLIOGRAPHY	104
VITA	129

LIST OF TABLES

Table 3.1 List of strains used in Chapter 3

Table 3.2 List of plasmids used in Chapter 3

Table 3.3 List of oligonucleotides used in Chapter 3

Table 3.4 List of antibodies used in Chapter

Table 3.5 List of CDD919 spores PCR-tested in an attempt to obtain
tpk1Δtpk2Δtpk3Δyak1Δ quadruple deletions

Table 4.1 List of strains used in Chapter 4

Table 5.1 List of strains used in Chapter 5

Table 6.1 List of strains used in Chapter 6

Table 6.2 List of oligonucleotides used in Chapter 6

LIST OF FIGURES

Figure 2.1 MtDNA encodes for most of the proteins that build up the components of OXPHOS.

Figure 2.2 Glucose activates cAMP-PKA signaling.

Figure 3.1 Reducing PKA activity by *PDE2* overexpression is beneficial for cells lacking mtDNA.

Figure 3.2 Deletion of *TPK3*, but neither other isoforms nor their combinations, rescued the growth defect of ρ^- cells.

Figure 3.3 Deletion of PKA activators, Gpa2p or Gpr1p, or a PKA catalytic isoform, Tpk3p, benefit cells lacking mtDNA.

Figure 3.4 Reduced glucose sensing diminishes the effects of mtDNA damage in yeast independent of the extracellular glucose concentration.

Figure 3.5 Characteristic transcriptional response to mtDNA loss is reversed with diminished PKA activity.

Figure 3.6 PKA inhibition does not increase protein import by mitochondria lacking mtDNA.

Figure 3.7 Reduced PKA activity allowed viability of mutants that depend on mtDNA for survival except *aac2Δ* and *tom70Δ*.

Figure 3.8 Phosphorylation mutants of TOM complex components do not have altered proliferation rates after their mtDNA is depleted.

Figure 3.9 Tom70p level does not significantly change in cells lacking mtDNA with or without *PDE2* overexpression.

Figure 3.10 Cells deleted of yeast Hsp90 isoforms, encoded by *HSP82* and *HSC82*, have increased proliferation rate when PKA activity is diminished.

Figure 3.11 Aac2p level is not altered when *PDE2* is overexpressed in cells lacking mtDNA.

Figure 3.12 Many transcriptional modulators that are regulated by PKA pathway are not the relevant targets of *PDE2* overexpression in cells lacking mtDNA.

Figure 3.13 PKA targets that are inhibitors of ribosome biogenesis are not involved in the mechanism of increasing proliferation rate of cells lacking mtDNA with *PDE2* overexpression.

Figure 3.14 Proliferation defect in cells deleted of the glycolysis regulator, Gcr2p, can be rescued by decreasing the activity of PKA.

Figure 3.15 Neither Snf1p nor Sip1p is required for the beneficial effects of *PDE2* overexpression in cells lacking mtDNA.

Figure 3.16 Cells deleted of TOR pathway regulatory components respond positively to diminished PKA activity after mtDNA depletion.

Figure 3.17 Cells lacking mtDNA with *PAT1* deletion exhibited an increased rate of survival.

Figure 5.1 Transfer of *PNT3-1* mtDNA to another nuclear background by cytoduction increases pentamidine resistance in the recipient background.

Figure 6.1 Deletion of *mgm1Δ*, but not *fzo1Δ* or *ugo1Δ* completely rescues the growth defect of *dnm1Δ mos1Δ* cells.

NOMENCLATURE

mtDNA Mitochondrial DNA

ETC Electron transport chain

tRNA transfer RNA

rRNA ribosomal RNA

WT Wildtype

EtBr Ethidium bromide

IM Inner membrane

IMS Inner membrane space

OM Outer membrane

$\Delta\Psi^{\text{mito}}$ Mitochondrial electrochemical potential

ρ petite

Δ Deletion

YEPD Yeast extract peptone dextrose

YEPGE Yeast extract peptone glycerol ethanol

Ura Uracil

His Histidine

Leu Leucine

Trp Tryptophan

Chapter 1

INTRODUCTION

Mitochondria are key organelles that take roles in a variety of cellular functions, including steroid and lipid synthesis ¹, the biosynthesis of iron-sulfur clusters (ISC) and heme ², and are well known for energy production via oxidative phosphorylation (OXPHOS) ³. The mitochondrion harbors a genome: mitochondrial DNA (mtDNA). Mitochondrial genomes are the reduced form of an ancestral prokaryotic genome. Most of the genetic information stored in this genome has been lost or transferred to the nucleus through the evolution of eukaryotes over billions of years ^{4,5}. Damage to mtDNA results in severe consequences for the cell, and is also linked to diseases including age-related neurodegenerative diseases such as Alzheimer's and Parkinson's ^{6,7}. Mutations in mtDNA may cause an array of diverse disorders, many of which are inherited. Moreover, mtDNA mutations correlate with, and may have a causal role in aging ⁸. Unfortunately, there are no means of treatment for mtDNA-associated diseases.

Although more than 99% of the genes required for mitochondrial function and architecture are located in the nucleus of the cell ⁴, mtDNA encodes for essential components of the energy-transducing system of mitochondria. Mitochondria form a dynamic network of interconnected double-membrane tubules ^{9,10}. The inner membranes (IMs) of mitochondria are well characterized by multisubunit complexes (CI-CIV) that make up the electron transport chain (ETC) which transfer electrons derived from oxidation of nutrients to reduce oxygen and trigger ATP production. ATP synthesis from ADP and inorganic phosphate (Pi) is driven by the proton gradient that is maintained by the proton pumping during electron flow, and depends on the activity of an enzyme called F₁F₀-ATP synthase (CV) ¹¹.

In cells carrying mtDNA mutations, the efficiency of electron transport chains (ETCs) is reduced, potentially leading to a variety of severe diseases in humans. In cells with no mtDNA, functional ETC is absent, since they cannot produce mtDNA-encoded ETC components. Facultative anaerobes, one of which is a yeast strain *Saccharomyces cerevisiae* can survive without OXPHOS when provided with an appropriate carbon source like glucose that enables them to produce ATP via glycolysis and fermentation.

“Petite” mutants in yeast are first described by Boris Ephrussi in 1949¹². Later these small, slow-growing colonies were shown to have deficiencies in respiration and lack cytochromes as a result of large deletions (ρ^-) or complete depletion (ρ^0) of their mtDNA¹². These petite mutations are common in *Saccharomyces cerevisiae*, spontaneously occurring at a frequency of $\sim 1\%$ per generation¹³, and *S. cerevisiae* cells are referred as petite-positive, since they can survive without a functional mtDNA¹⁴. Interestingly, some nuclear DNA mutations can result in petite-negative phenotype in yeast such as mutations in ATP/ADP translocase¹⁵. However, there are nuclear mutations that can predispose petite-negative yeast to survive on non-fermentable medium. Such mutations are also shown in the TOR pathway, highlighting the importance of signaling pathways in mitochondrial function¹⁶.

Experiments using the tractable eukaryote *Saccharomyces cerevisiae* allow us to investigate the connection between nutrient-sensing and mitochondrial function. My thesis focuses on the protein kinase A (PKA) pathway, which controls *S. cerevisiae* behavior according to glucose availability.

Chapter 2 provides a literature review about mitochondrial function and consequences of mtDNA loss. It briefly explains well characterized diseases associated with mitochondrial dysfunction, and discusses the current ineffective treatment approaches. Moreover, it supplies a general overview of glucose signaling, especially glucose signaling pathways, in yeast.

In Chapter 3, I demonstrate that reduced PKA signaling leads to improved fitness after mtDNA loss. There is significant evidence showing that diminished glucose sensation is beneficial for cells lacking the mitochondrial genome. I investigate the specific targets of PKA that may be relevant to the outcome of mtDNA damage.

Chapter 4 describes my contribution to a study linking the Pleiotropic Drug Resistance (PDR) pathway activation and mtDNA maintenance in *Saccharomyces cerevisiae* cells with disrupted mitochondrial dynamics.

Chapter 5 outlines my contribution to a study suggesting that pentamidine may block mitochondrial translation in many eukaryotes.

Chapter 6 discusses a possible link between mitochondrial dynamics and mitochondrial architecture that will further increase our understanding of mitochondrial function and mechanisms of mtDNA retention.

Chapter 2

LITERATURE REVIEW

2.1 Overview of mitochondria and mitochondrial DNA (mtDNA)

The cytoplasm of the eukaryotic cells is divided into various functionally different compartments, or membrane-bound organelles. Mitochondria are key organelles that play central roles in a wide variety of cellular functions, including energy provision via oxidation of nutrients through a mechanism called oxidative phosphorylation (OXPHOS)¹⁴, calcium homeostasis¹⁷, and synthesis of Fe-S clusters², heme¹⁸, and certain phospholipids¹⁹. Mitochondria are double-membraned organelles, further divided into four sub-compartments: the outer membrane (OM), the intermembrane space (IMS), the inner membrane (IM) and the matrix. Each compartment includes different sets of proteins taking unique roles in any of the essential processes performed by the mitochondria.

Unlike many other organelles, mitochondria harbor their own genome (mitochondrial DNA or mtDNA), which is the remnant of α -proteobacterial genome⁵. Mitochondrion is thought to have originated from acquisition of an endosymbiont²⁰. Through its evolution, the endosymbiont lost or transferred a massive fraction of genes to the nuclear chromosomes; only less than one percent of the endosymbiont's genome is thought to be retained on the mtDNA⁴. Actually, this is an evidently demonstrated on-going process for some lineages such as flowering plants²¹. Moreover, there are other studies experimentally supporting the possibility of mtDNA migration to the nucleus in yeast²². The mitochondrion-to-nucleus gene transfer leads to variability in mitochondrial gene content and coding capacity among different species. *Reclinomonas americana*, a protist, has the mtDNA with the greatest coding

capacity of 67 protein-coding genes, while *Plasmodium falciparum*, the causative agent of malaria from apicomplexans has only three coding genes in their mtDNA^{5,20}.

Mamallian mtDNA is extremely compact, composed of 13 protein-coding regions with no introns and some overlapping genes. All of the proteins are essential components of the cellular energy-producing apparatus, including 7 subunits or associated proteins of NADH dehydrogenase (ND1, ND2, ND3, ND4, ND4L, ND5, ND6: Complex I), cytochrome b (Complex III), 3 subunits of cytochrome oxidase (COI, COII, COIII: Complex IV), and two of the subunits of ATPase (ATP6, ATP8: Complex V)²³. Although there is a great size difference between the *Saccharomyces cerevisiae*, the budding yeast, (84 kb) and mammalian (16 kb) mtDNA, they encode proteins with similar functions. The mtDNA of *Saccharomyces cerevisiae* is composed of seven genes having functions in oxidative phosphorylation (*COX1*, *COX2*, *COX3*, *ATP6*, *ATP8*, *ATP9*, and *COB*), a gene coding for a ribosomal protein (*VAR1*), and mitochondrial small and large ribosomal RNA (rRNA) genes together with 24 transfer RNA (tRNA) genes²⁴. Unlike mammals, *S. cerevisiae* has a single-subunit NADH: ubiquinone oxidoreductase (Ndi1p) instead of multi-subunit NADH dehydrogenase (Complex I)²⁵.

Components of electron transport chain (ETC) are involved in the energy production mechanisms from various ranges of nutritional sources. The ETC is a series of four enzyme complexes (Complex I – Complex IV) and two coenzymes (ubiquinone and Cytochrome c), which act as electron carriers and proton pumps used to pump H⁺ ions out of the inner mitochondrial matrix and located on the inner membrane (IM) of mitochondria (Figure 2.1). The ETC enables the process of OXPHOS by coupling the transfer of electrons between a donor such as NADH and an acceptor such as O₂ with the transfer of protons, H⁺ ions, across the inner mitochondrial membrane.

OXPHOS is a metabolic pathway for ATP production, consuming O₂ in cells. The accumulation of the protons that are pumped into the inner membrane space of mitochondria creates a proton gradient with respect to the mitochondrial matrix, which establishes a proton-motive force with two components: a pH differential and an electrical membrane potential, referred as the mitochondrial electrochemical potential ($\Delta\Psi^{\text{mito}}$). Moreover, there is a protein complex (Complex V), F₁F₀-ATPase or ATP synthase, embedded in the inner mitochondrial membrane. It is made of two well defined protein entities: the F₁ sector, a soluble portion situated in the matrix, and the F₀ sector, bound to the IM. ATP synthase acts as a turbine

powered by the H^+ ions going into the mitochondrial matrix across the IM down its concentration gradient through the F_0 , which rotates the shaft of the complex, and thus ADP and inorganic phosphate (P_i) are favored to generate ATP in the matrix, in the F_1 sector. Also, ATP hydrolysis occurs through the same pathway in reverse. When F_1 -ATPase subunit γ rotates the $\alpha_3\beta_3$ hexamer (3 α and 3 β subunits of F_1 -ATPase), it provides energy for ATP synthesis²⁶. Modulation of the angle of the γ subunit is responsible for communicating the catalytic β subunits to hydrolyze ATP to power the rotation of the γ subunit²⁷. ADP and P_i have to be imported for ATP production, which depends on carrier proteins. Mitochondrial porins, the voltage-dependent anion channels (VDACs); in the OM allow passage of adenine nucleotides and P_i , while ADP import is facilitated by the ADP/ATP carriers.

Therefore, in a cell with intact mtDNA, $\Delta\Psi^{mito}$ is created by the cooperative activity of ETC coupled with the protons pumped out by F_1F_0 -ATPase, resulting in ATP synthesis. However, when ETC is inhibited, F_1F_0 -ATPase reverses to pump protons into the IMS by hydrolyzing ATP so that $\Delta\Psi^{mito}$ is maintained²⁸. In organisms do not depend on respiration for survival like *Saccharomyces cerevisiae*, some proteins encoded by nuclear DNA become essential for viability in the absence of mtDNA, such as Aac2p, since they are needed to maintain a reduced state of $\Delta\Psi^{mito}$ that will be further discussed in this chapter^{12,15}, (Figure 2.1).

MtDNA is a multi-copy extra nuclear genome, which also differs in copy number depending on the species and the cell type. Approximately, each cell contains between 10^3 and 10^4 copies of the mtDNA²⁹, replicated independently of the cell cycle by a nuclear encoded DNA polymerase, DNA polymerase γ ³⁰. It is critically important to maintain the normal level of mtDNA copy number for the proper function of mitochondria. Also, mitochondria constantly fuse and divide by the aid of small conserved GTPases, which enables them to maintain their shape, mass, function and integrity of their mtDNA³¹.

2.2 Defects in mtDNA are associated with human disease

Mitochondrial DNA accumulates mutations as well as nuclear DNA. Each somatic cell possesses thousands of copies of mtDNA, as compared to only two copies of nuclear DNA, and thus these mutations are generally compensated by the healthy mitochondria in the cell. More than 500 point mutations in mtDNA are found to be associated with diseases so far³².

Because of the high energy requirements of brain and muscle, mitochondrial diseases typically affect these body parts, and pathogenic mtDNA mutations are associated with classic mitochondrial diseases, myopathies, and neurodegenerative diseases. Besides point mutations, large-scale lesions in mtDNA are implicated in numerous human diseases.

Mitochondria are controlled by both the mitochondrial and nuclear genomes, and thus damage to either of these genetic materials may lead to mitochondrial disorders. Pathogenic mutations of the mtDNA are found in approximately 1: 5000 individuals¹¹. The mtDNA mutation rate is far higher than that observed in nuclear DNA. Although there are repair mechanisms for mtDNA mutations³³, they are not enough to prevent the oxidative damage sustained by the mitochondria. Disease phenotypes caused by the mtDNA mutations can be partly explained by threshold effect. The situation in which all mtDNA molecules are identical is referred as homoplasmy. However, most pathogenic mtDNA mutations are found in some genomes but not all, which is referred as heteroplasmy. Therefore, clinical symptoms in such cases are generally associated to the ratio of wild-type to mutant genome content, and this threshold is altered according to tissue type^{34,35}. An example of human diseases caused by mtDNA mutations is Leigh syndrome, a severe neurological disorder, affecting at least 1 in 40,000 newborns³⁶. It is recognized by progressive loss of mental and movement abilities and typically results in death within a few years, commonly by respiratory failure. Leigh syndrome is caused by certain mtDNA mutations. For example, mutations in the *MT-ATP6* gene, coding for a part of ATP synthase protein complex (complex V), block ATP production, and are found in approximately 20 percent of the people with the disease³⁷. The most common cause of the Leigh syndrome, however, is the defect in cytochrome c oxidase (complex IV) formation. The *SURF1* gene, encoded by nuclear DNA, is required for proper assembly of complex IV, and thus when it is mutated, complex IV formation is depleted, which impairs mitochondrial energy production and results in disease³⁷.

Inheritance of a large fraction of deleted mitochondrial genome results in several syndromes. They comprise three overlapping phenotypes inherited maternally and observed in a single family member or in various members of the family. One of these phenotypes is Kearns-Sayre Syndrome (KSS) which is a multisystemic disorder with an onset before 20 ages. The second instance, Pearson Syndrome, is lethal at infancy and characterized by sideroblastic anemia and exocrine pancreas dysfunction. A third example is Progressive External Ophthalmoplegia (PEO), a mitochondrial myopathy. It is defined by drooping of the

eyelids (ptosis), paralysis of the extraocular muscles (ophthalmoplegia), and proximal limb weaknesses with different grades of severity ^{34,38}.

Mitochondrial DNA depletion syndromes (MDS) are nuclear genetic disorders characterized by a severe reduction in mtDNA content. These diseases are caused by defects in nuclear genes that encodes mtDNA maintenance factors, functioning in either mitochondrial nucleotide synthesis (*DGUOK*, *SUCLA2*, *SUCLG1*, *TK2*, *RRM2B*, and *TYMP*) or mtDNA replication (*POLG* and *C10orf2*) ³⁹. These mutations lead to severe disorders, including muscle weakness, progressive encephalopathy, or liver failure, with poor diagnosis in most patients ³⁴. *POLG*-related disorders clearly demonstrate the variability of disorders that can result from mutation of a single gene. The gene encodes for the mitochondrial DNA replicase. One disease associated with *POLG* mutation is Alpers-Huttenlocher syndrome (AHS), characterized by early-onset progressive encephalopathy with intractable epilepsy and hepatic failure ⁴⁰. Another example is the ataxia neuropathy spectrum (ANS) characterized by ataxia, neuropathy and seizures ³⁹.

Besides from their link to human disease, mitochondria have been significantly valued in the aging studies. Mitochondrion has long been debated to take a very distinguished role in aging processes ^{8,23,41}. Mouse models that accumulate significant mtDNA damage were developed ^{42,43}, which suggest a casual role for mitochondria in aging, and contribute to the better understanding of mitochondrial aging in mammals. Later, mitochondrial dysfunction is implicated during the normal aging process ⁴⁴, and lesions in mtDNA were demonstrated to be more prevalent with age ⁴⁵. Moreover, 20 percent of healthy participants in the 1000 Genomes Project were shown to carry mutations associated with disease in some fraction of their mtDNA ⁴⁶. As explained before, this phenomenon is known as heteroplasmy. These mutations must be compensated with the copies of healthy mtDNA; however it is possible that there is a threshold beyond which the number of healthy mtDNA in cell cannot meet the energy needs of the cells, which could explain why all people with certain age suffer from some kind of an mtDNA-associated disease. Therefore, accumulation of mtDNA mutations and mitochondrial dysfunction may be of ultimate importance in elderly diseases if it is not causative ⁸.

2.3 There is no effective treatment for mtDNA-associated diseases

Currently, there is no means of a highly effective treatment of mitochondrial disease. Medications used for the treatment of mitochondrial diseases that will be discussed in this chapter are commonly focused on symptom-based management and maintenance of optimal health ⁴⁷. Although we will focus on the therapies that cannot cure but help individuals with mitochondrial dysfunction-associated diseases, the studies on the effectiveness of these therapies are also in controversy ⁴⁸. A recent study employing a systematic approach has revealed that not more than 3% of the publications on the treatment of mitochondrial diseases in the last fifty years include observations on more than five patients ⁴⁸.

Aside from reducing symptoms of the diseases, there are therapies using vitamins and cofactors that are evaluated in patients. However, the supportive treatment methods that will be exemplified in this section have been shown to be effective on either isolated patients or a small group of clinical trials. For instance, coenzyme Q10, CoQ10, is an integral component of the ETC, shuttling electrons between complexes. Promotion of this kind of enzymatic reactions benefits patients with reduced energy-provision capacity. Exogenous CoQ10 administration can be a way to treat a group of mitochondrial diseases such as Leigh syndrome, lactic acidosis, and some kinds of nephropathy ⁴⁹. However, recent studies conducted by higher doses of CoQ10 in blinded trials suggest that the positive results obtained before lack reproducibility and may partially be due to open-label trials that result in low-quality and a bias towards beneficial outcomes ^{48,50}.

Furthermore, administration of L-arginine, a semi-essential amino acid involved in creatine synthesis and nitric oxide production that acts as a neurotransmitter, is effective in patients with Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome, and those patients who responded to the therapy were demonstrated to have critically lower amounts of L-arginine than normal ⁵¹. MELAS can result from mutations in several genes including mitochondrial ones, and particularly affects the brain and nervous system.

Also, creatine produced by L-arginine combines with phosphate in the mitochondria to form phosphocreatine, and needed in tissues with high energy demand. Phosphocreatine is shown to help people having mitochondrial myopathies, which is also called ‘energy buffering’ therapy ⁵².

Finally, there are alternative strategies used in the treatment of mitochondrial diseases. One of them is external supplementation of intermediate factors to preserve defected complex activity: the biochemical 'bypass' system. For instance, succinate, a citric acid cycle intermediate partially bypasses complex I that leads improvement in a patient with a severe mtDNA deletion syndrome ^{48,53}. Other options include antioxidant therapies including cysteine, vitamins C and E, lipoic acid, and dimethylglycine that reduce toxic metabolites ⁴⁸.

However, current treatment methods are either effective for only a short time or actually toxic to patients ⁴⁷. No effective treatment for the mitochondrial dysfunction associated diseases has been found yet. Vitamin and cofactor supplementations that are discussed in this part can be efficient at maintaining the good health of the individual in limited instances ⁴⁷, but the proposed treatments have not been proved to have clinical efficacy or tested in controlled trials. Since there is no single protocol that is optimal for each patient having the same disease, it is crucially important to understand the mechanisms of mitochondrial dysfunction and investigate the ways to reverse its detrimental effects in cells. Understanding the biology of cells lacking mtDNA may contribute to the identification of druggable targets to treat these diseases.

2.4 *Saccharomyces cerevisiae* can survive after mtDNA loss

Most eukaryotes including humans depend on mtDNA for their survival, and called obligate aerobes. However, *Saccharomyces cerevisiae* cells can survive mtDNA loss ¹⁴.

Mutants lacking a functional mitochondrial (p^+) genome are called "petite" ¹². "Petite colonies" mutants in *S. cerevisiae* are first described by Boris Ephrussi and his colleagues in 1949 ⁵⁴, and the study of mitochondrial genetics in yeast owes its history to this study. They found that a cytoplasmic factor was either defective or lacking in these cells ^{54,55}, and further characterized cytoplasmic petite mutants as exhibiting slow growth ⁵⁶, respiration deficiency and lack of cytochromes *a* + *a*₃ and *b* ^{57,58}. Later, petite colonies were shown to arise spontaneously at a frequency of ~1%, and can be induced up to much higher frequencies by acriflavine ^{59,60}, which was previously thought to be a selective agent for petite mutants. The frequency of petite induction was so high to be caused by a Mendelian gene, also suggesting that a cytoplasmic factor is likely to be responsible for petite phenotype.

The genetic behavior of these mutants attracted intense interest. When they were crossed to a wild-type strain, the petite phenotype was not observed either in the resulting diploid or in the haploid progeny. For a chromosomal mutation in yeast, it is possible to obtain wild-type appearance in the diploids, and such mutations are called recessive with the current knowledge. However, recessive chromosomal mutations are expected to reappear following sporulation. Therefore, petite mutations were possibly due to lack or alteration of a self-replicating cytoplasmic factor, and considering the respiratory deficiency of petite mutants, this factor may reside in mitochondria⁶¹. These mutants are called neutral petites. Also, they demonstrated that some petite mutants in crosses with wild-type produces progeny of which variable non-Mendelian proportions are petite and referred them as suppressive petites⁶¹. This was thought to be caused by the suppressive activity of the altered cytoplasmic factor rather than its loss. This factor has since been known as ρ factor.

The following experiments lead to the discovery of mtDNA in yeast⁶² and confirmed the affected cytoplasmic factor in petite mutants (ρ) as mtDNA^{63,64}. The studies revealed two kinds of petite mutants: one lacking mtDNA⁶³, the other having reduced amount of mtDNA^{64,65}. Then, petite mutants that have extensive deletion of mtDNA are referred as ρ^- , while the petite mutants that have no mtDNA are called ρ^0 . The suppressive petites are suggested not to lose their mtDNA, but have an intensive damage to their mtDNA that may affect the progeny when transmitted. Spontaneously arising petites retain some of their mtDNA, however induced petites exhibit complete loss of mtDNA as a result of long acriflavin treatment. Currently, ethidium bromide (EtBr), a more affective DNA targeting agent, is used to rapidly deplete mtDNA in experimental studies⁶⁶.

Cells lacking mtDNA do not have the proteins encoded by the mtDNA, and thus their ETC is impaired which leads to a characteristic decreased inner membrane potential ($\Delta\Psi^{\text{mito}}$)²³. These cells cannot also generate $\Delta\Psi^{\text{mito}}$ by the reverse activity of F_1F_0 -ATPase, as would happen in lack of O_2 , since F_0 is partially encoded by mtDNA¹².

Some nuclear genes which are not essential for the survival of *S. cerevisiae* cells are required in the absence of mtDNA. When they are mutated or deleted in cells with no mtDNA, the cells are not viable, and this is called petite-negative phenotype. One of these genes encodes for ADP/ATP antiporter¹², encoded by *AAC2* in yeast. Cells disrupted in *AAC2* are deficient in respiration⁶⁷. The petite-negative *op1* mutants⁶⁸ were shown to have lesions in *AAC2* gene⁶⁹, resulting in their intolerance to mtDNA loss. This intolerance is due

to disruption of $\Delta\Psi^{\text{mito}}$ generation in ρ^- cells. The ADP/ATP translocase trades internal ADP³⁻ for external ATP⁴⁻, and increase the negative charge on the matrix side of the IM. This mechanism is preserved in respiratory deficient *S. cerevisiae*⁶⁷, and seems to be required for the maintenance of the reduced state of $\Delta\Psi^{\text{mito}}$ in ρ^- cells⁷⁰.

Second, catalytic and regulator subunits of i-AAA protease complex are required for viability of *S. cerevisiae* cells without mtDNA. The ATP dependent protease i-AAA is conserved from yeast to higher eukaryotes, and acts in the mitochondrial biogenesis and quality control network on the IM. Mutations in the gene *YME1* encoding the catalytic subunit of i-AAA protease complex were demonstrated to lead defects in mitochondrial function and morphology⁷¹, a higher rate of mtDNA migration to the nucleus than that in WT cells^{22,72} and lethality when mtDNA is depleted²². Moreover, the absence of the i-AAA complex adaptor proteins Mgr1 or Mgr3 is lethal for cells lacking mtDNA⁷³. *MGR1* mutants were shown to misassemble i-AAA protease and subsequently have defects in the IM protein biogenesis although they preserve some i-AAA activity^{73,74}.

Third, genes that are required for the viability of ρ^- cells include the genes encoding for α and β subunits of F₁-ATPase¹⁵. As explained before, ATP is synthesized by the activity of F₁ sector of F₁F₀-ATPase by using the energy gained by the proton movement from the IMS to the matrix through the F₀ entity. In *Saccharomyces cerevisiae*, all subunits of F₁ complex is encoded by nuclear genes, while F₀ complex is partially encoded by mtDNA¹². Mutations in the subunits of F₁-ATPase affect the growth of cells with no mtDNA, and *S. cerevisiae* cells disrupted in genes encoding α and β subunits of the F₁-ATPase, *ATP1* and *ATP2* respectively, exhibit petite-negative phenotype¹⁵. F₁-ATPase is suggested to be required for the production of intra-mitochondrial ADP, which is subsequently used by ADP/ATP antiporter to maintain $\Delta\Psi^{\text{mito}}$ in ρ^- cells¹².

Finally, some outer and inner membrane components including Tim9p, Tim10p, Tim12p, Tim54p and Mgr2p involved in mitochondrial protein import complexes are essential for the survival of ρ^- cells⁷³⁻⁷⁶. For instance, the TOM complex component, mitochondrial protein import receptor Tom70 protein⁷⁵ and a non-essential component of TIM22 complex Tim18 protein⁷⁷ were shown to be required in the absence of mtDNA. However, the cells lacking *TOM70* were not dependent on mtDNA for survival as strictly as *TIM18* mutants, shown by the viability of ρ^- cells after mtDNA loss on minimal growth medium⁷⁵. Both of these proteins together with Tim9p, Tim10p, Tim12p and Tim54p act as components of the TIM22

pathway required for the import of polytopic IM proteins into the mitochondria, and it is indicated that deficiency in this pathway may result in severe growth defects in ρ^- cells ⁷⁵.

Besides the proteins required for the survival of ρ^- yeast cells, which are also conserved in higher eukaryotes, there are mechanisms that promote fitness of the cells after mtDNA loss. Numerous observations suggest that *Saccharomyces cerevisiae* is able to survive mtDNA loss so long as a sufficient $\Delta\Psi^{\text{mito}}$ is generated. The petite-negative phenotype of *S. cerevisiae* cells deleted of *YME1* can be suppressed by point mutations in the α and γ subunits of F_1 -ATPase ⁷⁸. These mutations were shown to lead increased ATP hydrolysis activity resulting in an elevated $\Delta\Psi^{\text{mito}}$ ⁷⁹. Interestingly, expression of *YME1* in the petite-negative *Schizosaccharomyces pombe* cells can make these cells petite-positive ⁸⁰.

Likewise, a petite-negative yeast species *Kluyveromyces lactis* can survive mtDNA loss when it has similar changes in the α and γ subunits of F_1 -ATPase ⁸¹. Moreover, F_1 -ATPase activity in *S. cerevisiae* is severely defected by deletion of *ATP3*, encoding the γ subunit of F_1 -ATPase ⁸². These cells lose some of their mtDNA and have a significantly slow growth rate ⁸². The loss of mtDNA in *atp3* Δ cells is due to proton-leaking in defected F_1F_0 -ATPase, resulting in the dissipation of the $\Delta\Psi^{\text{mito}}$. The slow-growth phenotype of these cells can be suppressed by a mutation in the β subunit of F_1 -ATPase increasing ATP hydrolysis ⁸³ as well as deletion of genes encoding either the α subunit or the β subunit of F_1F_0 -ATPase, which are *ATP1* or *ATP2*, respectively ⁸². All of these mutations lead to generation of a greater the $\Delta\Psi^{\text{mito}}$ and in turn increase the proliferation rate of the cells ^{82,83}.

The suppressor function of F_1 associated ATPase activity or enhanced $\Delta\Psi^{\text{mito}}$ in yeast cells depending on mtDNA for their survival seems to be widely conserved. The trypanosome *Trypanosoma brucei* causes African trypanosomiasis known also as sleeping sickness in humans. *T. brucei* is a parasite that lives inside a host, which is either a tsetse fly (insect form) or a mammalian (bloodstream form), and depends on its mtDNA for survival in both insect and bloodstream forms. However, other closely related animal pathogens that live always in bloodstream, *T. evansi* and *T. equiperdum*, can survive although they lack functional mtDNA. While investigating the reasons for the essential function of mtDNA in *T. brucei*, single mutations were identified in F_1F_0 -ATPase subunit γ that enables the pathogen to live in the absence of mtDNA ⁸⁴. These mutations were shown to enhance $\Delta\Psi^{\text{mito}}$ depending on the highly active ADP/ATP carrier (AAC) in the absence of mtDNA ⁸⁴ that maintain the viability of *T. brucei* pathogens. Moreover, inhibition of F_1F_0 -ATPase subunit γ is lethal for

bloodstream form of *T. brucei* although it has little effect on *T. evansi* survival⁸⁵, indicating that the essential function of mtDNA in the bloodstream *T. brucei* is the requirement for F₁F₀-ATPase activity in order for the maintenance of the $\Delta\Psi^{\text{mito}}$. F₁-ATPase inhibition is lethal for *T. evansi* while insect form of *T. brucei* is not affected, and AAC inhibition significantly decreases $\Delta\Psi^{\text{mito}}$ in both bloodstream *T. brucei* and *T. evansi*⁸⁵. These results indicate that $\Delta\Psi^{\text{mito}}$ is maintained by the combined activity of F₁-ATPase and AAC in *T. evansi* lacking mtDNA, and enhanced $\Delta\Psi^{\text{mito}}$ may suppress the mtDNA dependence of the bloodstream form of *T. brucei*.

Finally, it is possible to generate ρ^0 human cells, first demonstrated by long-term exposure to EtBr as applied in *S. cerevisiae* cells⁸⁶. These cells were dependent on pyruvate and uridine for survival⁸⁶, since their ETC is impaired in the absence of mtDNA and metabolism of pyruvate to lactate is increased in cells with no mtDNA. Also, ρ^0 cells were successfully derived from different primates⁸⁷ and mouse⁸⁸ by treating them with different concentrations of EtBr for varying period of time. However, EtBr and other chemicals used to generate ρ^0 cells such as an anti-cancer drug ditercalinium (ddC)⁸⁹ have disadvantages, such as induction of mutagenesis and multi-drug resistance family gene expression that abolish the inhibitory effect on mtDNA replication⁹⁰. To overcome these drawbacks, recent studies suggest techniques that involve enzymatic targetting of mtDNA for depletion in mammalian cells to generate ρ^0 cells⁹⁰. Furthermore, the positive outcome of enhanced $\Delta\Psi^{\text{mito}}$ in cells lacking mtDNA may potentially be conserved in the mammalian cells, too. A recent genome-wide genetic screen that aims to identify genes making haploid human cells resistant to severe mitochondrial dysfunction revealed a similar result. Deletion of ATP1F1 protected cells from induction of ETC dysfunction induced by complex III inhibitor antimycin and allowed the viability of these cells via maintaining $\Delta\Psi^{\text{mito}}$ ⁹¹. ATP1F1 is an inhibitor of the ATPase activity of the F₁-F₀ ATP synthase, and activated to prevent ATP consumption when $\Delta\Psi^{\text{mito}}$ is decreased. However, in cells with dysfunctional ETC, the ones lacking ATP1F1 activity were shown to have increased $\Delta\Psi^{\text{mito}}$ while ATP levels are decreased compared to the WT cells⁹¹, indicating reverse F₁-ATPase activity. These findings strongly suggest that mechanisms enhancing $\Delta\Psi^{\text{mito}}$ in cells lacking mtDNA or having dysfunctional mtDNA can be widely conserved across diverse organisms.

2.5 *Saccharomyces cerevisiae* is a good model organism in which to study mitochondrial dysfunction

The preservation of mtDNA over an individual's lifetime depends on proper replication, segregation, and expression of functional genes during development and all subsequent mitotic cell divisions, and also protection from mtDNA damage. How can we better understand the biology of cells with mitochondrial dysfunction? Our current understanding underlying these processes has come from studies utilizing *Saccharomyces cerevisiae* as the model organism.

S. cerevisiae is the first eukaryotic organism to be sequenced⁹². The budding yeast is highly appreciated by geneticists as a model organism, since it is easy to grow with an approximately 90 min doubling time. Its accessibility in terms of classical and molecular genetics eases genetic manipulations in yeast. Most importantly, genes and pathways involved in mitochondrial biogenesis are conserved from yeast to humans⁹³. Moreover, new genome-wide technologies increase the utility of *S. cerevisiae*. Bar-coded deletion sets are available that enable us to uncover many genetic interactions in complex cellular mechanisms⁹⁴. Also, it is possible to conduct large-scale protein-protein interaction experiments, and the organism makes transcriptome, proteome and metabolomic analyses easier⁹⁵.

Mitochondrial functions have been preserved over evolution. Therefore, biogenesis and functions of mitochondria are widely studied in *S. cerevisiae*. First characterizations of OXPHOS system and initial observations of the mechanisms of mitochondrial and intra-mitochondrial protein transport were made in yeast^{96,97}. Likewise, biochemical assays that lead to the observation of DNA residing in mitochondria was first performed on highly purified yeast mitochondria⁶².

Homology between the human mitochondrial genes and *S. cerevisiae* enables its use as a model organism to investigate the effect of mutations in nuclear genes encoding OXPHOS components. There are mitochondrial diseases resulting from mutations that are first identified in yeast⁹⁸, and *S. cerevisiae* has been used as a model to study the function of proteins implicated in human disorders^{98,99}.

S. cerevisiae provides a powerful model organism to differentially study respiratory growth and mitochondrial genome maintenance and function, since it is possible to genetically dissect the pathways required for respiration and survival of the cells. *S. cerevisiae* generates ATP in

the cytosol via fermentation on carbon sources like glucose and fructose, leading to a reduction in the expression of genes required for the utilization of other carbon sources, referred as glucose repression ¹⁰⁰. These genes include many mitochondrial factors such as ETC components and TCA cycle enzymes. Moreover, mitochondrial size, volume and structure are altered according to the available carbon source in medium. When *S. cerevisiae* cells were grown on glucose, they exhibit a simpler mitochondrial network compared to that of cells grown in non-fermentable carbon sources such as glycerol ¹⁰¹. As OXPHOS is a major function of mitochondria, glycerol-grown cells have increased number of mitochondria that form a highly interconnected network ¹⁰¹. These nutritional effects in mitochondrial content and morphology ease the studies on the mitochondrial biogenesis machinery and mitochondrial function in *S. cerevisiae*.

Using the respiratory deficient mutants, petite mutants, a great source of data that explain mitochondrial genetics and cellular response to mtDNA loss are provided. Genome-wide deletion mutant analyses that identify respiratory mutants lead to assignment of novel functions to different proteins and identification of the molecular processes required for maintenance and expression of the mtDNA and respiratory growth in yeast ^{102,103}. For instance, besides proteins already known to be required for mtDNA maintenance such as Mgm1p regulating mitochondrial fusion and Doc1p involved cell cycle regulation, novel proteins such as Rrg5p with unknown functions are demonstrated to be essential for mtDNA maintenance ¹⁰².

Loss of mtDNA was shown to be associated with cell division defects. In a wide-scale screen for mutants producing petite cells, mutations in genes encoding thymidylate kinase and thymidylate synthase, *CDC8* and *CDC21* respectively, were found to arrest mtDNA replication that increase the frequency of petite formation ¹⁰⁴. However, a mutation in the cyclin-dependent kinase catalytic subunit encoded by *CDC28* had increased mtDNA stability and decreased frequencies in petite formation ¹⁰⁵, indicating a tight control mechanism for mtDNA replication during cell cycle.

One of the most dramatic effects of mtDNA loss is activation of a mitochondrial retrograde regulation ¹⁰⁶, providing communication between mitochondria and nucleus. The results were obtained by genome-wide transcriptional profiling of ρ^+ and ρ^- cells ¹⁰⁶. The mechanism including key signaling factors such as Rtg1p, Rtg2p and Rtg3p was found to signal mitochondrial dysfunction to the nucleus and result in altered nuclear gene expression ¹⁰⁷.

This signaling regulates mitochondrial biogenesis and protein import pathways to cope with the mitochondrial defects ^{108,109}.

The cellular responses to mtDNA loss and their consequences will be further discussed. These examples, however, highlight the importance of *Saccharomyces cerevisiae* as a model organism to study mitochondrial biogenesis and function. Therefore, we may search for novel ways to improve the fitness of cells lacking mtDNA in yeast. Identifying novel conserved molecular and biochemical targets modulation of which will benefit the cells having mitochondrial dysfunction will lead to the development of novel therapeutic approaches for the treatment of mitochondrial diseases.

2.6 Nutrient signaling pathways have roles in the response to mtDNA damage

Yeasts are facultative anaerobes, meaning that they can obtain energy from nutrients in one of the two ways: fermentation of sugars such as glucose or oxidation of fermentation products such as glycerol and ethanol ¹¹⁰. *Saccharomyces cerevisiae* responds to nutrient composition of the environment through complex interconnected signaling pathways. This response includes modifications in their transcriptional, metabolic and developmental programs. The mitochondria of yeast facilitate adaptations according to the metabolic events in cell ¹¹¹. Therefore, it is extremely important to reveal mechanisms by which mitochondrial function and nutrient signaling pathways are coordinated.

Glucose signals the repression of genes involved in respiration and stress response and blocks the use of alternate carbon sources, while enhancing the expression of genes involved in protein synthesis and growth ^{112,113}. Unique responses are given through highly complex signaling mechanisms sharing many common targets, so in this part, I will provide a simplified version of the response to glucose in yeast, which is highly conserved from yeast cells to human cells.

2.6.1 The cAMP-Protein Kinase A (PKA) Network

The cAMP-PKA pathway is one of the major ways to mediate extracellular glucose sensing. This pathway couples cell cycle progression to mass accumulation by repressing genes involved in stress response and stationary phase and activating genes involved in cell

proliferation^{112,113}. There are two branches in the glucose sensing system in cAMP-PKA pathway (Figure 2.2, A). First, extracellular glucose is sensed through a GPCR system composed of the Gpr1 protein and its associated GTP binding protein Gpa2, leading to the activation of PKA. Second, an intracellular mechanism employing glucose uptake results in activation of the Ras proteins, increasing PKA activity. However, double deletion of *RAS2* with either *GPR1* (*ras2Δ gpr1Δ*) or *GPA2* (*ras2Δ gpa2Δ*) is lethal and this lethality could be rescued by increasing PKA activity with *PDE2* deletion^{114,115}, strongly suggesting that activation of PKA pathway requires glucose sensing aside from the glucose uptake. It is important to emphasize that Ras proteins play a more significant role than Gpa2p in glucose response based on current observations, but both respond to glucose solely by modulating PKA activity.

PKA signaling is essential for cells to respond glucose. Uptake of glucose into the cells occurs through hexose transporters, encoded by the *HXT* genes (*HXT1* to *HXT4*) in yeast. These genes are upregulated 10- to 300-fold by glucose induction¹¹⁶. Ras1p/2p is activated by a currently unknown mechanism at the later steps of glucose metabolism. Deletion of Ras components in PKA signaling is lethal as well as *CYR1* (adenylyl cyclase) or *TPK1 TPK2 TPK3* (PKA) deletions. Ras proteins, Ras1 and Ras2, are small GTPases, and they cycle between GTP-bound and GTP-unbound state. Guanine nucleotide exchange factor (in short GEF, Cdc25/Sdc25 in yeast) facilitates GTP binding to Ras, while GTPase activating protein (in short GAP, Ira1/2 in yeast) hydrolyzes GTP to GDP. Ras protein is only active when bound to GTP, and the levels of GTP-Ras depend on the physiological properties of the cell, possibly responding to intracellular acidification. When glucose is added, Ras-GTP levels are upregulated in cell, which results in a significant transient increase in PKA activity¹¹⁷.

The glucose sensing GPCR signaling system was discovered by the sequence similarities of the proteins with their mammalian counterparts. Gpa2p is homologous to mammalian Gα subunit of the heterotrimeric G proteins¹¹⁸. Gpr1p is homologous to mammalian seven-transmembrane G-protein coupled receptors¹¹⁹. Gpr1p was discovered in a two-hybrid screen employing Gpa2p as a probe, which suggests that Gpr1p physically interacts with Gpa2p¹¹⁴. How Gpr1p senses glucose is still unknown. Currently, there is no identified ligand of Gpr1p, including glucose. However, when glucose is sensed, Gpr1p activates Gpa2p, acting as a GEF, and Gpa2p is inhibited by the function of Rgs2p, acting as a GAP, regulating glucose activation of cAMP/PKA pathway¹²⁰.

Activation of both Gpa2p and Ras in turn leads to the activation of adenylyl cyclase, encoded by *CYR1*, and Cyr1p catalyzes cAMP synthesis (Figure 2.3, B). The level of intercellular cAMP is balanced by the phosphodiesterase activity, encoded by *PDE1* and *PDE2*. PKA is a heterotetrameric protein activated by the increase of cAMP in eukaryotic cells. Yeast PKA is also composed of two catalytic subunits, encoded by *TPK1*, *TPK2*, or *TPK3* and a regulatory subunit, encoded by *BCY1*. The regulatory subunit Bcy1p regulates PKA activity by binding to the catalytic subunits at the substrate binding site in the absence of cAMP. Upon binding to cAMP, Bcy1p gets dissociated, and catalytic subunits become active in cell. PKA, then, phosphorylates its target proteins favoring fermentative growth and acting negatively on respirative growth, stationary phase and stress response.

Tpk1p, Tpk2p and Tpk3p are redundant catalytic subunits of PKA. They share a set of target proteins, but they also play distinct roles¹²¹. Tpk2p is shown to stimulate pseudohyphal morphogenesis, while Tpk1p and Tpk3p negatively control it¹²². Tpk1p takes negative roles in the biosynthesis of certain amino acids that may control iron levels and maintain DNA stability within mitochondria¹²³. Tpk2p downregulates genes involved in iron uptake and induce a class of genes involved in trehalose degradation, and may take unique roles for growth on non-fermentable carbon sources¹²³. Moreover Tpk2p and Tpk3p were shown to be associated with translation initiation factors and take regulatory roles in mRNA fate upon nutrient starvation¹²⁴. Tpk3p is also suggested to be specifically involved in the adjustment of cellular mitochondrial content according to the energy demand of the cell¹²⁵.

Moreover, Yak1p is an additional protein kinase that responds to glucose, but Yak1p activity triggers stress response and inhibits growth in contrast to PKA activity¹¹³. *YAK1* deletion can suppress complete loss of the Ras activity¹²⁶. Since Ras is known to be required for basal PKA activity in the cell, and Ras mutants exhibit almost no response to glucose¹²⁷, we may conclude that deletion of Yak1p can suppress PKA activity. One of the possible mechanisms of action of Yak1p may be inhibiting PKA by facilitating Bcy1p relocalization in response to glucose because Bcy1p is a target of Yak1p, and Yak1p phosphorylates Bcy1p and locates it to the cytoplasm in glucose starvation¹²⁸.

The metabolic signals are among the major components of the crosstalk in between mitochondrial structure and its functional changes in various physiological states of the cell¹²⁹. In higher eukaryotes, cAMP-PKA signaling is differentially distributed through PKA anchor proteins (AKAPs). Only compartments with AKAPs can efficiently signal PKA

stimuli. Uncovering such interactions is likely to reveal mechanisms of regulation of the vital mitochondrial processes by PKA. Although there is not much evidence, Rab32, a protein involved in mitochondrial fission, was shown to participate in PKA anchoring to the mitochondria¹³⁰. Moreover, there are AKAPs that are shown to target mitochondrial OM¹³¹, but future studies are required to assign dual roles for differentially anchored PKA in the regulation of mitochondrial function. Defects in cAMP compartmentalization are linked to neurodegenerative diseases associated with mitochondrial dysfunction¹³². Also, mitochondrial dysfunction in a cellular model of Parkinson's disease was shown to be reversed by locating PKA to mitochondria¹³³, highlighting the crucial role for PKA signaling in the regulation of mitochondrial function.

In yeast, however, proteins similar to AKAPs are not yet identified, but bioenergetics of the cell and, specifically, the relation of mitochondrial function to cell growth and survival is modulated by the cAMP-PKA pathway¹³⁴. Increased Pde2p activity and PKA down-regulation in non-respiring cells grown on glucose lead to enhanced activity of matrix-localized functions of mitochondria, which contributes to stabilization and folding of mitochondrial translation products¹³⁵, indicating a modulatory effect of cAMP-PKA signaling on mitochondrial functions. Moreover, in cells deleted of mtDNA, *FLO11* expression, required for both filamentous and invasive growth, was shown to be downregulated through the modulation of cAMP/PKA pathway¹³⁶. Another recent report demonstrated the regulation of autophagy by modulating the activity of the cAMP/PKA pathway in cells with dysfunctional mitochondria¹³⁷.

In conclusion, evidence supports regulatory links between mitochondrial function and PKA pathway which will be further discussed in chapter 3. Therefore, further studies on this relationship will shed light on how the modulation of cAMP and PKA can increase the fitness of the cells with mitochondrial dysfunction that may suggest novel ways of treatments for mitochondrial dysfunction-associated diseases.

2.7 Literature Review Figures

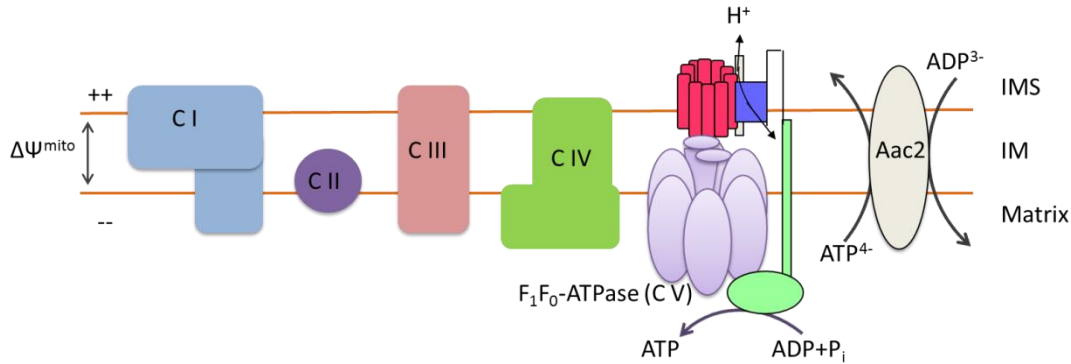


Figure 2.1 mtDNA encodes for most of the proteins that build up the components of OXPHOS. ATP is synthesized using energy derived from the redox reactions of the ETC. Electrons move down a chain of protein complexes (C I- C IV) and electron acceptors to oxygen, which creates a proton gradient across the membrane in the mitochondria called the mitochondrial IM potential ($\Delta\psi^{\text{mito}}$). ATP synthesis is driven by the ability of IM to pump H^+ ions and then harness the energy stored in H^+ gradient to spin the subunits of $\text{F}_1\text{F}_0\text{-ATPase}$ that forms a ring in the IM like a turbine to produce ATP. ADP/ATP carrier, Aac2p in yeast, is an antiporter required for the catalysis of the exchange of ADP for ATP across the IM, and required in the absence of mtDNA to maintain a reduced state of $\Delta\psi^{\text{mito}}$ that enables cells survive without mtDNA. At cellular pH, ATP has four negative charges (ATP^{4-}), while ADP has three negative charges (ADP^{3-}).

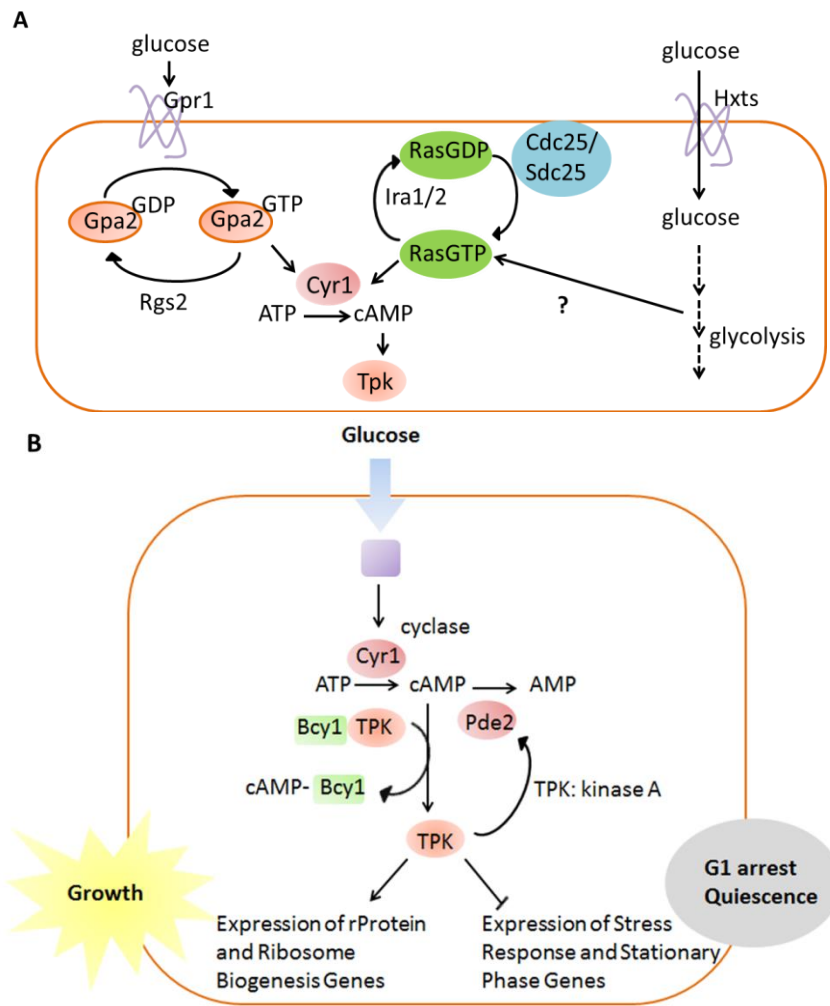


Figure 2.2 Glucose activates cAMP-PKA signaling. (A) Adenylyl cyclase (Cyr1) activity is triggered by the early steps of glucose signaling through extracellular glucose sensing via Gpr1 or intracellular glucose catabolism activating Ras proteins via an unknown mechanism (indicated by a question mark). The GPCR, Gpr1, stimulates guanine nucleotide exchange on Gpa2, while the Cdc25/Sdc25 proteins do that on Ras proteins. Rgs2 and Ira1/2 act as GAPs on Gpa2 and Ras proteins, respectively. The rise in cAMP in the cell by the activity of Cyr1, in turn activates PKA (in yeast Tpk isoforms of catalytic subunit). (B) Cyr1 acts as a cyclase, and cAMP levels rise in the cell, which is balanced by the activity of Pde2p. Cyclic AMP binds to the Bcy1 regulatory subunit of PKA. Upon binding, Bcy1 dissociates and free the activated catalytic subunits, Tpk1, Tpk2 and Tpk3. PKA activity triggers the expression of genes involved in growth and inhibition of genes associated with quiescence and cell-cycle arrest.

CHAPTER 3

REDUCED cAMP-DEPENDENT PROTEIN KINASE (PKA) SIGNALING CAN INCREASE THE FITNESS OF *SACCHAROMYCES CEREVISIAE* CELLS LACKING MTDNA

3.1 Introduction

Mitochondria are crucial to metabolic regulation and the modulation of a wide range of signaling events ^{107,138,139}. Likewise, nutrient signaling pathways have roles in response to mitochondrial dysfunction and mtDNA damage ^{111,136,137}.

Cell adapts to mitochondrial perturbations through the successful induction of the stress response, potentially the unfolded protein response, increase in DNA repair enzymes and morphological changes in mitochondria to keep mitochondria functional in order to maintain cellular homeostasis. An important fraction of the stress response system is directly responsive to nutrient signaling, and mitochondrial biogenesis is closely linked to PKA activity, which controls *S. cerevisiae* behavior according to glucose availability ¹¹³.

As reviewed in Chapter 2, mitochondrial dysfunction contributes to the etiology of diseases. Cilostazol is a FDA approved drug used in the treatment of intermittent claudication, which is a crampy leg pain caused as the major symptom of a serious arterial disorder, Peripheral Artery Disease (PAD). Cilostazol selectively inhibits phosphodiesterase III (*PDE3*), and increases intracellular cAMP levels ¹⁴⁰. Recent studies have shown that cilostazol treatment increases ATP content, mtDNA to nuclear DNA ratio, cytochrome B expression and mitochondrial mass in human umbilical vein endothelial cells (HUVECs) ¹⁴¹, which are indications of enhanced mitochondrial biogenesis. Furthermore, these indications of the promoted mitochondrial biogenesis are lost when a specific PKA inhibitor is in the medium, leading to the conclusion that cAMP increase stimulates PKA activity to increase mitochondrial biogenesis ¹⁴¹.

In Alzheimer's disease (AD), damaged mtDNA including both mtDNA mutations and mtDNA defects are shown to be involved ¹⁴², and also mitochondrial dysfunction is a prominent AD factor ¹⁴³. Experiments performed on cells over-expressing familial AD-causing APP mutant (APP^{swe}) have demonstrated a reduction in the gene expression of factors triggering mitochondrial biogenesis, including the mitochondrial transcription factor A (TFAM) that operates the transcription and the replication of mtDNA. This typical gene expression change indicating mitochondrial dysfunction was suppressed by the increase in cAMP levels in a dose-dependent manner, and this rescue is blocked in the presence of a specific PKA inhibitor ¹⁴⁴, again supporting the idea that PKA activity can alter gene expression to improve mitochondrial biogenesis.

Similarly, in yeast, mitochondrial function and biogenesis are regulated in response to environmental conditions by the regulation of several transcription factors. Some mammalian factors do not have direct counterparts in yeast; however, they may have functional homologs. For instance, Abf2p is needed for mtDNA maintenance, structure and expression ¹⁴⁵, and TFAM is able to complement its deficiency in yeast ¹⁴⁶. Recent studies on yeast revealed that cAMP/PKA signaling controls mitochondrial respiratory activity or leads to the unviability of cells through dysfunctional mitochondria in response to the nutritional composition of the environment ¹³⁹. Increased Tpk3p activity stimulates formation of dysfunctional mitochondria due to the altered gene expression that inhibits mitochondrial biogenesis, lead to defects in the ETC and block the stress response, suggesting a crucial link between the cAMP/PKA signaling, nutritional sensing and mitochondrial biogenesis ¹³⁹. In this chapter, we will look into mechanisms of how modulation of PKA pathway can benefit cells without mtDNA.

A brief overview of PKA as a complex target system transducing the glucose signal to promote growth is given in Chapter 2. However, glucose-controlled cellular response is also contributed by a complex network of signaling pathways such as the TORC1-Sch9 and Snf1p networks that share targets and cross-talk with PKA pathway ¹¹³. For instance, Target Of Rapamycin Complexes (TORCs) take roles in regulation of ribosome biogenesis, translation initiation, and entry into stationary phase in response to growth factors or nutrient composition of the environment ¹⁴⁷. Yeast cells possess the Tor/Sch9 pathway as a part of their glucose sensing mechanism. Sch9p is the functional orthologue of mammalian PKB/Akt and S6 kinase and is phosphorylated by TORC1. Genetic

evidence suggests that Sch9p activity has similar outcomes to PKA activity because *SCH9* overexpression can rescue the lethality of *ras1Δras2Δ*, *tpk1Δtpk2Δtpk3Δ* and *cry1Δ* cells¹⁴⁸. However, some common targets of PKA and Sch9p are demonstrated to be oppositely regulated on gene expression analysis¹⁴⁹. Moreover, Sch9p regulates the localization of the regulatory subunit Bcy1p and may alter the phosphorylation status of Bcy1p, which leads to the inactivation of PKA¹⁵⁰. Therefore, we could conclude that Sch9p works as a fine-tuning regulator of PKA in response to glucose signal.

Another nutrient-controlled cellular regulation mechanism includes Snf1p, the yeast homolog of mammalian AMP-activated protein kinase (AMPK). Yeast Snf1p is required for growth on less preferred carbon sources such as sucrose¹⁵¹, and directly responds to glucose limitation, working as a switch¹⁵². In the presence of glucose, Snf1p is auto-inhibited, while it is activated in the absence of glucose via further phosphorylating its conserved T-loop threonine (Thr210)¹⁵³. Snf1p activity has a pronounced importance in general stress response to high pH, high salinity, oxidative stress, and Tor inactivation¹⁵⁴.

The major glucose-sensing pathway is the PKA network that stimulates multiple protein phosphorylation events and has direct phosphorylation targets in cell. For instance, PKA activates 6-phosphofructo-2-kinase (PFK2), a key enzyme in gluconeogenesis and glycolysis, *in vivo*¹⁵⁵. Moreover, PKA can phosphorylate pyruvate kinase both *in vitro* and *in vivo* in yeast¹⁵⁶. Besides post-translational modifications of enzymes, PKA regulates gene expression at the transcriptional level as well. We will mainly focus on those targets in this chapter.

One of the PKA targets in cell is Rim15p, which is controlled directly in a negative way¹⁵⁷. Rim15 is a glucose-repressible protein kinase, and a common target of PKA, Sch9 and TOR pathways. Activation of Rim15p targets by TOR pathway requires neither protein phosphatase2A (PP2A) TOR effectors (Pph21p and Pph22p) or Sit4p (yeast PP6), deletion of which are beneficial for cells with damaged mtDNA¹⁶, nor PKA. In stress conditions, Sch9p mediates the response in a TORC1 dependent manner via phosphorylation and location of Rim15p at the cytosol¹⁵⁸. Relocation of Rim15p hampers the activity of downstream transcription factors. Also, Sch9p and TOR impinge on Rim15p localization through different mechanisms¹⁵⁹. Rim15 positively regulates three transcription factors that induce expression of genes involved in post diauxic shift and stress response.

Rim15p kinase stimulates Gis1p, a transcription factor required for gene regulation for carbon metabolism and the stress response in cells entering stationary phase ¹⁶⁰. Other targets of Rim15p are partially redundant transcription factors, Msn2p and Msn4p, required in yeast for general stress response ¹⁶¹. Msn2p and Msn4p are the master regulators of stress response to various environmental conditions like starvation, oxidative or osmotic stresses, enabling cells to adapt new conditions, and thus proliferate better. The regulation of Msn2p/4p cellular localization controls their transcriptional activity. Msn2p/4p are kept in the cytosol inactivated by PKA activity in normal conditions, however in the presence of stress factors, Msn2p/4p locates at the nucleus where they promotes the expression of stress response elements by binding stress response element (STRE) ^{161,162}.

Additionally, it has long been debated that calorie restriction can delay and attenuate changes with age, and expand the lifespan ¹⁶³. There is evidence indicating that the Ras/cAMP/PKA/Rim15/Msn2/4 and the Tor/Sch9/Rim15/Gis1 pathways are the crucial modulators of the calorie restriction-dependent stress resistance ¹⁶⁴. Furthermore, studies on *Saccharomyces cerevisiae* have shown that reducing kinase activities of TORC1, Sch9 and PKA increase chronological lifespan, making cells viable in stationary phase for a longer time in contrast to Rim15p which decreases the chronological lifespan when its kinase activity is reduced ¹⁶⁵, and there is also evidence that this regulation may be valid for replicative lifespan ^{166,167}.

An additional classical response to severe changes in the environmental properties is the heat shock response (HSR). This response is characterized by the increase in the level of heat shock proteins (HSPs), many of which are molecular chaperones ¹⁶⁸. HSPs prevent misfolding of proteins and aggregation in cytosol and help the cell to maintain the cell homeostasis. PKA regulates transcription of genes encoding small heat-shock proteins via Msn2p/4p-independent mechanisms as well as Msn2p/4p-dependent mechanisms ¹⁶⁹. Msn2p/4p regulates *HSP* expression by binding STRE ¹⁷⁰. Independent of Msn2p/4p, *S. cerevisiae* Heat Shock Factor, Hsf1p, is the chief transcriptional regulator that binds to the heat shock elements (HSEs) within *HSP* promoters, and induces stress response ^{171,172}. Unlike Msn2p/4p, Hsf1p is mostly found in nucleus and the activation of the expression of *HSF1* target genes is due to its altered binding stability to the promoter region, and possibly regulated by differential phosphorylation under varying stress conditions ¹⁷³.

Moreover, *HSF1*-inducible Hsp12p and Hsp26p levels are increased with diminished or eliminated activity of PKA, even in the absence of Msn2p/4p, suggesting a specific regulation of *HSP* expression¹⁶⁹. Although the mechanisms of Hsf1p activation is not established yet, both Rim15p and Yak1p phosphorylate Hsf1p *in vitro*^{174,175}, suggesting the regulation of the expression of Hsf1p target genes by two different pathways that are negatively regulated by PKA activity upon stress conditions.

Nutrient availability establishes a growth control in the cell by regulating translation and ribosome biogenesis^{112,113}. The yeast ribosome is composed of ribosomal proteins (RPs) encoded the RP regulon and ribosomal RNAs (rRNAs) encoded by the ribosomal DNA (rDNA) repeats. Also, there are other proteins involved in ribosomal assembly, encoded by the Ribi regulon including RNA polymerases I and III and other proteins required for many aspects of ribosome biogenesis. Naturally, it is a highly energy consuming process. In fact, 60% of total transcription is dedicated to ribosomal RNA, and 50% of RNA polymerase II transcription and 90% of mRNA splicing are dedicated to ribosomal proteins (RPs) in exponentially growing cells¹⁷⁶.

Since PKA activity correlates with fermentative growth, one could expect that PKA has targets that control cellular growth according to the nutrient availability. PKA is involved in nutrient control of both RNA polymerase II and III. Three transcriptional repressors Stb3p, Dot6p and Tod6p of Pol II-dependent RP and Ribi genes are controlled by both PKA and TOR pathways^{177,178}. TORC1/Sch9 dependent phosphorylation of these factors locate them at the cytoplasm in the presence of glucose¹⁷⁷. PKA can phosphorylate Dot6p and Tod6p and direct them to the cytoplasm, while export of Stb3p from the nucleus depends only on Tor kinase activity. However, a global transcriptional mediator, Sfp1p, controls a TORC1 positive regulation of ribosomal biogenesis¹⁷⁹. PKA phosphorylates Sfp1, and this phosphorylation is not sufficient to direct Sfp1 to the nucleus in the absence of glucose, but Sfp1 is directed to the nucleus in a PKA-dependent manner in the presence of glucose¹⁸⁰.

On the other hand, RNA polymerase III dependent transcription is negatively regulated by Maf1p. Maf1 transcription factor has multiple phosphorylation sites that locate it to different compartments triggered in response to nutrient composition of the environment. When it is dephosphorylated, it is located to the nucleus and prevent Pol III-dependent transcription to take place¹⁸¹. PKA, Sch9, Tor and casein kinase 2 (CK2) are the kinases

responsible for differential phosphorylation status of the Maf1. When phosphorylated by PKA, in particular by Tpk1, Maf1 is locked in the cytosol, and thus Pol III-dependent transcription occurs ¹⁸².

At the same time, PKA has targets involved in the mitochondrial protein import machinery, and contribute to the tight adjustment of the mitochondrial protein import in the case of a change in nutrient environment. Most of the nuclear encoded proteins are translated in the cytosol as precursors and directed to the mitochondria via the central way of entry into the mitochondria, the translocase of the OM (TOM complex) ¹⁸³. The TOM complex is composed of the central pore of translocation, Tom40p, the associated import receptors Tom20p, Tom70p, Tom22p, and the small subunits Tom5p, Tom6p, and Tom7p involved in the assembly and stability of the complex. PKA phosphorylates Tom70p, Tom40p and Tom22p, and thus has an important impact on the mitochondrial protein import capacity from different aspects ^{184–186}.

There are two important effects of phosphorylation of TOM complex components by PKA. First, it alters proper integration of Tom proteins to their destined places. When Tom40p is phosphorylated, the channel forming subunit can still bind to the membrane, but cannot be integrated properly ¹⁸⁵. Similarly, Tom22p precursor cannot be imported into mitochondria after its phosphorylation by PKA ¹⁸⁶. Second, besides its contribution to the control of the TOM complex biogenesis, PKA has regulatory roles on the complex function. Tom70p is the main import receptor for the metabolite carrier family including ADP/ATP carrier and phosphate carrier of the IM, and its phosphorylation by PKA impairs mature TOM complex function ¹⁸⁴.

In contrast to PKA, casein kinase 1 (CK1) promotes TOM complex biogenesis in response to glucose-driven signaling. CK1 promotes TOM complex assembly through Tom22p phosphorylation even in the presence of active PKA ¹⁸⁶. Although there is no evidence supporting differential regulation of the protein entry into mitochondria via altered phosphorylation status of the receptors, further investigation of such mechanisms may uncover a complex regulation system for the mitochondrial import in altered physiological conditions.

Once the precursors pass this stage, they are distributed to their destined sub-mitochondrial locations. Mainly, precursors with a N-terminal, cleavable presequence are sorted to the matrix or IM, which is mediated by the presequence translocase of the IM, the

TIM23 complex, while precursors carrying internal targeting signals are generally directed to the IM by the carrier translocase, TIM22 complex. There are also two other mechanisms of protein sorting in mitochondria, the machinery for intermembrane space import and assembly (MIA) and the sorting and assembly machinery (SAM) pathways, which are responsible for the specialized import and assembly of some IMS proteins and the assembly of β -barrel proteins on the OM, respectively^{187,188}.

In conclusion, taking advantage of the simplicity and the great conservation of cellular mechanisms of the *Saccharomyces cerevisiae*, it is possible to understand how the cells cope with the loss of mtDNA, and identify targets or increase the understanding of the biology of cells with mitochondrial defect, and thus lead to the identification of targets to cure mitochondrial diseases.

3.2 Results

3.2.1 Reducing cAMP/PKA pathway activity increases proliferation rate of yeast cells lacking mtDNA

Cells deleted of *PDE2* have a petite negative phenotype⁷³. In other words, cells require *PDE2* activity to survive in the absence of mtDNA. Pde2p hydrolyzes cAMP to AMP for balancing the cAMP: AMP ratio in the cell together with the adenylyl cyclase, Cyr1p, which converts ATP to cAMP. The levels of cAMP will increase in the cells when *PDE2* is deleted. A rise in the cAMP level will increase the PKA activity. When cAMP binds to the regulatory subunits of PKA, Bcy1p, the catalytic subunit of PKA, Tpk proteins, are dissociated from Bcy1p and activate its downstream targets as reviewed in Chapter 2 (Figure 2.2, B). So, we could explain the Pde2p dependence for the viability of cells when they lose their mitochondrial genome by hypothesizing that high PKA activity is harmful for cells lacking mtDNA. If so, we might expect that low PKA activity is beneficial for cells with no mtDNA. To test this possibility, we have overexpressed *PDE2* in wild type (WT) cells, and compared the proliferation rate of the cells with or without *PDE2* overexpression in the absence of mtDNA. *PDE2* overexpressing ρ^+ cells proliferate worse than WT ρ^+ cells, however, there is a significant proliferation increase in cells lacking mtDNA and having *PDE2* overexpressed compared to cells lacking mtDNA otherwise WT

(Figure 3.1). *PDE2* overexpression significantly increased the proliferation of ρ^- cells, suggesting downregulation of PKA pathway is beneficial for ρ^- cells.

TPK1, *TPK2* and *TPK3* encode isoforms of PKA in yeast, which take distinct and redundant roles in cell ¹⁸⁹. Therefore, we next asked which isoform is the relevant target. We compared the growth rate of single and double deletions of the three isoforms in the presence and the absence of mtDNA. We observed that *tpk3 Δ* ρ^- cells grow better than *tpk1 Δ* ρ^- or *tpk2 Δ* ρ^- cells (Figure 3.2), suggesting inhibiting a specific function of Tpk3 is beneficial for cells with no mtDNA. Double deletions do not grow better than *tpk3 Δ* ρ^- cells (Figure 3.2), supplying no additive beneficial effect to the cell proliferation. However, *tpk2 Δ tpk3 Δ* ρ^- cells proliferated much more slowly than *tpk3 Δ* ρ^- cells, and *tpk1 Δ tpk2 Δ* ρ^- cells were worse in proliferation than *tpk1 Δ* ρ^- and *tpk2 Δ* ρ^- cells that may suggest a shared function for Tpk1p and Tpk2p that could be important to maintain health in stress conditions. On the other hand, since the difference is not so significant, it may also be an artifact of deletion of two genes making cells less healthy. Since PKA is essential for the survival of *Saccharomyces cerevisiae*, *tpk1 Δ tpk2 Δ tpk3 Δ* cells are not viable ¹⁸⁹, but they are reported to live with the suppressor mutations *msn2 Δ msn4 Δ* or *yak1 Δ* . Although other groups used the *tpk1 Δ tpk2 Δ tpk3 Δ yak1 Δ* quadruple deletions to devoid of the PKA activity ¹⁹⁰, we were not able to obtain it among 30 tetrads tested for individual deletions of genes with PCR, possibly due to the use of different background strains. The genotypes of spores tested are listed in Table 3.5.

We have deleted key activators of the PKA pathway to inhibit PKA activity and test for beneficial effects in ρ^- cells. Components activating the adenylyl cyclase, Cyr1p, are the membrane associated glucose sensor, Gpr1p, and the small GTPase, Gpa2p. Deletion of either of these components greatly increases the proliferation rate of cells lacking mtDNA (Figure 3.3). Also, *tpk3 Δ* ρ^- cells are demonstrated to grow more slowly than *gpr1 Δ* ρ^- and *gpa2 Δ* ρ^- cells, suggesting that diminished PKA activity through upstream regulators of glucose sensing in cells lack of mtDNA has a more positive effect on their proliferation than deletion of the PKA catalytic subunit Tpk3p (Figure 3.3). In other words, *PDE2* overexpression does not benefit ρ^- cells solely through Tpk3p.

Since diminished PKA activity is beneficial for cells without mtDNA, we wondered if it is purely a reduction in glucose-sensing that provides benefits to ρ^- cells or the level of glucose matters as well. Previously, it was also reported that lowering glucose

concentration can strongly stabilize the nuclear genome of ρ^- cells, but not in ρ^+ cells¹⁹¹. Therefore, we have compared the growth rates of *WT*, *gpr1 Δ* , and *gpa2 Δ* cells with or without mtDNA on medium with different glucose concentrations. We demonstrated that reduction of glucose in the growth medium benefits cells lacking mtDNA, however we did not observe any additional benefit when we combine deletion of *GPR1* or *GPA2* with reduced glucose levels (Figure 3.4). This result suggests what is important to get the beneficial effect that we observed in the growth rate of ρ^- cells is purely the diminished glucose sensing through Gpa2p or Gpr1p.

3.2.2 Reduction of PKA activity reverses the nuclear transcriptional response associated with mtDNA damage

Loss of mtDNA results in a characteristic transcriptional signature within the nucleus. First, genes induced by the response to iron starvation are up-regulated^{192,193}. Secondly, inter-organelle communication is affected because of the altered gene expression of the components in the retrograde (RTG) pathway¹⁹⁴. Both of these responses are triggered by the reduction in the mitochondrial membrane potential^{192,195}. Therefore, we employed quantitative mRNA sequencing as an approach to observe if there is any effect of *PDE2* overexpression on the characteristic gene expression after mtDNA loss. We destroyed mtDNA of cells carrying an empty overexpression vector (plasmid b13) and cells in which *PDE2* is overexpressed from a plasmid (plasmid b89). We selected the genes mRNA levels of which are significantly upregulated upon mtDNA damage. Among them, we investigated targets, the mRNA levels of which return around the normal levels when *PDE2* is overexpressed. We observed that some genes involved in iron starvation or RTG pathway activation were at least two-fold upregulated in cells lacking mtDNA, as previously reported^{193,194,196}. However, diminished PKA activity through *PDE2* overexpression successfully attenuated this stereotypical transcriptional response to mtDNA depletion (Figure 3.5). The most remarkable hits were *FIT3* and *FIT2*, which were upregulated in ρ^- otherwise WT cells more than 45 fold, and ρ^- cells overexpressing *PDE2* upregulated their expression only around tenfold compared to WT ρ^+ cells. That means transcriptional response to mtDNA depletion through *FIT3* and *FIT2* expression was weakened by at least 80% by reduced PKA activity. Since the typical transcriptional

response to mtDNA loss is associated with reduced $\Delta\Psi^{\text{mito}}$, this result might suggest enhanced $\Delta\Psi^{\text{mito}}$ in cells overexpressing *PDE2*.

3.2.3 Reduction of PKA activity does not rescue the protein import deficiency of mitochondria with no DNA

The transport of preproteins into or across the IM of mitochondria is driven by $\Delta\Psi^{\text{mito}}$. In cells lacking mtDNA, $\Delta\Psi^{\text{mito}}$ is low^{70,197}, and thus mitochondrial protein import is impaired¹⁹⁸. Cox4p is a member of the mitochondrial ETC on the IM, and the localization of a fluorescent protein targeted by a Cox4 presequence is successfully used as an indication of mitochondrial protein import efficiency¹⁹². Since the presequence cannot be imported successfully into the mitochondria in ρ^- cells, the fluorescent signal is diffused in the cytosol. However, any mutation or modulation that will raise the $\Delta\Psi^{\text{mito}}$ results in the relocalization of the fluorescent signal to the mitochondria via the enhanced import of Cox4p presequence to the mitochondria^{16,192,199}.

ATP3-5 allele rises $\Delta\Psi^{\text{mito}}$ and rescues the mitochondrial protein import defect of ρ^- cells^{79,199} similar to the deletion of a *V₁V₀-ATPase* (vacuolar ATPase) component, *Vma2p*¹⁹⁹. Also, it has been shown that deletion of conserved protein phosphatases in ρ^- cells was able to relocate Cox4p (1-21)-GFP to the mitochondria¹⁶. Therefore, we checked the location of Cox4p (1-21)-GFP in ρ^- cells with *PDE2* overexpression or in cells deleted of *GPR1* or *GPA2* to see if reduced PKA activity was able to enhance the precursor import. We included *ATP3-5* ρ^- and *vma2Δ* ρ^- cells as positive controls in our microscopy experiments. More than 90% of the cells overexpressing *PDE2* or in cells deleted of *GPR1* or *GPA2*, mitochondrial morphology was not discernable due to diffused fluorescent signal in the cell. These cells resembled the negative controls of the experiment, the cells lacking mtDNA transformed with empty vectors. However, *ATP3-5* ρ^- and *vma2Δ* ρ^- cells were able to relocate Cox4p (1-21)-GFP observed with the distinguished fluorescent mitochondria (Figure 3.6 A, B), as previously reported.

3.2.4 A robust import pathway component for mitochondrial polytopic membrane proteins, Tom70p, is required for the beneficial effects of PKA reduction in cells without mtDNA

While searching for a mechanism that may explain how reduced glucose sensing benefits cells with mtDNA damage, we have tested the ability of *PDE2* overexpression to rescue the growth phenotype of several petite negative mutants. First, deletion of *ATP2* encoding for the β subunit of the F₁-ATPase results in petite-negative phenotype¹². Second, cells deleted of *MGR1*, encoding for a subunit of the i-AAA protease complex located in the mitochondrial IM, or *MGR2*, encoding for a TIM23 complex component located in the mitochondrial IM, require mtDNA to survive^{73,74}. Third, some components of the TIM22 pathway, the main mechanism for the import of polytopic IM proteins, including Tom70p are required for viability in the absence of mtDNA⁷⁵, as were discussed in Chapter 2. Therefore, we overexpressed *PDE2* in *aac2Δ*, *atp2Δ*, *mgr1Δ*, *mgr2Δ* and *tom70Δ* cells, and destroyed their mtDNA to see whether they would respond to *PDE2* overexpression or not. Cells deleted of *AAC2*, *aac2Δ*, are not viable upon mtDNA depletion regardless of the Pde2p levels (Figure 3.7 A), which was expected since cells highly depend on Aac2p for their survival in the absence of mtDNA, and no mutation or mechanism has been reported that reverse the detrimental effects of mtDNA loss in *aac2Δ* cells. PKA down-regulation allowed the viability of other mutants that depend on mtDNA for survival except one of them, *TOM70* (Figure 3.7 B, C).

Tom70p is phosphorylated at position Ser174 by PKA, which is thought to impair its receptor activity¹⁸⁴. Previously, it has been shown that replacement of serine (S) by alanine (A) at the PKA phosphorylation sites of Tom40p, Tom22p and Tom70p results in non-phosphorylatable mutant, while its replacement by glutamate (E) results in a phosphomimic mutant¹⁸⁴. Therefore, we have transformed *tom70Δ* cells with one of the plasmids, *pTOM70^{S174A}*, *pTOM70^{S174E}* or *pTOM70^{WT}*, and asked if the cells had any proliferation defect after mtDNA loss. Unlike *tom70Δ* ρ^- cells, cells with *TOM70* mutant carrying plasmids were proliferating at a comparable rate to cells with *pTOM70^{WT}* after mtDNA loss (Figure 3.8 A), indicating that phosphorylation status of Tom70p via PKA at the residue tested does not affect the proliferation of cells when their mtDNA is depleted.

We also transformed these cells with *PDE2* overexpression vector to see if they would positively respond to PKA downregulation. The *tom70Δ* cells carrying p*TOM70*^{WT} or p*TOM70*^{S174A} did not respond to *PDE2* overexpression with a significant increase in their proliferation rate (Figure 3.8 B). This might be a drawback of this particular experiment, since the cells are expected to keep two plasmids: one carrying *TOM70*, and the other carrying *PDE2*. Therefore, we performed an assay to test if a second plasmid affects the ability of *PDE2* overexpression to increase the proliferation rate using a WT strain (CDD463). This WT strain was transformed with the backbone plasmid (b3) which is used to construct WT and mutant *TOM70* plasmids, and then transformed with either b13 (empty 2μ vector) or b89 (2μ-*PDE2*). We observed a small increase in the proliferation rate of the cells carrying *PDE2* overexpression vector as the second plasmid (Figure 3.8 C), but this response was not as significant as in the cells carrying only one plasmid (Figure 3.1). Therefore, we could conclude that it is not possible to clearly detect if PKA reduction increases the fitness of the cells having mutations in *TOM70* with the plasmid-based assay.

Moreover, Tom22p and Tom40p are phosphorylated by PKA at the sites of Thr76 and Ser54, respectively ¹⁸⁴. We have tested if their phosphomutant forms have any growth defect when their mtDNA is depleted. However, we have concluded that phosphorylation status of other TOM complex components does not affect the proliferation of cells when their mtDNA is depleted (Figure 3.8 D).

Cells require Tom70p to exhibit the full benefits of PKA down-regulation. Therefore, we suspected if Tom70 protein levels were increased with *PDE2* overexpression. We used a strain having *TOM70* tagged with GFP in the chromosome. This strain was obtained from a library of 4156 yeast-GFP clones ^{200,201}. The chromosomally C-terminal tagged GFP fusion protein strains constructing the library covers approximately 75% of *S. cerevisiae* proteome ^{202,203}. We performed immunoblotting to detect the protein levels in cells lacking mtDNA with or without *PDE2* overexpression. Hexokinase, catalyzing glucose phosphorylation during its metabolism, was used as the loading control for protein levels. As expected, we were able to detect almost equal levels of hexokinase control in *TOM70::GFP* cells. Similarly, Tom70p levels were comparable in the presence or in the absence of mtDNA, and not altered upon *PDE2* overexpression (Figure 3.9). This is

consistent with the quantitative mRNA sequencing results since no significant increase in the transcript levels of *TOM70* was detected in the analysis.

Tom20p, Tom22p and Tom70p act as receptors for the import of different classes of precursor proteins into mitochondria ²⁰⁴. The Tom70p receptor activity is specifically critical for the import of particularly hydrophobic pre-proteins, such as the IM metabolite carrier family ²⁰⁵. Pre-proteins destined to mitochondria are associated to chaperones, Hsp70 (Ssa proteins in yeast) and Hsp90 (Hsp82 and Hsc82 in yeast), in cytosol, and Tom70p provides docking sites for these chaperone proteins ²⁰⁶. Hsp90 and Hsp70 work as a chaperone machine and prevents aggregation of newly synthesized pre-proteins in cytosol ²⁰⁷. Hsp90/Hsp70 docking onto Tom70p participates in their recognition by the Tom70p component of the TOM complex ^{206,208}, facilitating the entry of pre-proteins into mitochondria. Furthermore, cytosolic Hsf1p is kept in an inactive state in Hsp90-multichaperone complexes under nonstress conditions ¹⁴¹, suggesting a role for these chaperone proteins in the regulation of gene expression upon stress induction. Therefore, we wondered if Hsp90 and Hsp70 chaperones were contributing to the enhanced proliferation of cells lacking mtDNA via *PDE2* overexpression. In yeast, Hsp90 has two isoforms, encoded by *HSP82* and *HSC82*. We overexpressed *PDE2* in *hsp82Δ* and *hsc82Δ* cells and compared their proliferation rate after mtDNA loss to the ρ^- otherwise WT cells. Both *hsp82Δ* ρ^- and *hsc82Δ* ρ^- cells responded to *PDE2* overexpression with an increase in their growth rate (Figure 3.10), indicating that they are not the relevant targets in the cells that better cope with mtDNA loss when *PDE2* is overexpressed.

As was described in Chapter 2, the membrane potential of cells lacking mtDNA typically depends on both the ADP/ATP translocator encoded by *AAC2* and the ATP synthase ⁷⁰, and in the absence of mtDNA, $\Delta\Psi^{\text{mito}}$ is reduced. Experiments with Cox4p (1-21)-GFP might suggest that there is no significant increase in $\Delta\Psi^{\text{mito}}$ by the negative regulation of PKA through *PDE2* overexpression. However, since cells lacking mtDNA depend on Aac2p for their survival ¹² and Aac2p is a substrate of Tom70p ²⁰⁹, PKA may control Aac2p synthesis or import. To test this hypothesis, we performed immunoblotting to compare the protein levels of Aac2 in cells lacking *TOM70* in the presence or absence of *PDE2* overexpression. However, there is no detectable change in the protein levels of Aac2 when mtDNA is depleted in the presence or in the absence of *PDE2* overexpression

(Figure 3.11), indicating that increased fitness of *PDE2* overexpressing ρ^- cells is not due to enhanced Aac2p import into mitochondria.

3.2.5 Many transcriptional regulators involved in PKA pathway are not required for the beneficial effects of *PDE2* overexpression in cells lacking mtDNA

We found that reduced PKA activity improves fitness of cells lacking mtDNA. Since PKA adjusts growth in response to the quality and quantity of the nutrient composition of the environment through regulation of transcriptional activators and repressors^{112,113}, we investigated transcriptional targets, modulation of which by PKA pathway could potentially benefit the ρ^- cells. To figure out which components of the PKA pathway are involved in response to *PDE2* overexpression, we have deleted genes encoding for components that alters transcription depending on PKA activity and transformed them with *PDE2* overexpression vector. Cells proliferating faster in the presence of *PDE2* overexpression suggest that the gene tested does not provide the only mechanism, by which the cells are still positively responding to the *PDE2* overexpression.

Glucose-stimulated PKA activity results in repression of transcription factors, Msn2p/4p and Gis1p, involved in stress response and stationary phase entry through the activity of Rim15p kinase. Deletions of neither of these transcriptional regulators involved in PKA pathway prevented beneficial effects of PKA downregulation (Figure 3.12 A).

Moreover, Hsf1p binds to specific sequences in the promoter region of *HSP90* genes and mitochondrial chaperone genes^{210,211}, and regulates the expression of its targets via coordinating with Msn2p/4p depending on gene and stress type²¹². Therefore, diminished PKA activity might activate a stress response upon mtDNA damage through a Hsf1p-specific pathway. However, *S. cerevisiae* cells cannot survive in the absence of a basal Hsf1p activity, making deletion of the gene impossible to work with. So, we performed the proliferation assay using a strain harboring *hsf1::DAmP* allele. The hypomorphic, Decreased Abundance by mRNA Perturbation (DAmP) alleles can reduce mRNA levels significantly with modest growth defects²⁰⁰. The strain carrying a hypomorphic DAmP allele of *HSF1* was tested for growth after mtDNA depletion on fermentable medium. Cells containing the *hsf1::DAmP* allele were shown to proliferate better than WT cells in the

absence of mtDNA (Figure 3.12 B), suggesting that decreased Hsf1p activity is good for cells lacking mtDNA.

However, this unexpected result prevented us from testing the effect of *PDE2* overexpression in cells with reduced Hsf1p activity through *hsf1::DAmP* allele. As another approach, we tested the response of diploid ρ^- cells heterozygous for *HSF1* deletion to *PDE2* overexpression, and their response was positive (Figure 3.12 C), indicating that decreased Hsf1p activity does not provide the sole mechanism for the beneficial effects of PKA down-regulation in cells with no mtDNA.

Having considered cells carrying one *HSF1* allele exhibit approximately half-the Hsf1p activity of WT diploid cells, it is also reasonable to investigate the response of cells having much lower amount of Hsf1p activity. We made use of a single mutation within the DNA-binding domain of Hsf1p, by substituting proline to glutamine at amino acid position 215; previously referred as *EXA3-1*²¹³. We should note here that the mutation is first described as “a proline-to-glutamine substitution at amino acid position 214” in the original publication²¹³, however proline is actually located at the position 215 in the DNA binding domain of Hsf1p. Strains with the *EXA3-1* allele otherwise WT were shown to exhibit more than 10 fold reduction in the activity of Hsf1p under non-stress conditions, and the accumulation of the mRNAs of its targets was delayed²¹³. Therefore, by providing plasmid-borne mutated Hsf1p to the cells deleted of *HSF1*, we tested if cells with decreased Hsf1p activity had altered response to *PDE2* overexpression. Cells with the mutated version of Hsf1p and overexpressing *PDE2* had increased fitness upon mtDNA depletion compared to ρ^- cells with the mutated version of Hsf1p and carrying an empty overexpression vector (Figure 3.12 C). However, it was interesting that control cells with the plasmid-borne Hsf1p carrying an empty overexpression vector were not viable in the absence of mtDNA, but this severe growth defect could be rescued by *PDE2* overexpression (Figure 3.12 C).

Nutrient limitation was shown to signal through cAMP/PKA/Rim15/Gis1, Msn2p/4p or Hsf1p and result in the expression of various genes, including genes encoding small heat shock proteins, *HSP12* and *HSP26*^{157,214}. Our data and the results of others demonstrate, diminished PKA activity strongly increase the expression of *HSP26* and *HSP12*¹⁶⁹. Therefore, we further tested the response of *hsp12 Δ* ρ^- cells and *hsp26 Δ* ρ^- cells to *PDE2*

overexpression. Hsp12 and Hsp26 proteins were also shown not to be involved in the mechanism (Figure 3.12 D).

As briefly explained in the introduction part, PKA pathway stimulates ribosome biogenesis by altering the activity of RNA polymerases through negative regulators of Pol II dependent gene expression, Dot6p/Tod6p, and Pol III dependent gene expression, Maf1p.

Dot6/Tod6 proteins are key regulators of Ribi regulon involved in ribosome biogenesis. Ribi regulon is a group of genes expression of which is altered in response to environmental conditions or genetic perturbations. Dot6/Tod6 proteins are targets of TORC1/Sch9p in the control of Pol II- mediated transcription¹⁷⁷, and combined deletion of Dot6 and Tod6 proteins lead to a severe proliferation defect in the absence of mtDNA in *S. cerevisiae*²¹⁵. Since these effectors are also phosphorylated by PKA¹⁷⁸, we asked if cells would still exhibit increased proliferation rate by *PDE2* overexpression in the absence of Dot6p/Tod6p. Therefore, we have transformed *dot6Δtod6Δ* cells with the *PDE2* overexpression vector, and depleted their mtDNA. The cells responded positively to the diminished PKA activity (Figure 3.13), indicating that Dot6p/Tod6p cannot be the sole effectors in the mechanism of increasing fitness of ρ^- cells by down-regulating PKA.

Maf1p is a specific mediator of repression of the Pol III-dependent transcription, the transcription of tRNA genes¹⁸², involved in the nutritional and stress-signaling pathways. PKA negatively regulates Maf1p activity^{182,216}. Strains lacking PKA activity were shown to be hyper-repressible, in other words to exhibit reduced Pol III-dependent transcription¹⁸². Therefore, we wondered if PKA downregulation triggers Maf1p-dependent transcriptional repression to benefit the cells lacking mtDNA. However, *PDE2* overexpression increased the proliferation rate of *maf1Δ* ρ^- cells (Figure 3.13), indicating that altered activity of Maf1p via PKA downregulation is not the mechanism that increases the fitness of the cells with no mtDNA.

Rap1p, a multifunctional transcription factor, is a general regulator of ribosomal protein gene expression, and thus cellular growth rate. PKA-induced growth was shown to stimulate Rap1p transcriptional activity²¹⁷. Both Gcr1p and Gcr2p are Rap1p binding partners, and required for efficient Rap1p-inducible gene expression. Also, Tpk1p has negative genetic interaction with Gcr2p²¹⁸. Gcr2p is especially responsible for the regulation of glycolytic genes rather than RB genes²¹⁹, and there is not much information

on the regulation of this factor in response to glucose. We wondered if reduced glucose sensing was beneficial for cells lacking mtDNA, since glycolysis is downregulated. Hence, we deleted *GCR2* to downregulate the expression of glycolytic genes, and compared the growth rate of *gcr2Δ* ρ^- cells to control ρ^- cells. Deletion of *GCR2* was not beneficial for cells lacking mtDNA (Figure 3.14); suggesting down-regulation of glycolytic genes does not increase the fitness of the cells with no mtDNA. Also, we tested the response of *gcr2Δ* ρ^- cells to *PDE2* overexpression. Cells deleted of *GCR2* exhibited an increased rate of growth with PKA down-regulation in the absence of mtDNA (Figure 3.14), which makes Gcr2p an unlikely target in the beneficial effects of PKA activity reduction in cells lacking mtDNA.

As reviewed in Chapter 2, Snf1 protein kinase is a conserved modulator of cellular energy. Vacuolar localization of Snf1-Sip1 protein kinase is negatively regulated by PKA activity²²⁰. Moreover, Snf1p is required for the efficient rescue of mtDNA dependence of mutants by *SIT4* (the homolog of PP6) removal¹⁶. Knowing that Snf1p is involved in a mechanism that benefits cells lacking mtDNA, we tested if Snf1p or Sip1p were affected in the response to *PDE2* overexpression in cells with no mtDNA. Both *snf1Δ* ρ^- and *sip1Δ* ρ^- cells have increased fitness with *PDE2* overexpression (Figure 3.15), suggesting that they are not needed for the beneficial effects of reduced PKA activity in cells having defected mtDNA.

3.2.6 Cells with deletion of TOR pathway regulatory components respond to *PDE2* overexpression when mtDNA is damaged

Previously, it was shown that deletion of conserved phosphatases acting downstream of TOR kinase, phosphatase 2A (PP2A, in yeast Pph21p/Pph22p) or protein phosphatase 6 (PP6, in yeast Sit4p), increase the fitness of *S. cerevisiae* cells after mtDNA depletion, while deletion of their inhibitors Sds23p and Sds24p caused impaired proliferation of cells lacking mtDNA¹⁶. Since they are important factors for the survival of the cells in the absence of mtDNA, we tested if cells deleted of Sds23p or Sds24p would respond to *PDE2* overexpression. We observed that the proliferation rate of the *sds23Δsds24Δ* ρ^- cells was enhanced by *PDE2* overexpression (Figure 3.16). These phosphatases are controlled by the

$\alpha 4$ /Tap42 protein (in yeast, Tip41p interacts with Tap42p) ^{221,222}. Tip41p was identified as a multicopy suppressor of the petite-negative phenotype of a mutant defected in F₁ sector activity, and shown to benefit cells lacking mtDNA ⁷⁵. We hypothesized that reduction in PKA activity might result in the increase of Tip41p levels through an unknown mechanism, and thus benefits the cells lacking mtDNA. So, we tested the response of *tip41Δ* ρ⁻ cells to the *PDE2* overexpression. We detected *tip41Δ* ρ⁻ cells carrying empty vector were proliferating far better than WT ρ⁻ cells with empty vector, but still *tip41Δ* ρ⁻ cells positively responded to *PDE2* overexpression (Figure 3.16).

3.2.7 Increased P-body formation by the down-regulation of PKA activity is not the mechanism how *PDE2* overexpression benefits the cells with damaged mtDNA

Depletion of mtDNA is followed by repression of cytoplasmic translation in cells ¹⁰⁹. The mRNAs that are not actively being translated can form RNA granules called Processing bodies (P-bodies). The mRNAs that enter P-bodies can either be directed to decay or instead re-enter translation ²²³. Since P-bodies increase in number and size when free untranslated mRNA accumulates ²²⁴, it is possible that P-body formation is essential to cope with the stress in cells lacking mtDNA. There are several lines of evidence that support this idea. P-bodies were shown to have prolonged contacts with mitochondria ²²⁵. While disruption of P-bodies do not affect mitochondrion morphology and function, defects in mitochondrial activity prevents the P-body associated function ²²⁵.

PKA influences P-body formation by directly phosphorylating a conserved factor in P-bodies, Pat1p ²²⁶. Its phosphorylation disrupts Pat1 binding to Dhh1, which is an important protein-protein interaction for foci formation ²²⁶. Tom70p is physically interacted with Dhh1p to which Pat1p binds for proper P-body assembly ²²⁷ and it is possible that Tom70p works an organizational center for P-bodies. Moreover, Mkt1p which is a nuclease-like protein forming complexes with Pbp1p, was shown to localize P-bodies in varying stress conditions ²²⁸. Damage to mtDNA in strains carrying different MKT1 alleles were shown to have non-identical consequences for the cells ²²⁹.

By piecing together the evidence, we suggested a mechanism that down-regulation of PKA pathway in cells lacking mtDNA is beneficial, since PKA down-regulation diminishes Pat1p phosphorylation, resulting in more P-body formation. We compared the

growth of *pat1Δ* ρ^- cells to ρ^- otherwise WT cells. Interestingly, *pat1Δ* ρ^- cells proliferated far better than ρ^- otherwise WT cells (Figure 3.17). However, we would expect to see a significant growth rate decrease in *pat1Δ* ρ^- cells compared to ρ^- otherwise WT cells if PKA downregulation benefited cells lacking mtDNA by increasing P-body formation through Pat1p phosphorylation. Moreover, we have performed microscopy experiments with GFP tagged P-body or stress granule components. Unlike other stress conditions like glucose deprivation or salt stress that are shown to trigger P-body formation, we did not observe fluorescent puncta after mtDNA depletion under the microscope (data not shown). Those were initial observations suggesting that P-bodies are of limited importance in dealing with the stress of mtDNA loss, but it is possible that there could be complex mechanisms that are only triggered when PKA is modulated or nutritional conditions are changed, which needs further investigation.

3.3 Discussion

Mitochondria are connected to the rest of the cell in a complex network that provides the coordination of two genomes and the communication to maintain homeostasis of the cell for good health. Deletions or mutations in mtDNA are implicated in various human diseases and the aging process. Therefore, it is crucially important to understand the biology of cells lacking mtDNA. We used *Saccharomyces cerevisiae* as a model organism to reverse detrimental effects of mtDNA loss by modulating a nutrient signaling pathway, the cAMP-dependent PKA pathway. We found that reduced PKA signaling is beneficial for cells lacking mtDNA. Specifically, over-expression of the cyclic AMP phosphodiesterase Pde2p, removal of PKA isoform Tpk3p, or ablation of other proteins promoting PKA activity leads to improved proliferation of cells deleted of the mitochondrial genome.

However, these findings are heavily dependent upon yeast genetic background. The strains that we used in our experiments to test the beneficial effects of *PDE2* overexpression are derived from BY4743 diploid or BY4741 and BY4742 haploid strains. All three of these strains respond positively to PKA down-regulation, while *PDE2* overexpression has no significant effect on the proliferation rate of the cells from another yeast background, W303 (C. Dunn, unpublished results). Since there is strain background

specificity to the effect, the genetic differences in the two strains that may lead to such a response should be investigated. One reason for the observed effect might be differential mtDNA stability of these two backgrounds due to their allelic differences. For example, *MKT1* gene encodes for a nuclease-like protein, allelic variations of which affects mitochondrial genome stability. The S288C allele of *MKT1* is reported to reduce the formation of *petite* colonies and negatively affect the growth of *petite* cells²²⁹. The strains that we use in our study are in BY background, which are also derived from S288C strain²³⁰. Second, W303 is an ethanol-sensitive background, and this sensitivity is improved by the overexpression of *PDE2*²³¹. Therefore, *PDE2* overexpression may be harmful for W303 cells lacking mtDNA since they need to make fermentation to survive, which increase ethanol in the medium, but at the same time, they are overexpressing *PDE2* that makes them more sensitive to ethanol. Since only two different backgrounds are tested, we do not also know the loss of benefits provided by *PDE2* overexpression in W303 cells with no mtDNA is specific to W303 background. In other words, down-regulation of PKA may still increase the fitness of cells from other yeast backgrounds, which needs further investigation.

Our results show that Tpk3p is the relevant PKA isoform, downregulation of which benefits cells lacking mtDNA in response to reduced cAMP levels. However, the rescue of the phenotype in the absence of mtDNA is not as effective as *GPA2* or *GPR1* deleted ρ^- cells. As shown by other groups^{122,232}, this result suggests that altered cAMP levels result in the selective activation of PKA isoforms in response to varying cell conditions.

Several mechanisms could explain why *TPK3* deletion is good for cells lacking mtDNA, while deletion of other components does not affect the proliferation rate of ρ^- cells or simultaneous deletion of *TPK1* or *TPK2* with *TPK3* does not have any additive effect on the fitness of the ρ^- cells. First, the mechanism by which PKA reduction benefits ρ^- cells might be via a specific target controlled by Tpk3p. Diminished PKA activity through Tpk3p deletion can affect the function of the relevant target repression or activation of which would be important in the absence of mtDNA. Second, a set of targets may need to be affected when PKA is downregulated in cells with no mitochondrial genome for a cooperative beneficial response that significantly increases the proliferation rate of cells lacking mtDNA. In this case, a set of the affected factors may also be targets of Tpk1p or Tpk2p. However, when Tpk1p or Tpk2p is deleted individually, these targets

will be down-regulated or up-regulated, but deletion of them would not provide the benefits that Tpk3p deletion provides. In other words, deletion of Tpk3p may affect a group of genes including the set of genes which are also targets of Tpk1p and Tpk2p, and result in activation or repression of a system to benefit cells lacking mtDNA. Although we could speculate on many mechanisms to explain the observed phenotype, the specific physiological functions of different PKA isoforms are not revealed yet.

However, according to the global protein phosphorylation analysis performed in yeast, Tpk1p, Tpk2p, and Tpk3p recognized 256, 29, and 79 substrates, respectively ¹²¹. There was only eight common targets for all three isoforms, and less than one fourth of the targets recognized by two isoforms ¹²¹. This unique substrate specificity strongly suggests essential functions for each isoform in response to different physiological states of the cell, which remains to be examined. Consistently, we observed deletion of only one of the PKA isoforms, Tpk3p, is beneficial for cells lacking mtDNA, suggesting a distinct function for this protein down-regulation of which increases the proliferation rate of ρ^- cells. In fact, protein level of Tpk3p in glycerol-grown wild type cells were lower than in glucose-grown wild type cells ¹⁵⁰. This observation is contradictory to our findings in a sense. Since fermentative cells seem to require a larger amount of Tpk3p than respiratory cells need, one would expect cells lacking mtDNA, and thus have to make fermentation, will require more Tpk3p. However, we observed deletion of *TPK3* benefits ρ^- cells. This may suggest a nutrient dependent switch mechanism to alter PKA activity primarily through Tpk3p subunit, which is modulated differentially via different stress conditions.

Moreover, studies employing quantitative phosphoproteomics in perturbed yeast backgrounds have revealed many relationships between kinases and the regulation of their phosphorylation network, including Tpk3p ²³³. Not surprisingly, cells give a complex system wide response when specific kinases are disrupted rather than simple effects on the downstream components of a single signaling pathway. Therefore, it is highly probable that deletion of Tpk3p or down-regulation of PKA pathway can act through multiple mechanisms to improve the fitness of ρ^- cells.

Although our initial data suggest that beneficial effects of PKA downregulation in cells lacking mtDNA is not due to increased P-body accumulation in the cell, it is worth noting here that deletion of Tpk3p was shown to alter the kinetics of P-body generation, and Tpk3p accumulates into P-bodies during several stress conditions including glucose

deprivation¹²⁴. Moreover, we found that deletion of an essential P-body component, Pat1p, increases the proliferation rate of cells lacking mtDNA. Actually, this may suggest decreasing P-body formation may be beneficial for cells in the absence of mtDNA rather than its increase through a mechanism that needs to be uncovered.

Interestingly, *TOM70* deleted cells did not respond to *PDE2* overexpression. Previously, deletion of components in the mitochondrial import machinery were demonstrated to be synthetic lethal with the loss of mtDNA⁷⁵, and cells lacking mtDNA were reported not to be able to direct mitochondrial matrix proteins to their destined locations²³⁴. Aside from its role of promoting respiratory growth, mtDNA may be important for the maintenance of the mitochondrial protein import. Although *PDE2* overexpression cannot rescue petite-negative phenotype of *tom70Δ* ρ^- , overexpression of *TIP41*, a direct effector of yeast TORC1 that interacts with *TAP42* and negatively regulates PP2A²²², can rescue (data not shown). *TIP41* overexpression was shown to increase the fitness of cells lacking mtDNA as well as *PDE2* overexpression⁷⁵. This result indicates that PKA inhibition likely does not act through the *TAP42* axis of the TOR pathway. We may also conclude Tom70p is specifically involved in the mechanism of how PKA downregulation benefits ρ^- cells, and indispensable for the increased proliferation of cells lacking mtDNA in the presence of *PDE2* overexpression.

PKA is reported to be involved in the negative regulation of mitochondrial biogenesis by phosphorylating Tom proteins, Tom22, Tom40 and Tom70¹⁸⁴. One mechanism for the beneficial effects of *PDE2* overexpression on ρ^- cells would be the increased mitochondrial biogenesis and import efficiency which may also trigger enhanced import of carrier proteins such as Aac2p. This could be through different mechanisms, including: (1) decreased phosphorylation of Tom70p, and thus enhanced receptor activity, by PKA downregulation, (2) increase in Tom70 protein levels triggered by the reduction in cAMP levels through an unknown mechanism, (3) increase in Aac2 levels due to decreased cAMP concentration through an unknown mechanism. Our results suggest that none of these mechanisms are relevant in response to *PDE2* overexpression in cells lacking mtDNA.

Although we obtained evidence to clearly answer the questions asked, there are weaknesses that should be addressed. First, the protein import assays and mitochondrial biogenesis experiments that are conducted to explain the functional relevance of the phosphorylation sites of PKA on Tom22p, Tom40p and Tom70p were performed with

extracted mitochondria in rabbit reticulate lysate ^{184,185}. Although this *in vitro* system provides a ‘near to physiological environment’, it cannot represent an exact *in vivo* mechanism. Therefore, a novel *in vivo* system is required to test the functional relevance of these mutation sites and the effect of PKA activity. Second, in the experiments that we determine the level of Tom70p or Aac2p in different conditions, the proteins were extracted from whole cell lysates. In other words, we know that the protein levels stay the same in the cell in the presence or in the absence of mtDNA, and not affected by *PDE2* overexpression in agreement with the quantitative RNA sequencing. However, we don’t know if the protein levels of either Tom70 or Aac2 were altered specifically in the mitochondria. Extracting proteins from isolated mitochondria in different conditions will be a way to test this possibility. However, a better approach will be to develop a novel kinetic assay for testing the efficiency of the import of specific mitochondrial proteins in different conditions, which enables us perform a wide range of experiments to test the specific membrane potential dependence of proteins and the proteins and physiological conditions needed for proper import into the mitochondria.

In yeast, cells lacking mtDNA are not viable in the absence of some components of the protein import mechanism, including Tom70p ⁷⁵. A reasonable explanation may be the proteins that stay in cytosol can be toxic to the cell and when it is combined with another stress condition like mtDNA loss results in severe consequences for the cell. We demonstrated the requirement for Tom70p to get the beneficial effects of *PDE2* overexpression upon mtDNA depletion. Therefore, it might be possible that Tom70p provides docking sites for the proteins that cannot be imported into the mitochondria in the absence of mtDNA and prevent formation of massive protein aggregates that can be toxic to the cell. *PDE2* overexpression may boost this ability to further help cells increase their proliferation rate. This mechanism, however, cannot work through increased Tom70p levels, since we detected no significant change in both RNA and protein levels as shown by quantitative RNA sequencing and western blotting, respectively. Protein aggregation is implicated in mitochondrial dysfunction associated diseases ^{235,236} although it is still debated if aggregating proteins are the causes or the consequences of such diseases. Most of the proteins shown to aggregate in various diseases localize in the nucleus, cytosol or mitochondria. This could be a protective effort of the cell, and protein aggregates may be formed as a consequence of disease associated defects in cell. However, defects resulting

in protein misfolding and aggregation may be causing alterations that lead to human diseases, which require an intense further investigation.

We found that *PDE2* overexpression does not increase Cox4p (1-21)-GFP import into mitochondria, potentially suggesting that the beneficial effects of decreased PKA activity in cells with no mtDNA may not be through increasing $\Delta\Psi^{\text{mito}}$. One of the characteristic phenotypes of cells with no mitochondrial genome is their reduced $\Delta\Psi^{\text{mito}}$. Cells lacking mtDNA have around -55mV mitochondrial membrane potential ⁷⁰, while the $\Delta\Psi^{\text{mito}}$ for a cell with intact mitochondria is around -180mV ²³⁷. It is highly probable that there is not an exact threshold for the mitochondrial membrane potential beyond which the import is allowed although loss of $\Delta\Psi^{\text{mito}}$ is lethal. This idea is supported by the observation that the import of mitochondrial proteins has differential dependence on $\Delta\Psi^{\text{mito}}$ ²³⁸. Therefore, the import experiment that we have performed does not prove that $\Delta\Psi^{\text{mito}}$ is not altered by the *PDE2* overexpression. Our current approach will not be able to detect an increase in $\Delta\Psi^{\text{mito}}$ due to diminished PKA activity that allows the import of a set of proteins which would increase the fitness of cells lacking mitochondria, but this increase might not be the level that Cox4p (1-21)-GFP is imported. Therefore, a novel assay to test mitochondrial protein import, specifically the import of polytopic membrane proteins to understand the contribution of Tom70p to the phenotype is required.

Our transcriptional analysis represented the reversion of the characteristic transcriptional response to mtDNA depletion when *PDE2* is overexpressed in ρ^- cells. A large number of genes were found to be differentially regulated in cells treated with EtBr for 24 hr to deplete their mtDNA. According to the expression profile obtained, 114 genes were up-regulated and 95 genes were down-regulated at least 2-fold in cells lacking mtDNA ($p < 0.05$). These genes were enriched for genes involved in iron homeostasis, transition metal iron homeostasis, oxidation reduction process and glutamate metabolic process (Gene Ontology, GO, Enrichment, $p < 0.05$). Among these genes, we selected the ones expression of which are still up-regulated, but significantly reduced compared to ρ^- cells, or returned to the normal levels when *PDE2* is overexpressed in ρ^- cells. In our transcriptome analysis, ρ^- cells overexpressing *TIP41* are used as the positive control group, which was already shown to rescue the petite-negative phenotype ⁷⁵. The significant

hits that are differentially regulated upon *PDE2* or *TIP41* overexpression in ρ^- cells matched in these strains.

The group of genes expression of which is highly affected from mtDNA loss was similar to the transcriptional signature of iron starvation ¹⁹³, and these were the most significant hits expression of which were reduced with *PDE2* overexpression in ρ^- cells. Eight of the most significant hits, *FIT1*, *FIT2*, *FIT3*, *CCC2*, *FRE3*, *ARN2*, *TIS11*, and *ENB1*, have previously been implicated as iron-responsive genes ¹⁹⁶, and two of them are TCA cycle enzymes, *IDH1* and *IDH2* expressions of which are dependent on the RTG genes ¹⁰⁸.

Significant hits identified by the quantitative RNA sequencing other than the ones involved in iron regulon include *IDH1* and *IDH2*, encoding for TCA cycle enzymes. Their expression is dependent on the RTG genes only in cells with mitochondrial defect ¹⁰⁸, indicating that any contribution to mitochondrial health will potentially down-regulate their expression as in the case of reduced PKA activity.

The cellular and intercellular iron transport mechanisms are highly regulated to maintain cell homeostasis. Fre1p and Fre2p are plasma membrane ferric reductases that are responsible for the reduction of insoluble, extracellular iron Fe (III) to soluble Fe (II) ²³⁹. The multi-copper oxidase Fet3p that re-oxidizes Fe (II) to Fe (III) and the transporter Arn1p together with the iron permease Ftr1p build up a high-affinity iron transport system into the cell ^{240–242}. Ccc2p, the P type ATPase, is needed for loading copper onto Fet3p ²⁴³. Moreover, Fit1p, Fit2p, Fit3p, Fre3p, Arn2p, Tis11p and Enb1p are involved in the pathway of iron and copper transport across the cell membrane.

Tpk2p was shown to be specifically involved in the regulation of this iron transport system. In *TPK2* mutants, expression of *FTR1*, *FRE2*, *FET3* and *CCC2* was reported to be up-regulated ¹²³. Moreover, *TPK2* mutants proliferate better on medium that force cells to respire, while *TPK1* mutants proliferate worse, suggesting opposing functions in respiratory regulation for Tpk1 and Tpk2 proteins ¹²³. Although Tpk3p seems not to be involved in the mechanism of iron uptake, it is reported to be involved in respiratory function regulation ^{125,139}. Evidently, iron-responsive genes are mediated by PKA. Therefore beneficial effects of diminished PKA activity in cells lacking mtDNA might be through modulation of expression of these genes. In other words, reversion of the

characteristic transcriptional response of cells lacking mtDNA via *PDE2* overexpression may not be a consequence of the beneficial effects of PKA down-regulation in the ρ^- cells, but the direct cause that benefits the cells lacking mtDNA. Taking the beneficial effects of *TPK3* deletion in ρ^- cells into consideration, the potential roles of Tpk3p in the regulation of iron uptake and its specific contribution to the respiratory functions of the cell in stress conditions require further investigation.

Upregulated expression of the genes which are components of the iron regulon and of the RTG pathway is tightly linked to the reduction in the $\Delta\Psi^{\text{mito}}$ of ρ^- cells^{192,195}. Therefore, our RNA-sequencing results further support the idea that $\Delta\Psi^{\text{mito}}$ may be increased with decreased PKA activity, however not to the level that Cox4p (1-21)-GFP could efficiently be imported into the mitochondria.

Defects in mitochondrial iron metabolism leads to nuclear genome instability which may be one of the consequences of mtDNA loss for the cell^{191,192}. Other groups observed that mitochondrial Iron-Sulfur Cluster (ISC) biogenesis is impaired in cells lacking mtDNA²⁴⁴, and in cells lacking a mitochondrial membrane potential²⁴⁵. Dirick et al. also suggests that high glucose fosters the nuclear instability in ρ^- cells¹⁹¹. Therefore, evidence may indirectly support a mechanism that nutrient signaling pathways, particularly reduction in PKA activity or true glucose levels in the environment, will result in the increased nuclear genome stability of cells lacking mtDNA. This might subsequently trigger other mechanisms that mediate ISC biogenesis and mitochondrial iron homeostasis, resulting in higher fitness of ρ^- cells. Therefore, potential mechanisms integrating nutrient signaling pathways and nuclear genome stability worth investigating in cells lacking mtDNA. If mechanisms that may increase the proliferation rate of cells with no mtDNA via mediating the mitochondria-nucleus network to maintain genome stability are found, this may explain the mechanism of *PDE2* overexpression to reverse the transcriptional response of the cells lacking mtDNA.

Although PKA is frequently involved in the control of gene expression in response to glucose, none of the transcription factors or their modulators which are the targets of PKA are shown to be essential in the mechanism providing beneficial effects to the cells lacking mtDNA when PKA activity is reduced.

Hsf1p regulates the transcription of *HSP90* genes and mitochondrial chaperone genes including *HSP10*, *HSP60*, *HSP78*, *MDJ1*, and *SSC1*^{210,211} and other target genes such as *ELC1*, which is important in chronological life-span²⁴⁶. None of them were among the hits that are up-regulated upon mtDNA loss in the RNA-sequencing analysis.

However, we demonstrated that reduced Hsf1p activity increase the proliferation of ρ^- cells. *PDE2* is shown to be a multicopy suppressor of a temperature sensitive *HSF1* mutant²⁴⁷. This observation together with our findings may suggest a novel mechanism that involves Hsf1p and is responsive to mtDNA loss. Modulation of this Hsf1p-controlled mechanism might also benefit cells lacking mtDNA in a PKA-dependent or PKA-independent way. Therefore, other pathways activity of which are altered in cells carrying *hsf1::dAMP* allele with no mtDNA should be further investigated. In fact, Yak1p, which is negatively regulated by PKA, directly phosphorylates Hsf1p, increasing its promoter-binding capacity and subsequently stimulating the expression of Hsf1p target genes as a result of glucose starvation or *PDE2* overexpression¹⁷⁵. This suggests a potential pathway for PKA to differentially regulate Hsf1p activity.

Interestingly, when we provide the *hsf1* Δ cells plasmid-borne WT Hsf1p together with the *PDE2* overexpression vector and deplete their mtDNA, the cells exhibit severe growth defects, although cells carrying the mutated *HSF1* allele, *EXA3-1*, proliferate far better with *PDE2* overexpression. One possible explanation for such a result may be that the level of Hsf1p was higher than WT levels, since it is provided by a plasmid, and *PDE2* overexpression may activate a stress response pathway simultaneously in cells lacking mtDNA. This may result in uncontrolled upregulation of heat shock proteins, which may be detrimental for cell.

Moreover, Snf1p was shown to be required for the full benefits of the deletion of conserved phosphatases in cells lacking mtDNA¹⁶. Hsf1p, a direct target of Snf1p, induces the expression of some targets in glucose starvation like *HSP30*²¹⁰. *HSP30* was significantly upregulated in our RNA-sequence analysis (~ 10 fold increase in ρ^- cell, yet only ~ 6 fold increase in *PDE2* overexpressing ρ^- cells, $q < 0.05$). Hsp30p acts as the negative regulator of H^+ -ATPase (Pma1p) that is also identified on the mitochondrial surface²⁴⁸. It might influence the ability of the cell to maintain $\Delta\Psi^{mito}$ in the absence of mtDNA, and thus increase the proliferation of cells with mitochondrial damage. However, we were not able to detect any statistical change in the expression levels of Hsf1p targets,

Ssa1-4p, Hsp78p, Btn2p, Hsp104p and Sis1p^{174,175,249}, in our RNA-sequencing analysis. Therefore, mechanisms involving Hsf1p that would benefit cells lacking mtDNA should still be investigated although it seems unlikely that Hsf1p is the direct target of PKA down-regulation in cells lacking mtDNA, which increases the proliferation of ρ^- cells.

S. cerevisiae Ras proteins are affected by multiple factors^{250–252}. However, no modulatory protein or altered pathway conditions to activate Ras-PKA pathway are yet identified. How Ras is activated and how its activity alters transcriptional status and apparatus is still an area of keen interest. Evidence suggests Ras/cAMP/PKA targets within the RNA polymerase II holoenzyme²⁵³, providing a tight coordination of gene expression with cell growth. Therefore, *PDE2* overexpression may alter a Ras dependent mechanism through which it benefits the cells lacking mtDNA. *HXT4* gene expression induced by low levels of glucose¹¹⁶ is also regulated by Gcr1p and Gcr2p²⁵⁴. These glycolytic modulators are good candidates for the activation of Ras-PKA pathway, enhancing growth. However, we have shown that Gcr2p is not required in the mechanism by which *PDE2* overexpression benefits the cells lacking mtDNA. Therefore, it is unlikely that downregulated Gcr2p activity upon *PDE2* overexpression is the cause of beneficial effects that we observed in cells lacking mtDNA. However, the contribution of these proteins in the Ras-PKA pathway, regulation of which is known to depend on glycolytic pathway, requires further investigation.

PKA pathway is activated when cells sense glucose, but also involved in response to the starvation of different essential nutrients through multiple receptors. Current understanding of nutrient signaling in yeast suggests an intense crosstalk and functional redundancies between glucose signaling pathways and other nutrient signaling pathways, mainly the TOR pathway^{112,113}. Therefore, it is possible that altered transcriptional regulation could be of limited importance for explaining the beneficial effects of *PDE2* overexpression in cells without a mitochondrial genome. This is supported with the observation that yeast could still exhibit the transcriptional response to glucose when lacking functional PKA¹⁹⁰, suggesting that PKA activity may be dispensable to alter gene expression in response to glucose¹⁹⁰. It is though possible that a decrease in PKA activity helps cells cope with the stress of mtDNA loss through a currently unknown mechanism that in turn, activates a signaling network to reverse the characteristic transcriptional response in the cells lacking mtDNA.

3.4 Conclusion

In conclusion, our findings evidently demonstrate the beneficial effects of the downregulation of cAMP-PKA pathway in cells lacking mtDNA. Bringing evidence together, over-expression of the cAMP phosphodiesterase Pde2p, removal of PKA isoform Tpk3p, or deletion of other proteins promoting PKA activity, Gpr1p and Gpa2p, increase proliferation of cells deleted of the mitochondrial genome. Moreover, *PDE2* overexpression reduces the transcriptional response to mtDNA loss. Although we investigated the mechanism of how reduced glucose sensing is beneficial for ρ^- cells, our results do not fit in a single clear mechanism with the current knowledge.

We showed that in ρ^- cells with reduced PKA activity, Cox4p (1-21)-GFP import is not enhanced. Previous studies explaining mechanisms to benefit cells with no mtDNA demonstrated the rescue of mitochondrial protein import which is associated with $\Delta\Psi^{\text{mito}}$ increase^{16,199}. However, we argue that $\Delta\Psi^{\text{mito}}$ increase that is resulted from decreased PKA activity was not significant enough to be observed by the microscopy experiment that we conducted, which is supported by two pieces of evidence that we obtained. First, the characteristic transcriptional response which is associated with decreased $\Delta\Psi^{\text{mito}}$ due to mtDNA loss¹⁹² is suppressed in cells lacking mtDNA via *PDE2* overexpression. Second, over-expression of Pde2p also suppresses the inviability of several mutants that normally cannot survive mtDNA loss. One of these mutants was *ATP2* encoding for the β subunit of the F_1 sector of mitochondrial F_1F_0 -ATPase¹⁵. As reviewed in chapter 1, cells lacking mtDNA depends on F_1 -ATPase to maintain the reduced state of $\Delta\Psi^{\text{mito}}$, and mutations that lead to generation of a greater the $\Delta\Psi^{\text{mito}}$ increase the proliferation rate of the cells. Moreover, deletion of ADP/ATP transporter, Aac2p, is lethal for cells lacking mtDNA, since it significantly decrease $\Delta\Psi^{\text{mito}}$ ¹², and consistently cannot be rescued by *PDE2* overexpression.

We were able to identify an essential component of the mechanism, Tom70p. Our results suggest the protein, not especially its receptor function is required for the beneficial effects of PKA down-regulation in ρ^- cells, and the levels of Tom70p is not changed in ρ^- cells overexpressing *PDE2*. Therefore, a possible mechanism for *PDE2* overexpression in ρ^- cells would be enhanced carrier protein import into mitochondria that will lead to an increase in $\Delta\Psi^{\text{mito}}$. Although Aac2p levels were shown not to increase by *PDE2*

overexpression, its increased import may benefit the cells lacking mtDNA or there may be other IM proteins enhanced import of which could benefit ρ^- cells. Indirectly supporting this idea, it was argued before; TIM22 pathway including Tom70p involved in the polytopic IM protein import is sensitive to mtDNA loss ⁷⁵. Therefore, we may suggest a model for beneficial effects of PKA down-regulation in cells lacking mtDNA that increase $\Delta\Psi^{\text{mito}}$ via enhanced activity of protein transport into mitochondria through TIM22 pathway, which obviously needs further investigation.

Although we cannot define a direct beneficial way for diminished PKA activity in ρ^- cells, our results suggest several potential future investigations. First, the response of ρ^- cells lacking other TIM22 pathway components to *PDE2* overexpression can be tested via serial dilution assays to compare their proliferation rates. Second, contribution of PKA down-regulation to $\Delta\Psi^{\text{mito}}$ may be indirectly tested by asking if it could rescue other petite negative phenotypes such as *YME1* mutants that are shown to be viable upon increase in $\Delta\Psi^{\text{mito}}$ ⁷⁹.

Furthermore, to directly test the effect of diminished PKA activity on mitochondrial energetics, we may employ flow cytometry. Relative quantitation of mitochondrial membrane potential in ρ^+ and ρ^- cells overexpressing *PDE2* or not can be performed by the comparison of tetramethylrhodamine ethyl ester (TMRE) staining that assesses $\Delta\Psi^{\text{mito}}$. Although TMRE is generally used in cultured-cell-based experiments ^{91,255}, its successful use in yeast was also reported ²⁵⁶. In a controlled experiment in which TMRE signal is normalized to the total mitochondrial mass detected by MitoTracker Green FM in ρ^- cells overexpressing *PDE2* or not, we should be able to detect any change in $\Delta\Psi^{\text{mito}}$ that will benefit cells lacking mtDNA.

Finally, we should further investigate the specific targets of PKA that may be relevant to the outcome of mtDNA damage. One of the best ways to represent the potential metabolic processes and novel proteins affected from diminished PKA activity in the absence of mtDNA is the mass-spectrometric analysis of protein phosphorylation. In the analysis, we may employ ρ^+ cells, ρ^- cells and ρ^- cells overexpressing *PDE2* and compare the detected phosphopeptides in these cells to identify differentially regulated targets in cells lacking mtDNA with diminished PKA activity. Moreover, deletion of *Tpk3p* significantly increases the proliferation rate of cells with no mtDNA, and thus suggesting a specific role for *Tpk3p* in the consequences of mtDNA loss. Therefore, we may perform a

functional analysis using the deletion collection of yeast genome and test in which cells deletion of Tpk3p is not beneficial when their mtDNA is depleted. The disrupted genes in cells that do not respond to Tpk3p deletion in the absence of mtDNA can be relevant targets of PKA that are regulated in response to mtDNA damage. Likewise, we may also employ *PDE2* overexpression instead of Tpk3p deletion to search for PKA targets affected upon mtDNA loss. The results that we obtained will increase our understanding of the mechanism how reduced PKA activity increases the fitness of cells lacking mtDNA.

3.5 Materials and Methods

3.5.1 Yeast Strains and Culture Conditions

Yeast media recipes and preparation were described in ²⁵⁷. Genetic manipulations were achieved according to ²⁵⁸. Table 3.1 provides the genotypes of strains used and their construction details. Table 3.2 provides the construction details of the plasmids used. Oligonucleotides used for strain and plasmid construction are listed in Table 3.3. Serial dilution assays were performed by diluting cultures to an OD₆₀₀ of 0.1, followed by three additional fivefold dilutions. Four microliters of each dilution were spotted to appropriate solid medium. EtBr (Thermo Fisher Scientific) was used at a concentration of 25 µg/mL in liquid medium to destroy mtDNA ⁶⁶, and each plate was tested for the success of mtDNA depletion when the assay was over by replica plating onto YEPLac (respiratory) medium on which ρ^- cells are not viable. Pictures of plates were taken by using a Colony Doc-It system (UVP, Upland, CA).

3.5.2 Protein Extraction and Western Blotting

Overnight cultures were diluted in 5mL of appropriate medium that is indicated in the figure legends. These cultures were grown until the logarithmic phase of growth, and proteins were extracted from whole cell lysates for western blotting. Cells with mtDNA (ρ^+ cells) were grown in the appropriate growth medium, and cells with no mtDNA (ρ^- cells) were obtained by growing for 24 hr in medium with EtBr at a concentration of 25 µg/mL. Protein extraction was performed immediately when the cultures reached to OD₆₀₀ in

between 0.4-1.5 by boiling cultures, treating cells with NaOH, and resuspending pellets in an appropriate volume of SDS–PAGE sample buffer calculated according to the OD₆₀₀ of the cells collected. The buffers were prepared and the steps of protein extraction were performed essentially as described in ²⁵⁹.

4 mL 8% separating gel was prepared as follows: 1.07 mL 30% Acrylamide-Bisacrylamide (29:1) (AppliChem Panreac, ITW Companies), 1.33 mL 1.0 M Bis-Tris (pH6.5), 30 µL 10% APS (in ddH₂O) (Sigma Life Sciences), 12 µL TEMED (Sigma Life Sciences), 1.6 mL ddH₂O. The gel was poured in between the vertical glass plates, and sealed with n-butanol. After the solidification, n-butanol was removed with a paper by capillary action. 4 mL of stacking gel composed of 670 µL 30% Acrylamide-Bisacrylamide (29:1) (AppliChem Panreac, ITW Companies), 1.33 mL 1.0 M Bis-Tris (pH6.5), 20 µL 10% APS (in ddH₂O) (Sigma Life Sciences), 12 µL TEMED (Sigma Life Sciences), 2 mL ddH₂O and 5 µL bromophenol blue (2% in EtOH) was poured on top of the separating gel.

1 x MOPS-SDS Running Buffer (diluted from 20 x MOPS-SDS Running Buffer, National Diagnostics) was prepared to run proteins on Bis-Tris gels. 10 µL of protein extraction was loaded into the wells and run at 30 mA approximately for 1.5 hours. The proteins in the gel were transferred onto a PVDF membrane (Thermo Scientific) by the aid of a semi-dry transfer machine using 1x Transfer Buffer (3.03 g Tris Base, 14.4 g glycine, 100 mL 100% Methanol (VWR), qs to 1L ddH₂O) at 25 V for 35 min. When the transfer was completed, MemCode™ Reversible Protein Stain Kit was used for staining proteins on the PVDF membrane to see if the proteins were loaded in equal amounts. The membrane was then blocked with 15 mL 0.5% Milk Blocking Buffer Solution (2.25 g non-fat dried milk powder (AppliChem), qs to 45 mL with TBST) for 1h at room temperature by shaking at 50 rpm and changing the solution two times. 0.1% primary antibody solution (0.15 g nonfat-dried milk powder, 15 mL TBST, primary antibody) was prepared with antibodies listed in Table 3.4, and the membrane was incubated in primary antibody solution for two hours at room temperature or overnight at 4°C. Following primary antibody, the membrane was washed three times with TBST for 10 min by shaking at room temperature. 0.1% secondary antibody solution (0.15 g nonfat dried milk powder, qs to 15 mL with TBST) was prepared with the antibodies listed in Table 3.4. The membrane was incubated in secondary antibody solution for an hour at room temperature by shaking. The

unbound antibody was removed with two times 10 min TBST washes followed by a 10 min wash with TBS. For visualization, the ECL reagent SuperSignal West Pico Chemiluminescent or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) was used as substrate in a volume enough to cover the membrane, and the Gel Logic 2200 Imaging System (Carestream Health) was used to recover the images. The membrane was exposed to luminescence until it gets saturated. For probing the loading control (hexokinase protein), the first and secondary antibodies used for the protein of interest was removed by four times 20 min TBST washes, and the same blot was used to target hexokinase protein (using hexokinase antibody, a gift from R. Jensen) to be used as a control of total protein loaded.

3.5.3 Preparation of Cultures for RNA Extraction

Strain BY4743 (WT) transformed either with b13 (empty 2 μ vector), b28 (2 μ -*TIP41*) or b89 (2 μ -*PDE2*) were diluted from overnight cultures in SC-URA medium. Cells deleted of mtDNA (ρ^- cells) were obtained by growing overnight cultures in SC-URA medium with EtBr (Thermo Fisher Scientific) at a concentration of 25 μ g/mL for 24 hours at 30°C, and harvested in the logarithmic phase. BY4743 (WT) ρ^+ cells with b13 (empty 2 μ vector) were diluted to 0.05 OD₆₀₀ units and grown until the logarithmic phase (OD₆₀₀ between 0.40-0.55) before harvesting.

3.5.4 RNA Isolation, cDNA Library Preparation and RNA-Sequencing (performed by Assoc. Prof. I. Halil Kavaklı and Mehmet Tardu)

Total RNA was extracted from 5 OD₆₀₀ units (~100 million cells) cell culture by using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. After quality and quantity measurement, it was treated with RNase-free DNase I (Thermo Scientific, USA) at a concentration of 1 U/ μ g to remove residual genomic DNA. MRNAs were purified from the 2 μ g total RNA using oligo (dT) magnetic beads and fragmented using fragmentation buffer by using TruSeq mRNA Sample Preparation Kit (Illumina, USA). The cleaved short RNA fragments were used for the first-strand cDNA synthesis using random hexamer-primer, and then the second strand was synthesized by using DNA

polymerase I and RNase H. The double-stranded cDNAs were purified with AMPure XP beads (Beckman Coulter, USA) and eluted with resuspension buffer followed by 3' end adenine nucleotide addition. Finally, sequencing adaptors were ligated to the fragments and cDNA fragments were enriched by PCR amplification. Enriched cDNA libraries were used for cluster generation and sequencing. Paired-end sequencing of the cDNA libraries was performed using the Illumina Miseq sequencing platform (Illumina, USA). All sequence data were at 2×75 bp length. The high quality reads were saved in fastq files. The fluorescent images process to sequences, base calling and quality value calculation were performed by the Illumina data processing pipeline (v1.5).

3.5.5 Transcriptome Analysis

Sequencing data from two of the paired ends were mapped against the *Saccharomyces cerevisiae* S288C genome (assembly sacCer3, April 2011) on the Galaxy web server²⁶⁰ using Tophat version 2.0.6, followed by determination of gene-expression levels using Cuffdiff version 2.2.1²⁶¹ with geometric library normalization, multi-read correction, and bias correction based on the sacCer3 genome. Transcript assignments were made using the Ensembl R64.1.1 GTF file for *Saccharomyces cerevisiae*.

3.5.6 Microscopy

To obtain ρ^- cells, cells were patched onto plates with 25 $\mu\text{g/mL}$ EtBr and incubated at 30°C for 2 d. Overnight cultures were started in appropriate medium that is indicated in figure legends, and next day diluted again in selective medium and incubated at 30°C until logarithmic phase of growth. An Eclipse 80i fluorescent microscope fixed with a 100X Plan Flour objective (Nikon, Tokyo, Japan) was used for visualization. Cells having clearly visible fluorescent signal (more than 200) were scored blind to genotype. Pictures were taken with a DS-Qi1Mc camera (Nikon, Tokyo, Japan). Automatic exposure times are used and images were captured using NIS-Elements software version AR 3.1.

3.6 Figure legends

Figure 3.1 Reducing PKA activity by *PDE2* overexpression is beneficial for cells lacking mtDNA. Pde2p catalyzes hydrolysis of cAMP, which activates PKA. Strain BY4742 (WT) carrying either plasmid b13 (empty 2 μ vector) or b89 (2 μ -*PDE2*) were tested for proliferation rate difference between ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in SC-URA medium, and incubated for 2 d (ρ^+ and ρ^-) at 30°C.

Figure 3.2 Deletion of *TPK3*, but neither other isoforms nor their combinations, rescued the growth defect of ρ^- cells. Strains BY4742 (WT), CDD884 (*tpk1 Δ*), CDD885 (*tpk2 Δ*), CDD886 (*tpk3 Δ*) CDD908 (*tpk1 Δ tpk3 Δ*), CDD922 (*tpk2 Δ tpk3 Δ*) and CDD923 (*tpk1 Δ tpk2 Δ*) were tested for proliferation rate difference between ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in YEPD medium, and plates were incubated for 2 d (ρ^+ and ρ^-) at 30°C.

Figure 3.3 Deletion of PKA activators, Gpa2p or Gpr1p, or a PKA catalytic isoform, Tpk3p, benefit cells lacking mtDNA. Strains BY4742 (WT), CDD849 (*gpa2 Δ*), CDD850 (*gpr1 Δ*) and CDD886 (*tpk3 Δ*) were tested for proliferation rate difference between ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells, ρ^+ and ρ^- , were incubated at 30°C for 2 d and 3 d, respectively, and they were grown in YEPD medium.

Figure 3.4 Reduced glucose sensing diminishes the effects of mtDNA damage in yeast independent of the extracellular glucose concentration. Strains BY4742 (WT), CDD849 (*gpa2 Δ*) and CDD850 (*gpr1 Δ*) were tested for proliferation rate difference between ρ^+ (+mtDNA) and ρ^- (-mtDNA) on plates with different glucose concentration. ρ^+ and ρ^- cells were grown overnight in YEPD liquid medium with 0.2% dextrose before spotting. ρ^+ and ρ^- cells were spotted on the same YEPD plates with 2%, 0.5% and 0.2% dextrose concentrations. Plates were incubated for 2 d (ρ^+ and ρ^-) at 30°C.

Figure 3.5 Characteristic transcriptional response to mtDNA loss is reversed with diminished PKA activity. Expression of genes selected for analysis were increased more than twofold in ρ^- strain BY4743 (WT) transformed with b13 (empty 2 μ vector) in comparison with ρ^+ strain BY4743 (WT) transformed with b13 (empty 2 μ vector) and were reported as increased under iron starvation¹⁹⁶. Gene-expression levels of ρ^- strain BY4743 (WT) transformed with b13 (empty 2 μ vector) and ρ^- *PDE2* overexpression strain, BY4743 (WT) transformed with b89 (2 μ -*PDE2*) were normalized to that of cells from ρ^+ BY4743

(*WT*) transformed with b13 (empty 2 μ vector), log₂ transformed, and plotted. Activation of iron-responsive genes (*FIT3*, *FIT2*, *FIT1*, *CCC2*, *FRE3*, *ARN2*, *TIS11*, and *ENB1*) and RTG signaling pathway targets (*IDH1*, *IDH2*) in ρ^- cells were reversed or significantly weakened by *PDE2* overexpression in cells lacking mtDNA.

Figure 3.6 PKA inhibition does not increase protein import by mitochondria lacking mtDNA. (A) *PDE2* overexpressing cells cannot import Cox4p (1-21)-GFP efficiently. Strain CDD463 (*WT*) carrying b148 (pRS314-*COX4* (1-21)-GFP) was transformed with a second plasmid b13 (empty 2 μ vector), b89 (2 μ - *PDE2*), b146 (pRS316- *ATP3-WT*) or b147 (pRS316- *ATP3-5*). Cells were grown in SC-URA-TRP medium with EtBr. (B) Cells deleted of *GPR1* or *GPA2* cannot import Cox4p (1-21)-GFP efficiently. Strains BY4742 (*WT*), CDD496 (*vma2 Δ*), CDD849 (*gpa2 Δ*) and CDD850 (*gpr1 Δ*) were transformed with b46 (*COX4* (1-21)-GFP-*LEU2*). Cells were grown in SD-LEU medium with EtBr. Microscopy and quantification were performed as explained in the Materials and Methods.

Figure 3.7 Reduced PKA activity allowed viability of mutants that depend on mtDNA for survival except *aac2 Δ* and *tom70 Δ* . (A) Proliferation defect in cells deleted of *AAC2* cannot be rescued by *PDE2* overexpression. Strains BY4741 (*WT*) and CDD632 (*aac2 Δ*) were transformed with either plasmid b10 (empty 2 μ vector) or b99 (2 μ -*PDE2*), and tested for petite negative phenotype. Cells were grown in SD-HIS medium, and plates were incubated for 3 d at 30°C. (B) Proliferation defect in cells deleted of *TOM70* cannot be rescued by *PDE2* overexpression. Strain CDD913 (*tom70 Δ*) was transformed with either plasmid b13 (empty 2 μ vector) or b89 (2 μ -*PDE2*), and tested for petite negative phenotype. Cells were grown in SC-URA medium, and plates were incubated for 2 d (ρ^+ and ρ^-) at 30°C. (C) *MGR1*, *MGR2* and *ATP2* mutants, depending on mtDNA for their survival, respond to *PDE2* overexpression upon mtDNA loss. Strains CDD11 (*mgr1 Δ*), CDD13 (*mgr2 Δ*) and CDD215 (*atp2 Δ*), CDD463 (*WT*) were transformed with either plasmid b13 (empty 2 μ vector) or b89 (2 μ -*PDE2*), and tested for petite negative phenotype. Cells were grown in SC-URA medium, and plates for CDD11 and CDD13 cells were incubated for 5 d, and plates for CDD215, CDD463 were incubated during 6 d for ρ^- and 3 d for ρ^+ at 30°C.

Figure 3.8 Phosphorylation mutants of TOM complex components do not have altered proliferation rates after their mtDNA is depleted. (A) Cells carrying PKA phosphorylation-site mutant forms of Tom70p proliferate at comparable levels to WT

Tom70p carrying cells. Strain CDD913 (*tom70Δ*) transformed with b110 (pRS314-*TOM70* WT), b111 (pRS314-*TOM70* S174A) or b112 (pRS314-*TOM70* S174E) and tested for proliferation rate difference between ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in SC-TRP medium, and plates were incubated for 2 d (ρ^+ and ρ^-) at 30°C. (B) The proliferation rate of *tom70Δ* cells carrying either WT Tom70 plasmid or Tom70^{S174A} mutant plasmid was comparable even when *PDE2* is overexpressed. Strain CDD913 (*tom70Δ*) transformed either with plasmid b110 (pRS314-*TOM70* WT) or b111 (pRS314-*TOM70* S174A) were transformed with a second plasmid either with plasmid b13 (empty 2μ vector) or b89 (2μ-*PDE2*), and tested for proliferation rate difference between ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in SC-TRP-URA medium, and plates were incubated for 2 d (ρ^+ and ρ^-) at 30°C. (C) WT cells carrying *PDE2* overexpression vector as the second plasmid do not respond to PKA downregulation as effectively as the cells carrying 2μ-*PDE2* as the only plasmid. Strain CDD463 (WT) carrying plasmid b3 (pRS314) was transformed with a second plasmid either with plasmid b13 (empty 2μ vector) or b89 (2μ-*PDE2*), and tested for proliferation rate difference between ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in SC-TRP-URA medium, and plates were incubated for 2 d (ρ^+ and ρ^-) at 30°C. (D) Phosphorylation status of Tom22p or Tom40p does not affect the proliferation of cells when their mtDNA is depleted. Strains CDD866 (*tom22Δ+ pTOM22* WT), CDD867 (*tom22Δ+ pTOM22* T76A), CDD868 (*tom22Δ+ pTOM22* T76E), CDD869 (*tom40Δ+ pTOM40* WT), CDD870 (*tom40Δ+ pTOM40* S54A) and CDD871 (*tom40Δ+ pTOM240* S54E) were tested for proliferation rate difference between ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in YEPD medium, and plates were incubated for 2 d (ρ^+ and ρ^-) at 30°C.

Figure 3.9 Tom70p level does not change significantly in cells lacking mtDNA with or without *PDE2* overexpression. Strain CDD926 spores confirmed as *TOM70::GFP* transformed either by empty vector or 2μ- *PDE2*, and proteins extracted from ρ^+ (mtDNA+) and ρ^- (mtDNA-) cells as explained in Materials and Methods. Cells were grown in SC-URA medium. Hexokinase protein was used as the loading control, and a CDD926 spore devoid of GFP tag was used as the negative control.

Figure 3.10 Cells deleted of yeast Hsp90 isoforms, encoded by *HSP82* and *HSC82*, have increased proliferation rate when PKA activity is diminished. Strains BY4742 (WT), CDD902 (*hsc82Δ*) and CDD909 (*hsc82Δ*) were transformed with either plasmid b13

(empty 2 μ vector) or b89 (2 μ -*PDE2*), and were tested for proliferation rate difference ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in SC-URA medium, and plates were incubated for 2 d (ρ^+ and ρ^-) at 30°C.

Figure 3.11 Aac2p level is not altered when *PDE2* is overexpressed in cells lacking mtDNA. Strain CDD859 transformed either by empty vector or 2 μ - *PDE2* and proteins extracted from ρ^+ (mtDNA+) and ρ^- (mtDNA-) cells as explained in the Materials and Methods. Cells were grown in SC-URA medium. Hexokinase protein was used as the loading control, and BY4742 (*WT*) was used as negative control for FLAG tag.

Figure 3.12 Many transcriptional modulators that are regulated by PKA pathway are not the relevant targets of *PDE2* overexpression in cells lacking mtDNA. (A) Glucose induced PKA activity results in repression of transcription factors, Msn2p/4p and Gis1p through the activity of Rim15p kinase, and ρ^- cells proliferate far better with *PDE2* overexpression in the absence of any of these proteins. Strains CDD463 (*WT*), CDD841 (*rim15 Δ*); BY4742 (*WT*), CDD801 (*gis1 Δ*) and BY4741 (*WT*), CDD838 (*msn2 Δ msn4 Δ*) were transformed with either plasmid b13 (empty 2 μ vector) or b89 (2 μ -*PDE2*), and were tested for proliferation rate difference between ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in SC-URA medium, and plates were incubated for 2 d (ρ^+ and ρ^-) at 30°C. (B) Strains with the *hsf1::DAmP* allele proliferate better than *WT* cells in the absence of mtDNA. BY4741 (*WT*), CDD910 (*hsf1::DAmP*) were tested for proliferation rate difference between ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in YEPD medium, and plates were incubated for 2 d (ρ^+ and ρ^-) at 30°C. (C) Cells with decreased Hsf1p activity positively respond to *PDE2* overexpression. Strains BY4743 (*WT*), CDD853 (*hsf1 Δ / HSF1*), CDD872 (*hsf1 Δ + pHSF1-WT*), CDD873 (*hsf1 Δ + pHSF1-P215Q*) were transformed with either plasmid b13 (empty 2 μ vector) or b89 (2 μ -*PDE2*), and were tested for proliferation rate difference between ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in SC-URA medium, and plates were incubated for 2 d (ρ^+ and ρ^-) at 30°C. (D) *HSP12* or *HSP26* deleted cells respond to *PDE2* overexpression by increased proliferation rate when their mtDNA is depleted. Strains CDD463 (*WT*), CDD534 (*hsp26 Δ*), CDD542 (*hsp12 Δ*) were transformed with either plasmid b13 (empty 2 μ vector) or b89 (2 μ -*PDE2*), and were tested for proliferation rate difference between ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in SC-URA medium, and plates were incubated for 2 d (ρ^+ and ρ^-) at 30°C.

Figure 3.13 PKA targets that are regulators of ribosome biogenesis are not involved in the mechanism of increasing proliferation rate of cells lacking mtDNA with *PDE2* overexpression. Strains BY4741 (*WT*), CDD928 (*maf1Δ*) and CDD289 (*WT*), CDD567 (*dot6Δtod6Δ*) were transformed with either plasmid b13 (empty 2μ vector) or b89 (2μ-*PDE2*), and were tested for proliferation rate difference between ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in SC-URA medium, and plates were incubated for 2 d (ρ^+ and ρ^-) at 30°C.

Figure 3.14 Proliferation defect in cells deleted of the glycolysis regulator, Gcr2p, can be rescued by decreasing the activity of PKA. (A) Deletion of *GCR2* is not beneficial for cells lacking mtDNA. Strains BY4741 (*WT*) and CDD941 (*gcr2Δ*) were tested for proliferation rate difference between ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in YEPD medium, and plates were incubated for 3d for ρ^- and 2d for ρ^+ . (B) Overexpression of *PDE2* increases the proliferation rate of the ρ^- cells deleted of *GCR2*. Strains BY4741 (*WT*) and CDD941 (*gcr2Δ*) were transformed with either plasmid b13 (empty 2μ vector) or b89 (2μ-*PDE2*), and were tested for proliferation rate difference ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in SC-URA medium, and plates were incubated for 3 d at 30°C.

Figure 3.15 Neither Snf1p nor Sip1p is required for the beneficial effects of *PDE2* overexpression in cells lacking mtDNA. Strains CDD463 (*WT*), CDD604 (*snf1Δ*), CDD842 (*sip1Δ*) were transformed with either plasmid b13 (empty 2μ vector) or b89 (2μ-*PDE2*), and and were tested for proliferation rate difference between ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in SC-URA medium, and plates were incubated for 2 d (ρ^+ and ρ^-) at 30°C.

Figure 3.16 Cells deleted of TOR pathway regulatory components respond positively to diminished PKA activity after mtDNA depletion. Strains BY4742 (*WT*), CDD874 (*tip41Δ*) and BY4741 (*WT*), CDD921 (*sds23Δsds24Δ*) were transformed with either plasmid b13 (empty 2μ vector) or b89 (2μ-*PDE2*), and were tested for proliferation rate difference between ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in SC-URA medium, and plates were incubated for 2 d (ρ^+ and ρ^-) at 30°C.

Figure 3.17 Cells lacking mtDNA with *PAT1* deletion exhibited an increased rate of survival. Strains BY4742 (*WT*), CDD879 (*pat1Δ*) were tested for proliferation rate

difference ρ^+ (+mtDNA) and ρ^- (-mtDNA). Cells were grown in YEPD medium, and plates were incubated for 2 d (ρ^+ and ρ^-) at 30°C.

3.7 Figures

Figure 3.1 Reducing PKA activity by *PDE2* overexpression is beneficial for cells lacking mtDNA.

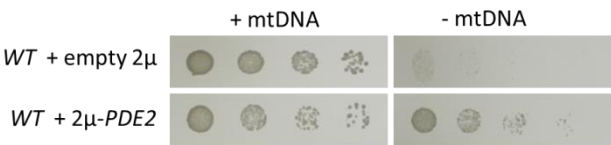


Figure 3.2 Deletion of *TPK3*, but neither other isoforms nor their combinations, rescued the growth defect of ρ^- cells.

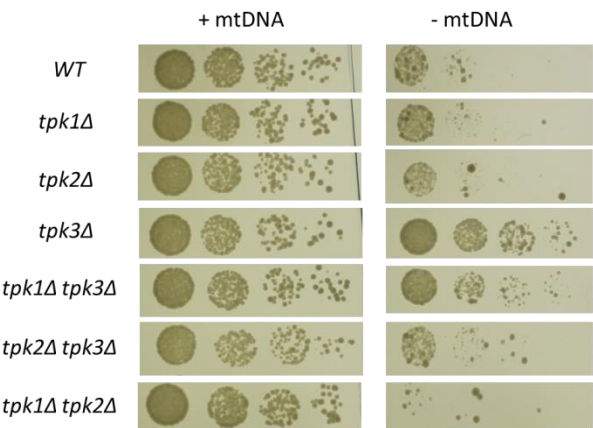


Figure 3.3 Deletion of PKA activators, Gpa2p or Gpr1p, or a PKA catalytic isoform, Tpk3p, benefit cells lacking mtDNA.

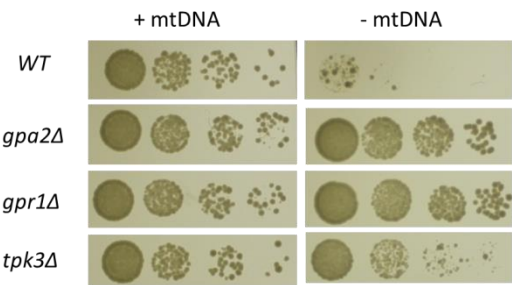


Figure 3.4 Reduced glucose sensing diminishes the effects of mtDNA damage in yeast independent of the extracellular glucose concentration.

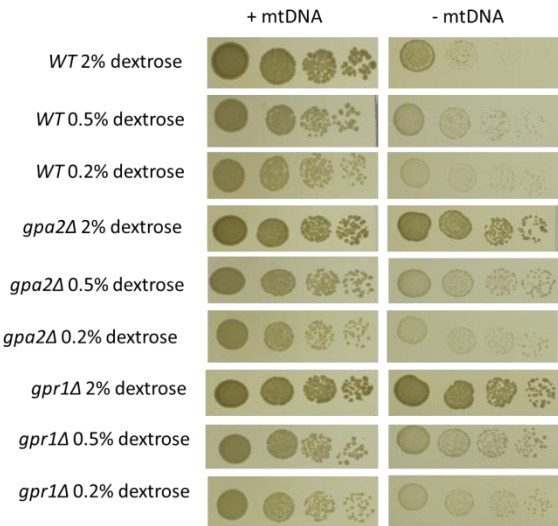


Figure 3.5 Characteristic transcriptional response to mtDNA loss is reversed with diminished PKA activity.

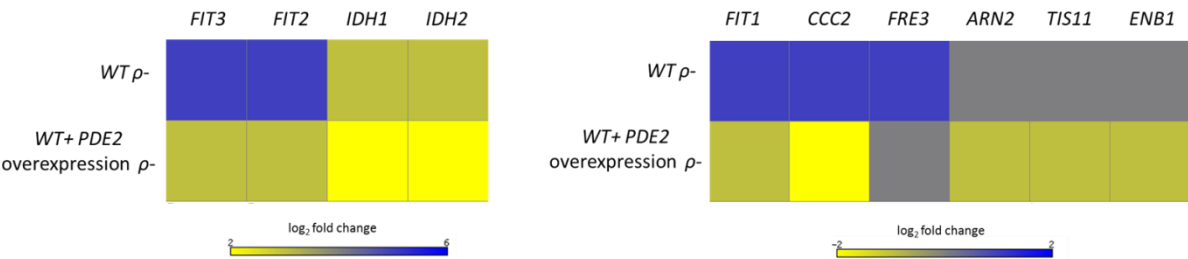


Figure 3.6 PKA inhibition does not increase protein import by mitochondria lacking mtDNA.

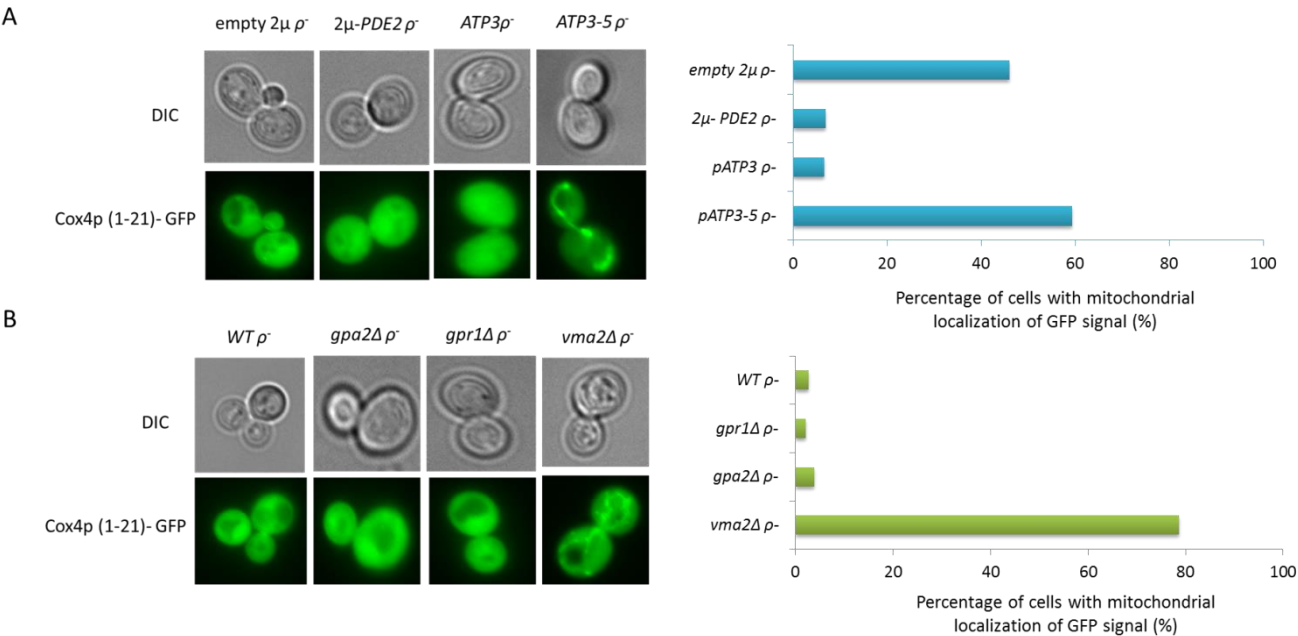


Figure 3.7 Reduced PKA activity allowed viability of mutants that depend on mtDNA for survival except *aac2* Δ and *tom70* Δ .

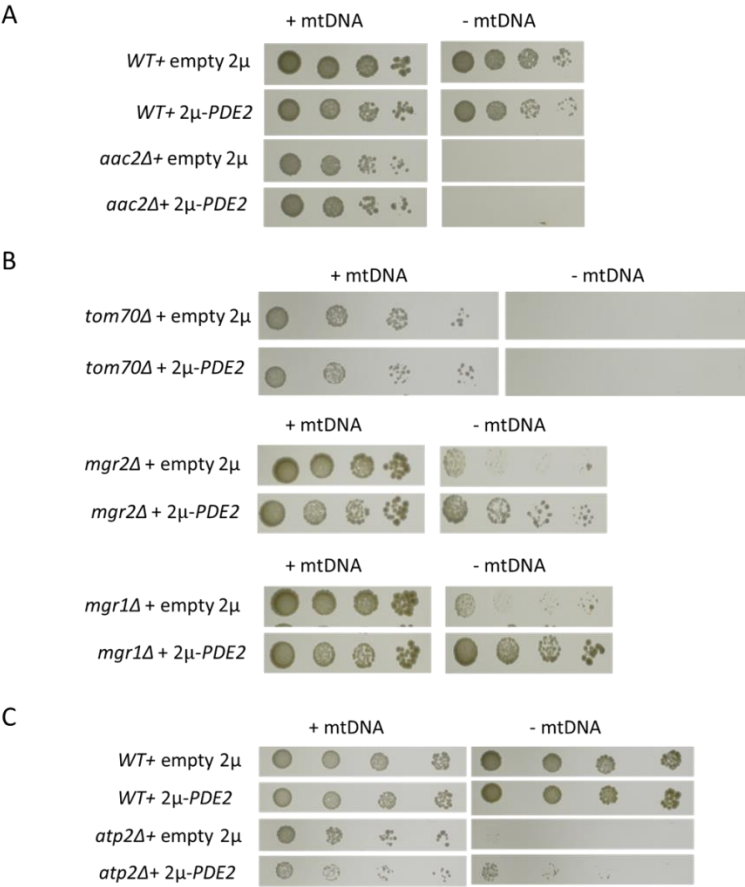


Figure 3.8 Phosphorylation mutants of TOM complex components do not have altered proliferation rates after their mtDNA is depleted.

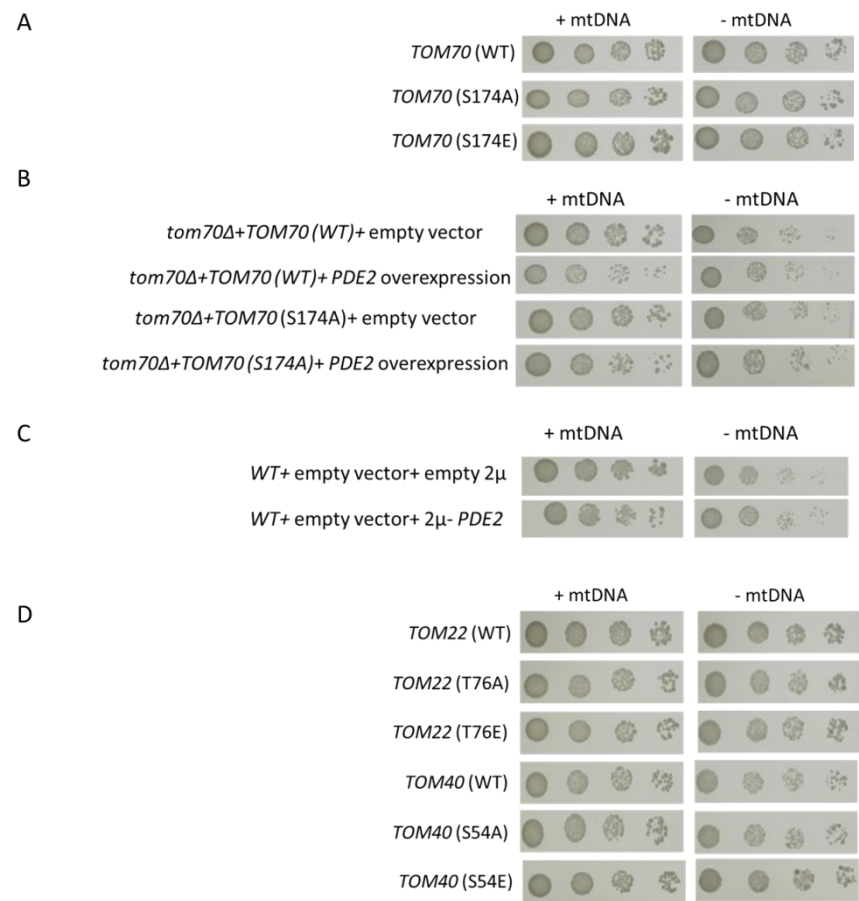


Figure 3.9 Tom70p level does not change significantly in cells lacking mtDNA with or without *PDE2* overexpression.

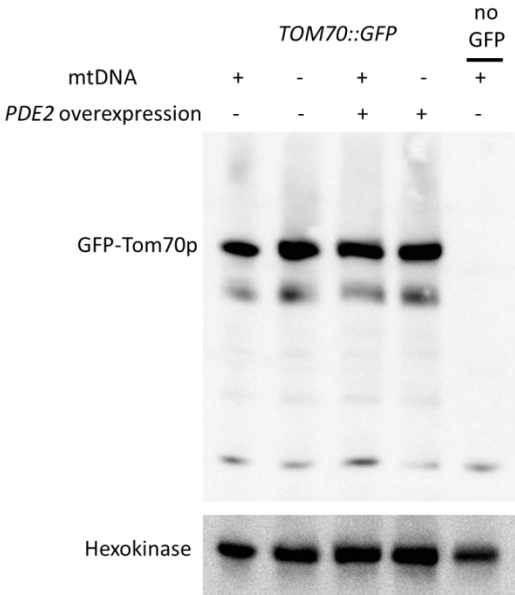


Figure 3.10 Cells deleted of yeast Hsp90 isoforms, encoded by *HSP82* and *HSC82*, have increased proliferation rate when PKA activity is diminished.

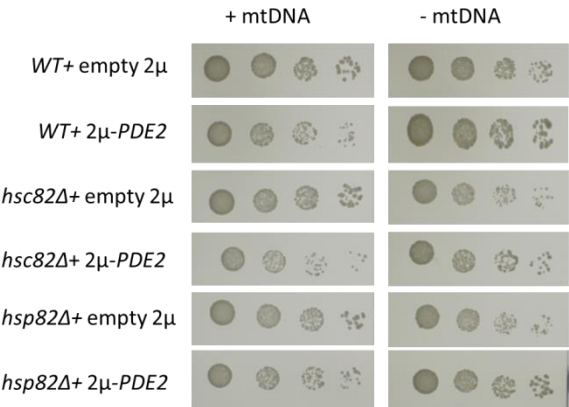


Figure 3.11 Aac2p level is not altered when *PDE2* is overexpressed in cells lacking mtDNA.

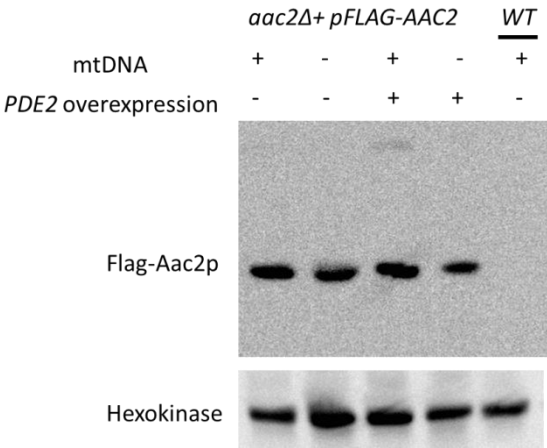


Figure 3.12 Many transcriptional modulators that are regulated by PKA pathway are not the relevant targets of *PDE2* overexpression in cells lacking mtDNA.

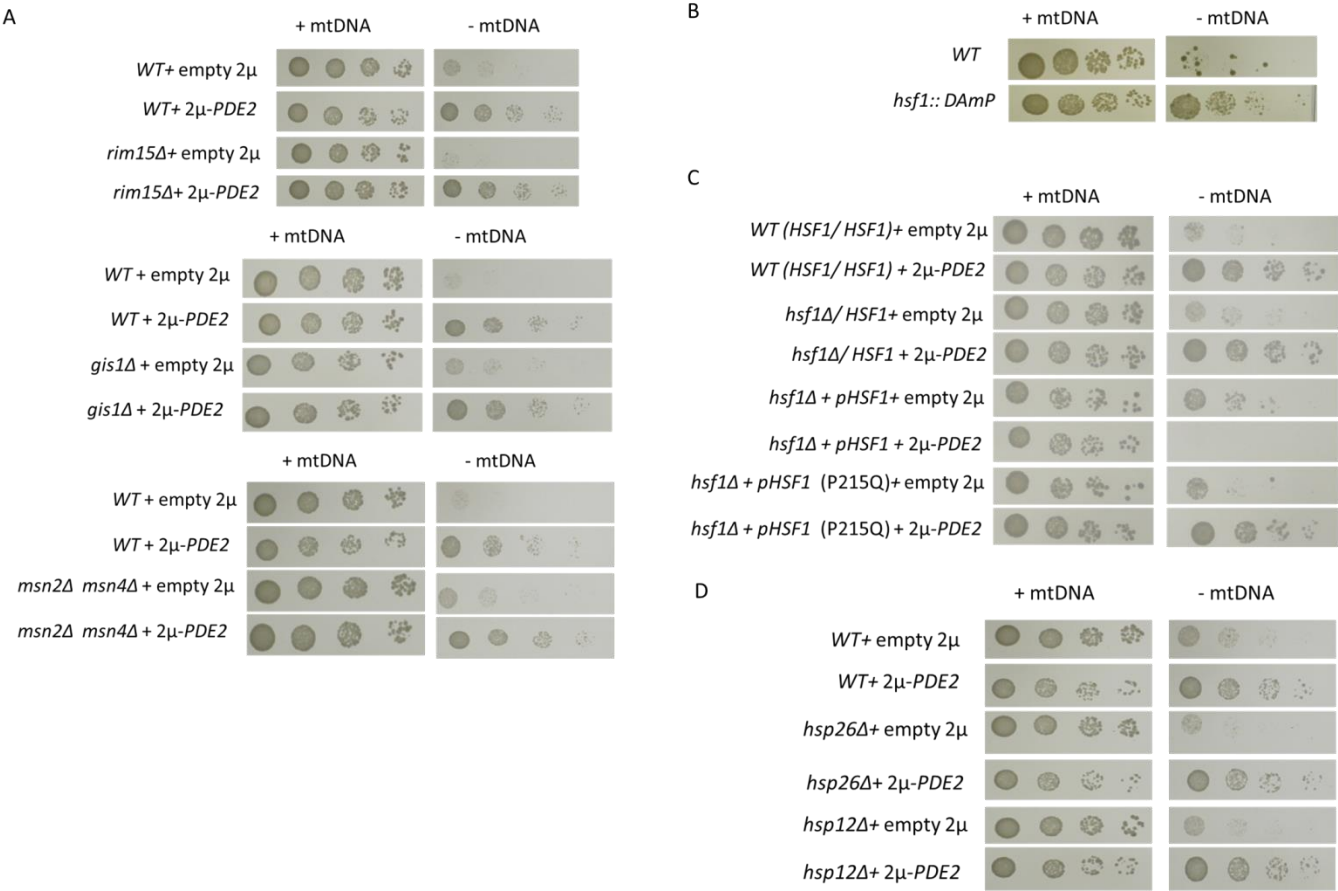


Figure 3.13 PKA targets that are inhibitors of ribosome biogenesis are not involved in the mechanism of increasing proliferation rate of cells lacking mtDNA with *PDE2* overexpression.

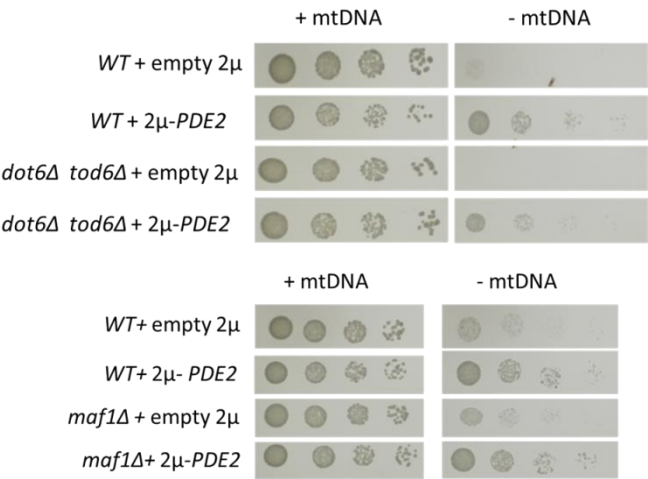


Figure 3.14 Proliferation defect in cells deleted of the glycolysis regulator, Gcr2p, can be rescued by decreasing the activity of PKA.

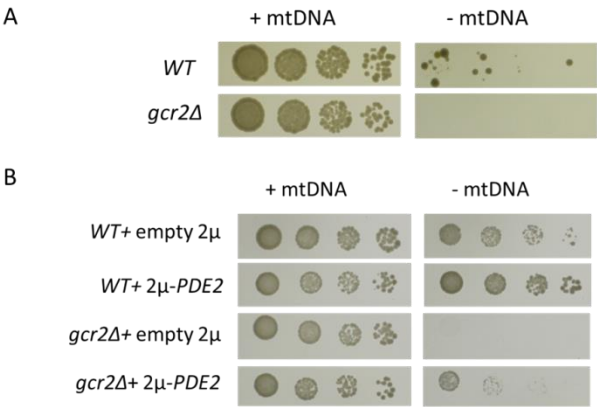


Figure 3.15 Neither Snf1p nor Sip1p is required for the beneficial effects of *PDE2* overexpression in cells lacking mtDNA.

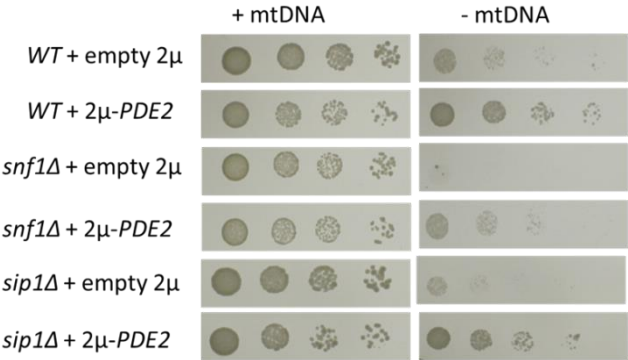


Figure 3.16 Cells deleted of TOR pathway regulatory components respond positively to diminished PKA activity after mtDNA depletion.

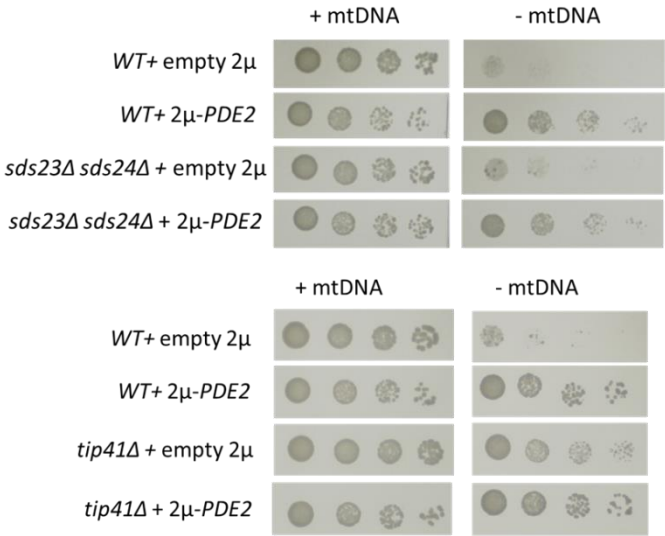
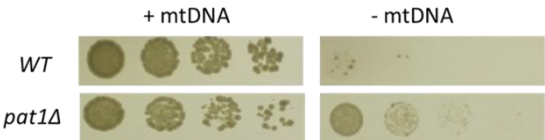


Figure 3.17 Cells lacking mtDNA with *PAT1* deletion exhibited an increased rate of survival.



3.8 Tables

Table 3.1 List of strains used in Chapter 3

Strain	Genotype	Source	Parental strain(s)	Method Used
BY4741	<i>MATa his3Δ1 leu2Δ0</i> <i>met15Δ0 ura3Δ0</i>	EUROSCARF Y00000		
BY4742	<i>MATα his3Δ1 leu2Δ0 lys2Δ0</i> <i>ura3Δ0</i>	EUROSCARF Y10000		
BY4743	<i>MATa/MATα his3Δ1/ his3Δ1</i> <i>leu2Δ0/leu2Δ0</i> <i>met15Δ0/MET15</i> <i>LYS2/lys2Δ0 ura3Δ0/</i> <i>ura3Δ0</i>	EUROSCARF Y20000		
RJ1854	<i>MATa/MATα</i> <i>met15Δ0/met15Δ0</i> <i>his3Δ200/his3Δ200</i> <i>leu2Δ0/leu2Δ0</i> <i>trp1Δ63/trp1Δ63</i> <i>ura3Δ0/ura3Δ0</i> <i>atp2Δ::LEU2/ATP2</i> <i>sit4Δ::HIS3/SIT4</i>	74		
CDD11	<i>MATa his3Δ1 leu2Δ0</i> <i>met15Δ0 ura3Δ0</i> <i>mgr2Δ::kanMX4</i>	BY4741 EUROSCARF Y02154		
CDD13	<i>MATa his3Δ1 leu2Δ0</i> <i>met15Δ0 ura3Δ0</i> <i>mgr1Δ::kanMX4</i>	BY4741 EUROSCARF Y03451		
CDD24	<i>MATα/MATa leu2Δ0/leu2Δ0</i> <i>lys2Δ0/LYS2Δ0</i>	BY4743 EUROSCARF		

	<i>met15Δ0/MET15</i> <i>ura3Δ0/ura3Δ0</i> <i>his3Δ1/his3Δ1</i> <i>tom70Δ::kanMX4/TOM70</i>	Y27244		
CDD215	<i>MATa leu2Δ0 his3Δ</i> <i>met15Δ0 trp1Δ63</i> <i>atp2Δ::LEU2</i>	16		
CDD236	<i>MATa leu2Δ0 lys2Δ0</i> <i>ura3Δ0 his3Δ1</i> <i>mig1Δ::kanMX4</i>	EUROSCARF Y14403		
CDD248	<i>MATa/MATa</i> <i>msn2Δ::kanMX4/MSN2</i> <i>msn4Δ::URA3/MSN4</i>	215		
CDD249	<i>MATa leu2Δ0 ura3Δ0</i> <i>his3Δ200 trp1Δ63 met15Δ0</i> <i>sit4Δ::HIS3 atp2Δ::LEU2</i>	constructed by Dr. C. Dunn	RJ1854	Sporulation
CDD280	<i>MATa met15Δ</i> <i>msn2Δ::kanMX4</i> <i>msn4Δ::URA3</i>	constructed by Dr. C. Dunn	CDD248	Sporulation
CDD289	<i>MATa leu2Δ0 ura3Δ0 his3Δ</i> <i>TRP1 LYS2 MET15</i>	constructed by Dr. C. Dunn	CDD236, CDD249	CDD236 crossed to CDD249, resulting diploid sporulated
CDD315	<i>MATa/MATa</i> <i>tom70Δ::kanMX4/TOM70</i> <i>vps16Δ::URA3/VPS16</i>	constructed by Dr. C. Dunn	CDD24	disrupted <i>VPS16</i> with <i>URA3</i> using primers 179/180 and template pRS306, then sporulated the resulting diploid
CDD338	<i>MATa tom70Δ::kanMX4</i> <i>LYS2 MET15 TRP1</i>	constructed by Dr. C. Dunn	CDD315	sporulation
CDD463	<i>MATa leu2Δ0 his3Δ200</i> <i>met15Δ0 trp1Δ63 ura3Δ0</i> <i>LYS2</i>	16		

CDD496	<i>MATα vma2Δ::kanMX4</i>	EUROSCARF Y13266		
CDD528	<i>MATα leu2Δ0 his3Δ ura3Δ0 met15Δ0 trp1Δ63</i>	215		
CDD534	<i>MATα hsp26Δ::kanMX4</i>	EUROSCARF Y13209		
CDD542	<i>MATα hsp12Δ::kanMX4</i>	EUROSCARF Y17070		
CDD546	<i>MATα rim15Δ::URA3 atp2Δ::LEU2 met15Δ0 LYS2</i>	215		
CDD567	<i>MATα dot6Δ::kanMX4 tod6Δ::kanMX4 MET15 LYS2</i>	215		
CDD570	<i>MATα HSP12- 13MYC::kanMX6</i>	215		
CDD604	<i>MATα leu2Δ0 his3Δ met15Δ0 trp1Δ63 ura3Δ0 snf1Δ::TRP1</i>	16		
CDD632	<i>MATα met15Δ0 his3Δ leu2Δ0 trp1Δ63 ura3Δ0 aac2Δ::URA3</i>	16		
CDD801	<i>MATα gis1Δ::HIS3 MET15 lys2Δ0 TRP1</i>	constructed by Dr. C. Dunn	CDD546, CDD570	CDD546 crossed to CDD570, and <i>GIS1</i> is disrupted with <i>HIS3</i> in the resulting diploid using primers 618/619 and template pRS303, followed by sporulation
CDD827	<i>MATα/MATα aac2Δ::kanMX4/aac2</i>	EUROSCARF Y23056		

CDD838	<i>MATα msn2Δ::kanMX4 msn4Δ::HIS3 LYS2 TRP1 met15Δ</i>	This study	CDD280	URA3 allele replaced using primers 144/145 and template pRS303
CDD841	<i>MATα rim15Δ::HIS3 trp1Δ63 met15Δ LYS2</i>	This study	CDD463	disrupted <i>RIM15</i> with <i>HIS3</i> using primers 307/308 and template pRS303, followed by sporulation
CDD842	<i>MATα sip1Δ::HIS3 trp1Δ63 met15Δ LYS2</i>	This study	CDD463	disrupted <i>SIP1</i> with <i>HIS3</i> using primers 447/448 and template pRS303, followed by sporulation
CDD849	<i>MATα gpa2Δ::kanMX4</i>	EUROSCARF Y10152		
CDD850	<i>MATα gpr1Δ::kanMX4</i>	EUROSCARF Y13731		
CDD851	<i>MATα/MATα tom40Δ::kanMX4/ TOM40</i>	EUROSCARF Y20789		
CDD852	<i>MATα/MATα tom22Δ::kanMX4/ TOM22</i>	EUROSCARF Y22945		
CDD853	<i>MATα/MATα hsf1Δ::kanMX4/ HSF1</i>	EUROSCARF Y24440		
CDD857	<i>MATα tom70Δ::kanMX4 trp1Δ63 MET15 LYS</i>	constructed by Dr. C. Dunn	CDD338, CDD463	CDD338 crossed to CDD463, and the resulting diploid sporulated
CDD859	<i>MATα aac2Δ::kanMX4 MET15 lys10 + plasmid b84 (pRS313-FLAG-AAC2)</i>	This study	CDD827	CDD827 transformed with plasmid b84 (pRS313-FLAG-AAC2), followed by sporulation
CDD866	<i>MATα tom22Δ::kanMX4 MET15 LYS2 + plasmid b103 (pRS316-TOM22 WT)</i>	This study	CDD852	CDD852 transformed with plasmid b103 (pRS316-TOM22 WT), followed by sporulation

CDD867	<i>MATa tom22Δ::kanMX4</i> <i>MET15 LYS2</i> + plasmid b104 (<i>pRS316-TOM22</i> <i>T76A</i>)	This study	CDD852	CDD852 transformed with plasmid b104 (<i>pRS316-TOM22 T76A</i>), followed by sporulation
CDD868	<i>MATα tom22Δ::kanMX4</i> <i>MET15 LYS2</i> + plasmid b105(<i>pRS316-TOM22 T76E</i>)	This study	CDD852	CDD852 transformed with plasmid b105 (<i>pRS316-TOM22 T76E</i>), followed by sporulation
CDD869	<i>MATα tom40Δ::kanMX4</i> <i>MET15 LYS2</i> + plasmid b106(<i>pRS316-TOM40 WT</i>)	This study	CDD851	CDD851 transformed with plasmid b106 (<i>pRS316-TOM40 WT</i>), followed by sporulation
CDD870	<i>MATα tom40Δ::kanMX4</i> <i>MET15 LYS2</i> + plasmid b107(<i>pRS316-TOM40 S54A</i>)	This study	CDD851	CDD851 transformed with plasmid b107 (<i>pRS316-TOM40 S54A</i>), followed by sporulation
CDD871	<i>MATα tom40Δ::kanMX4</i> <i>MET15 LYS2</i> + plasmid b108(<i>pRS316-TOM40 S54E</i>)	This study	CDD851	CDD851 transformed with plasmid b108 (<i>pRS316-TOM40 S54E</i>), followed by sporulation
CDD872	<i>MATa hsf1Δ::kanMX4</i> + plasmid <i>pRS315- HSF1</i> (WT) <i>LYS2 MET15</i>	This study	CDD853	CDD853 transformed with plasmid <i>pRS315- HSF1</i> (WT), followed by sporulation
CDD873	<i>MATα hsf1Δ::kanMX4</i> + plasmid <i>pRS315- HSF1</i> (P215Q) <i>LYS2 MET15</i>	This study	CDD853	CDD853 transformed with plasmid <i>pRS315- HSF1</i> (P215Q), followed by sporulation
CDD874	<i>MATα tip41Δ::kanMX4</i> <i>LYS2 MET15</i>	This study	BY4743	<i>TIP41</i> disrupted in BY4743 with <i>kanMX4</i> using primers 752/753 and template plasmid b81, followed by sporulation
CDD879	<i>MATa pat1Δ::kanMX4 lys40</i> <i>MET TRP1</i>	This study	BY4743	<i>PAT1</i> disrupted in BY4743 with <i>kanMX4</i> using primers 779/780 and template plasmid b81, followed by sporulation

CDD883	<i>tom70Δ::kanMX4/ TOM70</i>	This study	BY4743	<i>TOM70</i> disrupted in BY4743 with <i>kanMX4</i> using primers 758/759, and template plasmid b81
CDD884	<i>MATα tpk1Δ::kanMX4 lys2Δ0 MET15 TRP1</i>	This study	BY4743	<i>TPK1</i> disrupted in BY4743 with <i>kanMX4</i> using primers 589/590 and template b81, followed by sporulation
CDD885	<i>MATα tpk2Δ::kanMX4 lys2Δ0 MET15 TRP1</i>	This study	BY4743	<i>TPK2</i> disrupted in BY4743 with <i>kanMX4</i> using primers 592/593 and template b81, followed by sporulation
CDD886	<i>MATα tpk3Δ::kanMX4 lys2Δ0 MET15 TRP1</i>	This study	BY4743	<i>TPK3</i> disrupted in BY4743 with <i>kanMX4</i> using primers 595/596 and template b81, followed by sporulation
CDD897	<i>MATα tom70Δ::kanMX4 lys2Δ0 MET15 TRP1</i>	constructed by Dr. C. Dunn	CDD883	Sporulation
CDD898	<i>MATα/MATα his3Δ200/his3Δ200 leu2Δ0/leu2Δ0 met15Δ0/met15Δ0 trp1Δ63/trp1Δ63 ura3Δ0/ura3Δ0 LYS2/LYS2</i>	constructed by Dr. C. Dunn	CDD463, CDD528	CDD463 crossed to CDD528
CDD899	<i>MATα hsf1::dAMP::kanMX4 cyh2 his3Δ1 met15Δ0 ura3Δ0 leu2Δ0 TRP1 LYS2</i>	200,201		
CDD902	<i>hsc82Δ::kanMX4 lys2Δ0 MET15 TRP1</i>	This study	BY4743	<i>HSC82</i> disrupted in BY4743 with <i>kanMX4</i> using primers 851/852 and template plasmid b81, followed by sporulation
CDD908	<i>MATα tpk1Δ::kanMX4 tpk3Δ::kanMX4 lys2Δ0 MET15 TRP1</i>	constructed by Dr. C. Dunn	CDD884, CDD886	CDD884 crossed to CDD886, and the resulting diploid sporulated

CDD909	<i>MATa hsp82Δ::kanMX4</i>	This study	BY4742	<i>HSP82</i> disrupted in BY4742 with <i>kanMX4</i> using primers 854/855 and template plasmid b81
CDD910	<i>hsf1::DAmP::kanMX4 his3Δ met15Δ0 ura3Δ0 leu2Δ TRP1 CYH2 LYS2</i>	constructed by Dr. C. Dunn	CDD528, CDD899	CDD528 crossed to CDD899, and the resulting diploid sporulated
CDD911	<i>MATa sds24Δ::kanMX4 met15Δ0 LYS2 trp1Δ63</i>	This study	CDD463	<i>SDS24</i> disrupted in CDD463 with <i>kanMX4</i> using primers 187/188 and template b81
CDD913	<i>MATa tom70Δ::kanMX4 trp1Δ63 met15Δ0 LYS2</i>	constructed by Dr. C. Dunn	CDD528, CDD897	CDD528 crossed to CDD897, and the resulting diploid sporulated
CDD919	<i>MATa/MATa tpk1Δ/ TPK1 tpk2Δ/ TPK2 tpk3Δ/ TPK3 yak1Δ::HIS3/ YAK1</i>	This Study	CDD885, CDD908	CDD885 crossed to CDD908, and <i>YAK1</i> is disrupted in the resulting diploid with <i>HIS3</i> using primers 717/718 and template pRS303
CDD921	<i>sds23Δ::kanMX4 sds24Δ::kanMX4 met15Δ0 LYS2 trp1Δ63</i>	constructed by Dr. C. Dunn	CDD240, CDD911	CDD240 crossed to CDD911, and the resulting diploid sporulated
CDD922	<i>tpk2Δ::kanMX4 tpk3Δ::kanMX4 lys2Δ0 MET15 TRP1</i>	constructed by Dr. C. Dunn	CDD919	Sporulation
CDD923	<i>tpk1Δ::kanMX4 tpk2Δ::kanMX4 lys2Δ0 MET15 TRP1</i>	constructed by Dr. C. Dunn	CDD919	Sporulation
CDD926	<i>TOM70-GFP:: HIS3MX6 ura3Δ leu2Δ met15Δ lyp1Δ TRP1 LYS2 CYH2</i>	constructed by Dr. C. Dunn,	(<i>TOM70-GFP</i>) strain ^{200,201} , CDD528	(<i>TOM70-GFP</i>) strain crossed to CDD528, and the resulting diploid sporulated
CDD928	<i>maf1Δ::kanMX4 trp1Δ63 LYS2 met15Δ0</i>	This study	CDD898	<i>MAF1</i> disrupted in CDD898 with <i>kanMX4</i> using primers 872/873, and template b81, followed by sporulation

CDD941	<i>gcr2Δ::kanMX4</i>	EUROSCARF Y12013		
--------	----------------------	---------------------	--	--

Table 3.2 List of plasmids used in Chapter 3

Plasmid number	Plasmid name	Reason of use	Source	Method used for construction
b2	pRS313	<i>ARS-CEN-HIS3</i>	258	
b3	pRS314	<i>ARS-CEN-TRP1</i>	258	
b4	pRS315	<i>ARS-CEN-LEU2</i>	258	
b5	pRS316	<i>ARS-CEN-URA3</i>	258	
b6	PRS303	<i>ARS-HIS3</i>	258	
b9	pRS306	<i>ARS-URA3</i>	258	
b10	pRS423	<i>2μ- HIS3</i>	258	

b13	pRS426	2μ - <i>URA3</i>	258	
b21	pRS315- <i>ATP3</i> (old M487)	control for b22 (pRS315- <i>ATP3</i> - 5)	73	
b22	pRS315- <i>ATP3</i> -5 (old M488)	petite-negative phenotype supression	73	
b28	pRS426- <i>TIP41</i>	petite-negative phenotype supression	75	
b46	pHS12 (old M285)	<i>COX4(1-21)</i> - <i>GFP-LEU2</i>	262	
b81	pRS306K	gene disruption with <i>kanMX4</i>	EUROSCA RF 30646	
b84	pRS313- FLAG- <i>AAC2</i>	detection of N- term FLAG- tagged Aac2p	constructed by Dr. C. Dunn and G. Garipler	<i>NotI</i> cut pRS313 cotransformed into CDD68 with PCR products of <i>AAC2</i> promoter amplified with primers 518/519 and <i>AAC2</i> gene with overlapping FLAG amplified with primers 520/521, followed by isolation and purification
b89	pRS426- <i>PDE2</i>	overexpression of <i>PDE2</i>	constructed by Gülkız Baytek	<i>NotI</i> cut pRS426 cotransformed into CDD3 with PCR product of <i>PDE2</i> amplified with primers 614/615 using a template gDNA, followed by isolation and purification

b99	pRS423- <i>PDE2</i>	overexpression of <i>PDE2</i>	This study	<i>NotI</i> -HF cut pRS423 cotransformed with PCR product of <i>PDE2</i> amplified with primers 614/615 using template CDD71 gDNA, followed by isolation and purification
b103	pRS316- <i>TOM22</i> (WT)	expression of <i>TOM22</i>	This study	<i>NotI</i> -HF cut pRS316 cotransformed into CDD3 with PCR products of <i>TOM22</i> amplified with primers 720/721 using template CDD71 gDNA, followed by isolation and purification
b104	pRS316- <i>TOM22</i> (T76A)	<i>TOM22</i> phosphomutant	This study	<i>NotI</i> -HF cut pRS316 cotransformed into CDD3 with PCR products of <i>TOM22</i> portions with primers 727/721 and 720/726 using template CDD71 gDNA, followed by isolation and purification
b105	pRS316- <i>TOM22</i> (T76E)	<i>TOM22</i> phosphorylation mimic	This study	<i>NotI</i> -HF cut pRS316 cotransformed into CDD3 with PCR products of <i>TOM22</i> portions with primers 729/721 and 720/728 using template CDD71 gDNA, followed by isolation and purification
b106	pRS316- <i>TOM40</i> (WT)	expression of <i>TOM40</i>	This study	<i>NotI</i> -HF cut pRS316 cotransformed into CDD3 with PCR products of <i>TOM40</i> amplified with primers 722/723 using template CDD71 gDNA, followed by isolation and purification
b107	pRS316- <i>TOM40</i> (S54A)	<i>TOM40</i> phosphomutant	This study	<i>NotI</i> -HF cut pRS316 cotransformed into CDD3 with PCR products of <i>TOM40</i> portions with primers 722/730 and 731/723 using template CDD71 gDNA, followed by isolation and purification
b108	pRS316- <i>TOM40</i> (S54E)	<i>TOM40</i> phosphorylation mimic	This study	<i>NotI</i> -HF cut pRS316 cotransformed into CDD3 with PCR products of <i>TOM40</i> portions with primers 733/723 and 722/732 using template CDD71 gDNA, followed by isolation and

				purification
b110	pRS314- <i>TOM70</i> (WT)	expression of <i>TOM70</i>	This study	<i>NotI</i> -HF cut pRS314 cotransformed into CDD463 with PCR product of <i>TOM70</i> amplified with primers 724/725 using template CDD71 gDNA, followed by isolation and purification
b111	pRS314- <i>TOM70</i> (S174A)	<i>TOM70</i> phosphomutant	This study	<i>NotI</i> -HF cut pRS314 cotransformed into CDD463 with PCR products of <i>TOM70</i> amplified with primers 724/734 and 735/725 using template CDD71 gDNA, followed by isolation and purification
b146	pRS316- <i>ATP3</i> (WT)	control for pRS316- <i>ATP3</i> -5	This study	<i>ATP3</i> PCR product amplified using primers 11/12 from b21 recombined into <i>NotI</i> -HF cut pRS316
b147	pRS316- <i>ATP3</i> -5	increase mitochondrial potential of cells lacking mtDNA	This study	<i>ATP3</i> -5 PCR product amplified using primers 11/12 from b22 recombined into <i>NotI</i> -HF cut pRS316
b148	pRS314- <i>COX4</i> (1- 21)- GFP	label mitochondria	This study	<i>COX4</i> (1-21)-GFP PCR product amplified using primers 11/12 from b46 recombined into <i>NotI</i> -HF cut pRS314
-	pRS315- <i>HSF1</i> (WT)	expression of <i>HSF1</i>	This study	<i>NotI</i> -HF cut pRS315 cotransformed into CDD3 with PCR product of <i>HSF1</i> amplified with primers 742/743 using template CDD71 gDNA, followed by isolation and purification
-	pRS315- <i>HSF1</i> (P215Q)	expression of <i>HSF1</i> DNA- binding domain mutant	This study	<i>NotI</i> -HF cut pRS315 cotransformed into CDD3 with PCR products of <i>HSF1</i> portions with primers 742/740 and 741/743 using template CDD71 gDNA, followed by isolation and purification

Table 3.3 List of oligonucleotides used in Chapter 3

Number	Sequence
11	GTAAAACGACGGCCAGTGAATTG
12	CGCCAAGCTCGGAATTAACCCTCAC
144	TTCGGCTTTTTTTTCTTTTCTTCTTATTAAAAACAATATAAGATTGTACTGAGAGTGCAC
145	TAGCTTGTCTTGCTTTTATTTGCTTTTGACCTATTTTTTCTGTGCGGTATTTACACACCG
179	ACAGACGGATAGGCCTCCCTTTTGTTCGCCTAATAAAAAGATTGTACTGAGAGTGCAC
180	TATGCGGCTAGATATCCACAACGTCTATAAATTATGAGTTCTGTGCGGTATTTACACACCG
187	TTAGTTTAAACCCTCTTTTTTGGCCGCTTCAAGACTGTCCTGTGCGGTATTTACACACCG
188	TAAACGTTAAAATTCAAACACAATAGTTGTTTCGC
307	CTCATTTGATAGAATAGATAAGCCCAGTAGAGGAAGACAGAGATTGTACTGAGAGTGCAC
308	ATTCAGTTATTTTTTTTAATTATCTTTATCTTAAAATTTACTGTGCGGTATTTACACACCG
447	GAAAGTTGAACTGTCATATTATATAGTTGTTGCAGCCGCCAGATTGTACTGAGAGTGCAC
448	GAATTAATAGAGTTCGTGAGAATCATTGCGAATTGAGATTCTGTGCGGTATTTACACACCG
518	GACTCACTATAGGGCGAATTGGAGCTCCACGCCCAACTGATAACGAAATAATTTCTTTTC
519	CTTGTTCATCGTCATCCTTGTAATCCATGGCTATTTGCTTATATGTATGTTAATGTATG
520	ATGGATTACAAGGATGACGATGACAAGTCTTCCAACGCCCAAGTCAAAAC
521	ATATCGAATTCCTGCAGCCCGGGGGATCCAGTTCTCTAGGTGCGACGTCTATGAACAAC
589	GAATTATAGCTGATTGTGTGAAAGAATCTTTTTTTTGGGTAGATTGTACTGAGAGTGCAC
590	AATATAGATACGAGAGGAAAATACAACAAAACATTAGTCACTGTGCGGTATTTACACACCG
592	TTCACCTCAGGTAACTCACATACTGTTGAAAATTGTGCGGTAGATTGTACTGAGAGTGCAC
593	GTACTTGAAAATTGTTTTTGTGTTTTTGGTTCATGGAACCTGTGCGGTATTTACACACCG
595	ATATCGGTGGTTGTACAAGGAAAGAGCGAGCCTGCACAAAAGATTGTACTGAGAGTGCAC
596	CAATTATCCCACTGAACCTCCTTAAAATTCTTTCATTAAACTGTGCGGTATTTACACACCG
614	CGAATTCCTGCAGCCCGGGGGATCCACTAGATCTGGCATAATAAAGTTGATTGTCTTGAC
615	CTCACTAAAGGGAACAAAAGCTGGAGCTCCATTTTCATGAACTTACGGGCTTGTATG
618	GAAGAATAGCTACAAAACAGACTACAATGGAAATCAAGCAGATTGTACTGAGAGTGCAC
619	GAACCCATTTTGTATATCATTTTCTTGACCTATGATTCAGCTGTGCGGTATTTACACACCG
717	CACATGCTAATTTGAGCAATCATGAACTCATCCAATAATAAGATTGTACTGAGAGTGCAC
718	ATGAAATGGGTAGTTAAGAAAAGTCAGACAAAATAACGATCTGTGCGGTATTTACACACCG
720	GACTCACTATAGGGCGAATTGGAGCTCCACCAAAAAGAGCTAATCAACTCCTTGAACCTTA

721 ATATCGAATTCCTGCAGCCCGGGGGATCCATGAAATATGTACAAGTTTATACATTTGTTT
722 GACTCACTATAGGGCGAATTGGAGCTCCACGACTCATCACTTGAAGAAGGGATACGC
723 ATATCGAATTCCTGCAGCCCGGGGGATCCATTCTTCTAAAAAAGAAAAATAGAATTTAAT
724 GACTCACTATAGGGCGAATTGGAGCTCCACCTTGATAGAATTATATTTTTAAACTAGGA
725 ATATCGAATTCCTGCAGCCCGGGGGATCCAACCTATCTTTTTTGTATTTGACCAGATTTTT
726 AGCTAGTAAAACCAAAAAAATTAGAAATTGCTTGTCTCTTACCTGGGGGGACAATGTCT
727 AGACATTGTCCCCCAGGTAAGAGACAAGCAATTTCTAATTTTTTTGGTTTTACTAGCT
728 AGCTAGTAAAACCAAAAAAATTAGAAATCTCTTGTCTCTTACCTGGGGGGACAATGTCT
729 AGACATTGTCCCCCAGGTAAGAGACAAGAGATTTCTAATTTTTTTGGTTTTACTAGCT
730 CCACGGTACCGGGATTGACCAGCTCCAAAGCTTGTCTGTGAGAATGCAATTGTTTGTAC
731 GTACAAACAATTGCATTCTCACAGACAAGCTTTGGAGCTGGTCAATCCCGGTACCGTGG
732 CCACGGTACCGGGATTGACCAGCTCCAACCTTTGTCTGTGAGAATGCAATTGTTTGT
733 ACAAACAATTGCATTCTCACAGACAAGAGTTGGAGCTGGTCAATCCCGGTACCGTGG
734 CCGCAAATTTTCCTAGCCCTTCGTTAGCAGCAGCTCTTCTCAGTAAAACTTTTGAGTAG
735 CTACTCAAAAGTTTTACTGAGAAGAGCTGCTGCTAACGAAGGGCTAGGAAAATTTGCGG
740 AGCGAAATTGGAATGTTTAAAATATTTTTGTAAAATTTGGTGCACAAATTCCTCCCTATT
741 AATAGGGAGGAATTTGTGCACCAAATTTTACAAAAATATTTTAAACATTCCAATTTGCT
742 GACTCACTATAGGGCGAATTGGAGCTCCACTACTTACGTTGTCAAAGACAGAATTGTTTG
743 ATATCGAATTCCTGCAGCCCGGGGGATCCATTGTCGTAGATTTTTCTGTTATTTTTTTG
752 ACCTAAGGGCAGCTTTAGACACAACAGCTCCCCAGAAAAAAGATTGTACTGAGAGTGCAC
753 CGTGTATGTATTTGTACGTATTGTTTTGTATATTTGATTGCTGTGCGGTATTTACACCG
758 GAAGTGAAATTACAGCTCACATCTAGGTTCTCAATTGCCAAGATTGTACTGAGAGTGCAC
759 TTTGTCTTCTCCTAAAAGTTTTTAAGTTTATGTTTACTGTCTGTGCGGTATTTACACCG
779 AGGTTTTAACCGGAAGTAAGAGCAGCAAGAAGCACTAGCAAGATTGTACTGAGAGTGCAC
780 AAAATACATGCGTAAGTACATTAAAATTACAGGAAAAATCCTGTGCGGTATTTACACCG
851 ACGCTACAGAACCAATAGAAAAATAGAATCATTCTGAAATAGATTGTACTGAGAGTGCAC
852 AATTTATATAATATATAAAACATGAAGGCGAAAAAAGAGACTGTGCGGTATTTACACCG
854 AGAGTCCTATAAACAAAAGCACAAACAAACACGCAAAGATAGATTGTACTGAGAGTGCAC
855 ATGTTTTGTTTATAACCTATTCAAGGCCATGATGTTCTACCTGTGCGGTATTTACACCG
872 ATTACTCAAACGGATTTTTTTGCCTAAAGAATCACGACAAGATTGTACTGAGAGTGCAC
873 AGGTGTAAGACAAGGAAAATTCACAAATTAAAGTTTAAACTGTGCGGTATTTACACCG

Table 3.4 List of antibodies used in Chapter

Antibody	Company/ Origin	Details	Dilution used
Primary monoclonal anti-FLAG M2, produced in Mouse	SIGMA	Lot# SLBG5673V (F1804-200UG)	1:10000
Primary GFP (D5.1) XP® Rabbit mAb	Cell Signaling	Lot:2 (2956S)	1:5000
Primary antibody for Hex, produced in rabbit	a kind gift from Prof. Robert Jensen at Johns Hopkins School of Medicine	dH2616 in 25% Glycerol	1:20000
Secondary Anti-rabbit IgG, HRP linked antibody	Cell Signaling	Lot:25 (#7074)	1:10000
Secondary Anti-mouse IgG, HRP linked antibody	Cell Signaling	CST-7076	1:10000

Table 3.5 List of CDD919 spores PCR-tested in an attempt to obtain *tpk1Δtpk2Δtpk3Δyak1Δ* quadruple deletions

Spore Number	<i>tpk1Δ</i>	<i>tpk2Δ</i>	<i>tpk3Δ</i>
2b	-	-	+
2d	-	+	+
3a	-	+	+
3d	-	+	+
4a	+	+	-
4b	-	-	+
4c	+	-	not tested
4d	-	+	-
5d	+	+	-
6c	-	+	-
6d	-	+	-
7c	-	-	+
7d	-	+	+
8a	-	-	not tested
8c	+	+	-
9c	+	-	not tested

10a	+	+	-
11b	-	+	-
11d	-	+	-
13a	+	-	not tested
14a	+	+	-
14d	+	-	+
16b	+	-	not tested
16c	+	+	-
17d	+	-	not tested
18c	+	-	not tested
19c	+	-	not tested
19d	+	-	not tested
20a	+	-	not tested
20d	+	-	not tested
21c	+	-	not tested
22b	-	+	not tested
23a	-	+	not tested
23d	+	-	not tested
24c	+	-	not tested
44b	-	-	+
44c	+	+	-
49b	+	+	-
49c	+	-	+
63c	-	+	-

CHAPTER 4

ACTIVATION OF THE PLEIOTROPIC DRUG RESISTANCE PATHWAY CAN PROMOTE MITOCHONDRIAL DNA RETENTION BY FUSION-DEFECTIVE MITOCHONDRIA IN *SACCHAROMYCES CEREVISIAE*

4.1 Introduction

Mitochondria are dynamic organelles, and their function depends on proper structure and position within the cell, which is provided by the integration of various parallel pathways ²⁶³. In many eukaryotic cell types, mitochondria move along cytoskeletal tracks and constantly fuse and divide, the rate of which determines mitochondrial shape, abundance and size ^{264,265}. Recently, it has been clearly revealed that this dynamic behavior is crucial for many mitochondrial functions in cell life and death. For instance, mitochondrial fusion is essential for the proper respiratory activity of the mitochondria and linked to cell survival ²⁶⁶. Moreover, mitochondrial fusion defects may also be found in neurodegenerative diseases such as Parkinson's and aging ²⁶⁷. Due to its complex architecture, mitochondrial fusion, OM fusion followed by IM fusion, occurs coordinately in a highly regulated manner. In yeast, at least three proteins are required for mitochondrial fusion: Fzo1, Ugo1 and Mgm1. Deletion of any of these components result in mtDNA loss ^{268–271}, and this loss results from the imbalance of fusion-fission cycle, since cells lacking both mitochondrial fusion and division preserve their mtDNA ²⁷⁰. Fzo1p is mainly responsible for OM fusion ²⁷², while IM fusion is driven by Mgm1p ²⁷³. The human counterparts of the dynamin related GTPases, Fzo1p and Mgm1p are the mitofusin proteins (MFN1/MFN2) and OPA1, respectively, which are directly related to human diseases. *MFN2* gene mutation is the cause of the most frequent form of autosomal dominant axonal Charcot-Marie-Tooth disease, CMT2A ²⁷⁴, while mutations in *OPA1* result in autosomal dominant optic atrophy (ADOA) ²⁷⁵. Ugo1p binds to both Fzo1p and Mgm1p, and thus enables mitochondrial fusion by coordinating their function ²⁷⁰.

Recently, a new mitochondrial disease gene *SLC25A46* that encodes for Ugo1-like protein has been identified in human cells ²⁷⁶. *SLC25A46* protein is located on the OM of mitochondria, connected to Mitofilin/Fcj1, and suggested to take roles in mitochondrial fusion, and thus this protein may potentially be the human counterpart of Ugo1p ²⁷⁶.

Similar to mitochondrial fusion, mitochondrial division machinery is dependent on dynamin-family GTPases. Dnm1p, Drp1 in higher eukaryotes, is mainly responsible for mitochondrial division, mediating membrane dynamics according to cell physiology ²⁷⁷. How the mitochondrial fission machinery works is much less understood in mammalian than that in yeast. However, the resolved crystal structure of mitochondrial fission complex in yeast reveals a requirement for Fis1, Mdv1, and Dnm1 proteins, and involves Caf4p ²⁷⁸. Fis1 is a mitochondrial outer membrane protein, required for Dnm1 recruitment to the OM by the aid of adaptor proteins, Mdv1 or Caf4 ^{279–281}.

An altered balance in mitochondrial fusion- fission cycle, promoting either excessive fusion or fission, can negatively affect cell function and viability. Deletion of essential components in this cycle is possible in yeast due to the fact that mitochondrial function is not essential for growth on glucose-containing media, and that gives us the opportunity to search for new components important in mitochondrial dynamics by performing growth tests on fermentable and non-fermentable media. Although previous studies employed selection for mutants that respire when mitochondrial fusion is inhibited to discover new components in the mitochondrial division machinery ^{279,282}, in the study of Mutlu et al., a unique selection approach was performed on fermentable medium for cells that cannot fuse mitochondria but maintain their mtDNA in a petite-negative background, and respiration was not required ²⁸³. I contributed to the study by generation of some strains, the genotypes of which are provided in Table 4.1.

4.2 Results and Discussion

A novel selection approach for unique components or pathways that impinge on mitochondrial dynamics demonstrated that up-regulation of the Pleiotropic Drug Resistance (PDR) pathway can help cells lacking mitochondrial fusion maintain their mtDNA under certain conditions ²⁸³. However, the mechanism of how PDR activation provides the maintenance of mtDNA when mitochondrial fusion is blocked is still

unknown. In Mutlu *et. al.*, activation of PDR pathway was shown to suppress mtDNA loss only in the petite-negative background of *aac2Δ* ²⁸³. Therefore, either the specific loss of AAC2 or the conditions that make cells unable to survive in the absence of mtDNA triggers the beneficial effects of PDR pathway activation when mitochondrial fusion is blocked. This may suggest a mechanism of protection to maintain the survival of the cell. Actually, since PDR activation could rescue the proliferation defect of *aac2Δ fzo1Δ* cells, understanding the mechanism of PDR activation in these cells may enable us to explain why the mtDNA is lost upon mitochondrial fusion blockage.

4.2.1 Defects in the Mitochondrial-Endoplasmic Reticulum-Cortex Anchor (MECA) do not rescue the growth phenotype of petite-negative cells disrupted in mitochondrial fusion

Num1p is identified in a screen for the genes that become essential in the absence of the mitochondrial fusion and division ²⁸⁴. Later, Num1p was shown to act as an anchor for mitochondria that ensures mitochondrial retention in each cell division. It forms a tether complex including Mdm36p, functioning in mitochondrial distribution ²⁸⁵, and these proteins make a complex called mitochondria-ER-cortex anchor (MECA). MECA associates three organelles to each other: mitochondrion, endoplasmic reticulum and plasma membrane, working in correct distribution of mitochondrial network, structure and position. We tested if deletion of either Num1p or Mdm36p would allow cells keep their mtDNA in the background that we employed in our selection approach that lack mitochondrial fusion and depend on mtDNA for their survival, *fzo1Δ aac2Δ*. However, deletion of neither Num1p nor Mdm36p rescues the proliferation defect of *fzo1Δ aac2Δ* cells ²⁸³. This result suggest that the components that have indirect roles in mitochondrial division, and deletion of which will partially balance mitochondrial fusion-fission in the cells lacking mitochondrial fusion is unlikely to be selected in our selection approach.

Since, the activation of PDR pathway significantly rescues the growth defect of *fzo1Δ aac2Δ* cells; this result strongly supports the close relation of PDR pathway activity in mitochondrial dynamics. Previous studies connecting PDR pathway with mitochondrial function revealed a regulatory mechanism that activates *PDR* genes in response to mtDNA loss ²⁸⁶. Pdr3p, but not Pdr1p, activity is enhanced in cells lacking mtDNA. When Pdr3p

was deleted, the activation of *PDR5* expression in response to mitochondrial signals was repressed²⁸⁶. Loss of the mtDNA induces *PDR5* expression, overexpression of which is triggered by Pdr1p and Pdr3p²⁸⁶. The expression of *PDR5* was demonstrated to be increased in *fzo1Δ* cells, but not in *atp2Δ* cells. *ATP2* deletion results in petite negative phenotype such as *AAC2*. Our results suggest that *PDR1* activation let the *aac2Δfzo1Δ* cells survive. Therefore, there may be differential mitochondrial or cellular signals that separately stimulate Pdr1p and Pdr3p, providing a complex regulatory mechanism that links multiple signaling pathways to mitochondrial dynamics and function.

4.2.2 Severe proliferation defects of *aac2Δ fzo1Δ* cells perturbed in sphingolipid biosynthesis do not favor growth assays to ask if PDR activation leads to increased sphingolipid biosynthesis

A possible mechanism to explain how the activation of PDR pathway enables cells lacking mitochondrial fusion to keep their mtDNA can include a modulation in sphingolipid biosynthesis. The PDR pathway was shown to transcriptionally regulate the level of proteins that function in different steps of sphingolipid biosynthesis in yeast²⁸⁷. Since sphingolipids are important structural components of membranes, it is not surprising that the transcriptional modulation of sphingolipid biosynthesis may alter mitochondrial dynamics. Therefore, we deleted *SUR4* to investigate if enhanced sphingolipid biosynthesis can rescue the proliferation defect of *fzo1Δ aac2Δ* cells. However, *aac2Δ sur4Δ* cells proliferated so slowly that we were not able to perform initial experiments that could possibly reveal connections between PDR pathway, sphingolipid biosynthesis and mitochondrial dynamics²⁸³.

Table 4.1 List of strains used in Chapter 4

Strain	Genotype	Source
CDD744	<i>MATα/MATa ura3-1/ura3-1 trp1Δ2/trp1Δ2 ade2-1/ade2-1 leu2-3,112/leu2-3,112 his3-11,15/his3-11,15 can1-100/can1-100 aac2Δ::URA3/AAC2 sur4Δ::kanMX4/SUR4</i>	283
CDD750	<i>MATa ura3-1 trp1Δ2 ade2-1 leu2-3,112 his3-11,15 can1-100 cyh2 fzo1Δ::HIS3 aac2Δ::URA3 num1Δ::kanMX4 pFZO1-CYH2-TRP1</i>	283

CHAPTER 5

MUTATION OF THE MITOCHONDRIAL LARGE RIBOSOMAL RNA CAN PROVIDE PENTAMIDINE RESISTANCE TO *SACCHAROMYCES CEREVISIAE*

5.1 Introduction

Pentamidine, an aromatic amidine, has been a common drug used to prevent and treat *Pneumocystis jirovecii* or trypanosomatid infections for over than 60 years²⁸⁸. *Pneumocystis pneumonia* (PCP) caused by *Pneumocystis jirovecii* is the most common opportunistic infection observed in people with HIV, and has been the major killer of HIV-infected people for years²⁸⁹. Interestingly, pentamidine exhibits anticancer activities by suppressing the growth of human melanoma cells in mice²⁹⁰, and may suggest a potential therapeutics in cancer treatment. Although its true mechanism of action is still unknown, pentamidine inhibits the topoisomerases of trypanosomes, but does not have much effect on mammalian topoisomerases²⁹¹. Pentamidine accumulates in the mitochondria²⁹², and resistance to the drug is due to the ability to exclude it from the organelle²⁹³. In trypanosomatids, pentamidine was demonstrated to diminish the mitochondrial electrochemical potential²⁹⁴, which may suggest a mechanism of action to disable the target to pump the drug out of the organelle. Moreover, pentamidine-induced mitochondrial dysfunction²⁹¹ and inhibition of mitochondrial translation²⁹⁵ may cause severe side-effects on people under pentamidine treatment²⁹⁶. Identification of its potential mitochondrial targets, thus, may help developing new ways to deal with significant patient toxicity.

An approach to investigate the relevant target(s) of pentamidine would be to recover dominant mutations that provide resistance to *Saccharomyces cerevisiae* when treated with the drug. By this way, we have isolated dominant mutations, one of which was located at the mtDNA, that make yeast cells resistant to pentamidine, and identified the mutation by next generation sequencing. The mutation altered a conserved base at the mitochondrial

large ribosomal RNA. I contributed to the study by performing an assay to test the degree of resistance of the strains which are listed in Table 5.1.

5.2 Results and Discussion

Previous studies on pentamidine provided conditions where proliferation of cells is severely affected ²⁹⁷, and certain dominant alleles were shown to regulate resistance to pentamidine ²⁹⁸. By taking advantage of this, diploid yeast cells that are able to live under the reported conditions were selected. Spontaneously arising dominant mutations were expected to enable cells survive under the selection conditions used. A candidate allele, referred as *PNT3-1*, was not inherited in Mendelian fashion ²⁹⁹, and thus we suspected if the mutation was located at mtDNA. Initial experiments demonstrated that depletion of mtDNA in cells carrying *PNT3-1* allele prevented the inheritance of the resistance to the resulting diploid strain after mating ²⁹⁹.

To clearly show that *PNT3-1* mutation was located to the mtDNA, cytoduction was performed. Cytoduction is a popular tool to determine cytoplasmic characters ³⁰⁰. Cytoduction is the term used for the autonomous cytoplasmic factor transfer from one cell (donor) to another (recipient). In our study, new strains were constructed by transfer of mitochondrial genomes either from WT $\rho+$ or WT $\rho+[PNT3-1]$ strains to a different nuclear background. The resulting strains were tested for their pentamidine resistance.

We observed a higher basal pentamidine resistance in the recipient background (CK01) than the donor background (W303), but it was clear that cells receiving mtDNA from WT $\rho+[PNT3-1]$ strains proliferated far better than cells receiving mtDNA from WT $\rho+$ strains, exhibiting around a two fold increase in pentamidine resistance (Figure 5.1). However, we observed varying results with different cytoductant strains although *PNT3-1* allele receiving cells were clearly more resistant to pentamidine (Akdogan, unpublished data), and the results for the donor strains were not highly consistent as in Figure 5.1. Therefore, we optimized the conditions by using different concentrations of pentamidine and switched to solid medium rather than liquid medium. These results were replicated, and it was shown more clearly that pentamidine resistance was increased two to three fold in the strains cytoduced with mtDNA harboring a *PNT3-1* allele ²⁹⁹.

Next-generation sequencing analysis revealed a single base alteration within the gene encoding the mitochondrial 21S ribosomal RNA of the *PNT3-1* allele carrying strain²⁹⁹. Although our results strongly support a mechanism of action for pentamidine through inhibition of mitochondrial translation, the site that is mutated in *PNT3-1* allele, C1955 in the mitochondrial large ribosomal RNA is not conserved in any trypanosomes affected by the drug²⁹⁹.

Since the true action mechanism of pentamidine is not known, the trypanocidal effect of pentamidine might be due to addition of unspecific actions in the other cellular mechanisms that the drug interferes with rather than inhibition of a single specific target. However, the blockage of mitochondrial translation can also be the ultimate effect that leads to severe defects for the cell, but this inhibition may be caused by different ways in different organisms. First, pentamidine interferes with intron splicing in both *Pneumocystis carinii*³⁰¹ and *Candida albicans*³⁰², against which pentamidine is clinically used. Second, it is possible that direct inhibition of mitochondrial translation at a single base in yeast mitochondrial ribosomal RNA might cause the proximal defect in pentamidine treated cells, since a single mutation is identified in the yeast large mitochondrial RNA that increase resistance against pentamidine. Third, experiments on different *Candida* strains demonstrated that a strain is more susceptible to pentamidine as the number of introns increases³⁰². Still, any of these effects of pentamidine will lead to defects in mitochondrial translation, and thus this may help to explain the adverse effects of pentamidine on patients.

In conclusion, since pentamidine has a broad range of antimicrobial activity, new drug combinations can be provided to patients with the identification of pentamidine targets which will not limit the use of this drug due to its side effects in the future.

5.3 Figure Legend

Figure 5.1 Transfer of *PNT3-1* mtDNA to another nuclear background by cytoduction increases pentamidine resistance in the recipient background. Although the CK01 recipient nuclear background is more resistant to pentamidine than the W303 nuclear background, pentamidine resistance is demonstrated to further increase following *PNT3-*

1 mtDNA transfer via cytoduction. WT cells (W303 $\rho^{+[WT]}$, CDD725), cells carrying *PNT3-1* mutation (W303 $\rho^{+[PNT3-1]}$, CDD726), cells receiving W303 mitochondria after cytoduction (CK01 $\rho^{+[WT]}$, CDD727) and cells receiving W303 mitochondria with *PNT3-1* mutation after cytoduction (CK01 $\rho^{+[PNT3-1]}$, CDD728) were grown overnight in non-fermentable medium (YEPGE) with different pentamidine concentrations (0, 0.2, 0.5, 1, 2, 5, 10 $\mu\text{g/ml}$), starting at 0.01 OD₆₀₀ unit. On the following day, their proliferation rate was tracked by measuring OD₆₀₀ with 150 min intervals (data not shown), and approximately 24 hours later their survival ratio is calculated, which is provided in the plots.

5.4 Figure

Figure 5.1 Transfer of *PNT3-1* mtDNA to another nuclear background by cytoduction increases pentamidine resistance in the recipient background.

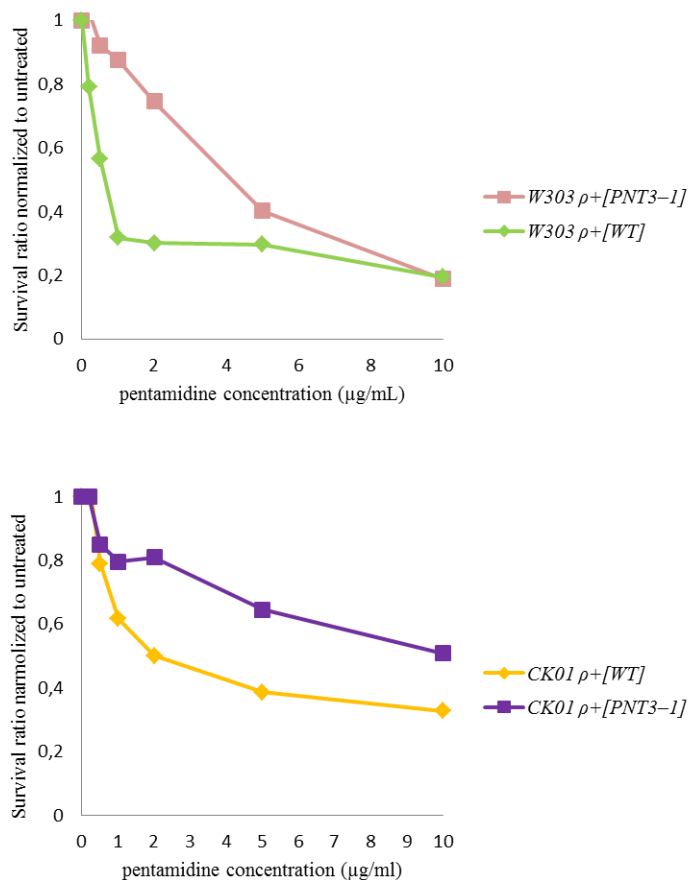


Table 5.1 List of strains used in Chapter 5

Strain	Genotype	Source
CDD725	<i>MATα ura3-1 trp1Δ2 ade2-1 leu2-3,112 his3-11,15 can1-100 ρ⁺</i>	299
CDD726	<i>MATα ura3-1 trp1Δ2 ade2-1 leu2-3,112 his3-11,15 can1-100 ρ⁺[PNT3-1]</i>	299
CDD727	<i>MATα kar1-1 leu1 can^R cyh2 ρ⁺</i>	299
CDD728	<i>MATα kar1-1 leu1 can^R cyh2 ρ⁺[PNT3-1]</i>	299

CHAPTER 6

UNDERSTANDING THE MECHANISMS OF MITOCHONDRIAL ARCHITECTURE AND FUNCTION

6.1 Introduction

Mitochondria are double membrane bound organelles, which are termed as mitochondrial inner membrane (IM) and outer membrane (OM) as reviewed in Chapter 2. The IM is further divided into two domains, inner boundary membrane (IBM) and cristae membrane, where the respiratory chain complexes forming large super complexes and F_1F_0 -ATP synthase are located. Cristae and IBM are physically separated by crista junctions (CJ). Recently, a large inner membrane complex, mitochondrial contact site and cristae organizing system, MICOS (also called MitOS or MINOS) has been discussed in separate publications^{201,303,304}. The complex is made of Fcj1/ Mitofilin (with new nomenclature Mic 60, MINOS2), Mos1 (with new nomenclature Mic10, Mio10, MINOS1), and at least four more proteins³⁰⁵. The MICOS complex contributes to tethering of cristae to OM, shaping of the CJs, membrane trafficking, regulation of the fusion of cristae with IBM. This complex is of central importance for the regulation of mitochondrial dynamics, biogenesis and their relation to mitochondrial architecture^{263,304}.

As summarized in Chapter 4, mitochondrial fusion–fission cycle is an ongoing process in cells, and mitochondrial morphology is controlled by a balance of these two events. It was reported that simultaneous deletion of Dnm1p, major component of the mitochondrial division machinery, and Mos1p slows down the proliferation rate of yeast, and *mos1Δ dnm1Δ* cells barely grow on non-fermentable medium although single deletion of either *DNM1* or *MOS1* does not affect the viability of the cells³⁰⁶. Both Fcj1p and Mos1p are required for the maintenance of CJs and may be involved in mtDNA nucleoid distribution and function³⁰⁶. However, the significantly limited viability of *mos1Δ dnm1Δ* cells, in contrast to *fcj1Δ dnm1Δ* cells may suggest a unique role for Mos1p in mitochondrial

fusion-fission cycle, which links mitochondrial dynamics and mitochondrial architecture through a component of the MICOS complex.

6.2 Results

6.2.1 Separate deletions of the fusion components in *mos1Δdnm1Δ* cells resulted in varying effects on the growth rate

If mitochondrial dysfunction and poor proliferation in *mos1Δdnm1Δ* is because of fusion-fission cycle disruption, the deletion of a fusion component, Mgm1p, Fzo1p or Ugo1p, is expected to rescue the growth defect on non-fermentable media. Deletions of the fusion components, *MGM1* or *FZO1* resulted in different degrees of rescue phenotypes, while *UGO1* deletion further decreased the growth rate on non-fermentable media, and even on fermentable medium (Figure 6.1 A, B). We also tested the effect of the deletion of another component Pcp1p/Ugo2p in *mos1Δdnm1Δ* cells, which is required for Mgm1p biogenesis, and cells lacking Pcp1p/Ugo2p were shown to partially fuse their mitochondria³⁰⁷. Deletion of *UGO2* has partially increased the proliferation rate of *mos1Δdnm1Δ* cells, which may associate their proliferation defect to destroyed fusion-fission cycle (Figure 6.1 B). We then asked whether increased growth defect of *dnm1Δmos1Δugo1Δ* is really because of the deletion of *UGO1* in *mos1Δdnm1Δ* cells or the acquisition of another mutation made the cells so sick by chance events. Therefore, we compared the growth rates of different isolates which are confirmed to be *mos1Δdnm1Δugo1Δ*. The result clearly shows that *mos1Δ dnm1Δ ugo1Δ* cells have a significantly slower growth rate on both fermentable and non-fermentable media (Figure 6.2).

6.3 Discussion

6.3.1 Ugo1p loss results in severe proliferation defect in *dnm1Δmos1Δ* cells

Surprisingly, *dnm1Δ mos1Δ ugo1Δ* cells have significantly reduced growth rate even on fermentable media compared to *dnm1Δ*, *dnm1Δmos1Δ*, *dnm1Δmos1Δmgm1Δ*, *dnm1Δmos1Δfzo1Δ* and their growth is further restricted on fermentable media (Figure 6.1

A, B). This result strongly suggests that Ugo1p deletion alters cell physiology or mitochondrial morphology independent of mitochondrial fusion, suggesting a role for Ugo1p other than simply functioning as an adaptor that links Fzo1p and Mgm1p. Distinct regions of Ugo1p directly bind to Fzo1p and Mgm1p, and then Ugo1p dimerizes to link these two GTPases in mitochondrial fusion^{308,309}.

Ugo1 is a mitochondrial transport family protein with no characterized role in membrane trafficking. Ugo1p contains putative carrier domains (PCDs) like mitochondrial carrier proteins (MCPs) such as the ADP/ATP carrier (Aac2p). For proper transport of mitochondrial carrier proteins, a consensus sequence in PCDs is required. Mutations in one of the two PCDs of Ugo1p were shown to disrupt mitochondrial fusion³¹⁰, indicating that the other domain might have a different function than being involved in fusion. Moreover, recent studies found additional transmembrane domains of Ugo1p³¹⁰ although it was long believed to be a single-pass transmembrane protein. Therefore, Ugo1p may be assigned different functions besides being involved in fusion, one of which could affect modulation of the import of proteins into mitochondria.

There are instances of proteins involved in MICOS, like Fcj1p, having dual roles in mitochondrial function. First, Fcj1p/Mitofilin interacts with the translocase of the outer membrane β -barrel proteins (TOB)/sorting, assembly machinery (SAM) complex and Ugo1p, providing a physical contact to the OM³¹¹. Also, it was shown that the TOB/SAM complex affects cristae morphology and CJ formation³¹¹. Based on this knowledge, it is strongly speculated that the interaction of the C-terminus of Fcj1p/Mitofilin with the TOB/SAM complex provides a mechanism for how CJs are located in the mitochondrial structure. Second, Fcj1p/ Mitofilin is reported to promote protein import by recruiting Mia40p (IM import receptor) via MIA (mitochondrial IMS assembly) pathway, independent of its role in mitochondrial membrane organization³⁰³. Furthermore, a mammalian study demonstrated that SAM mutants have abnormal cristae morphology³¹². Therefore, we could conclude that proteins functioning in IM structure and in cell trafficking are linked to each other. A similar concerted action of Ugo1p and Mos1p might further integrate proteins functioning in fusion and in mitochondrial transport as a large complex, and is worthy of further investigation.

MICOS subunits including Mos1p are integrated in or associated with the IM. The MICOS complex is made of sub-complexes that independently localize to CJ and are

connected via adaptor proteins, and absences of MICOS sub-complexes rather than individual MICOS subunits cause defects in cells ³¹³. Moreover, they have contact sites that anchor MICOS and the CJs to the OM. In yeast, Fcj1/Mitofilin containing complexes of IM are reported to interact with the OM complexes such as SAM, TOM, porin and Ugo1p ^{304,308,311}. The growth defect that we observed, however, is unlikely to be due to the prevention of Fcj1p-Ugo1p interaction, since there are also other interactions with the OM. Therefore, Ugo1p might be an essential component of the MICOS complex. Any possible genetic or physical interaction between Ugo1p and Mos1p needs to be examined further. These studies may assign unique functions for Ugo1p working with Mos1p as a sub-complex in keeping membranes together and supplying cristae stability, simultaneous deletion of which could result in severe mitochondrial morphological defects.

6.3.2 *FZO1* deletion partially rescue the proliferation defect in *dnm1Δmos1Δ* cells

Although we haven't seen a significant growth defect in *dnm1Δmos1Δ* cells in contrast to the reported proliferation inhibition ³⁰⁶, we observed that deletion of fusion components, Fzo1p or Mgm1p, is beneficial for *dnm1Δmos1Δ* cells (Figure 6.1 A,B). It should also be noted here that we think simultaneous deletion of *DNM1* and *MOS1* in a different background used in the study of Itoh et al. is likely to be the reason for varying results ³⁰⁶. However, we observed that the rescue of *dnm1Δmos1Δfzo1Δ* is partial compared to *dnm1Δmos1Δmgm1Δ* (Figure 6.1 A). One explanation could be that Fzo1p deleted cells could still fuse their mitochondria, which will be highly unexpected. Although the growth defect of cells lacking division components can be rescued by *FZO1* deletion ³¹⁴, it cannot be concluded that mitochondrial fusion is completely blocked in *fzo1Δ* cells. Moreover, Fzo1p is suggested to be a main component of fusion, deletion of which blocks mitochondrial fusion, depending on microscopy experiments ²⁶⁸. Any mitochondrial fusion event that goes on in the cells in the absence of Fzo1p is extremely difficult to detect with colocalization experiments. A novel genetic approach that will enable us to see if the mitochondria of two cells fuse when *FZO1* is deleted will prove if mitochondrial fusion is completely blocked in *fzo1Δ* cells, which will enable us to correctly interpret the results that we obtained.

6.4 Figure Legends

Figure 6.1 Deletion of *mgm1Δ*, but not *fzo1Δ* or *ugo1Δ* completely rescues the growth defect of *dnm1Δ mos1Δ* cells. (A) For spot tests, CDD729 (*dnm1Δ mos1Δ*), CDD732 (*dnm1Δ*), CDD733 (*dnm1Δ mos1Δ mgm1Δ*), CDD738 (*dnm1Δ mos1Δ fzo1Δ*), CDD742 (*dnm1Δ mos1Δ ugo1Δ*) were grown to logarithmic growth phase and diluted to an OD at 600 nm of 0.1, then further diluted in 10 fold increments and 4 μl of each dilution spotted onto appropriate medium. (2 days of incubation for YEPD, 4 days of incubation for YEPGE at 30 °C) (B) For streak test, 4μL of overnight culture of CDD729 (*dnm1Δ mos1Δ*), CDD732 (*dnm1Δ*), CDD733 (*dnm1Δ mos1Δ mgm1Δ*), CDD737 (*dnm1Δ mos1Δ ugo2Δ*), CDD738 (*dnm1Δ mos1Δ fzo1Δ*) or CDD742 (*dnm1Δ mos1Δ ugo1Δ*) was streaked on non-fermentable media (3 days of incubation at 30 °C). YEPGE: non-fermentable media, YEPD: fermentable media.

Figure 6.2 *dnm1Δ mos1Δ ugo1Δ* cells have dramatically reduced growth rate on both fermentable and non-fermentable medium. CDD729 (*dnm1Δ mos1Δ*), CDD732 (*dnm1Δ*), CDD742 (*dnm1Δ mos1Δ ugo1Δ*, isolate #1) and a separately isolated *dnm1Δ mos1Δ ugo1Δ* strain (*dnm1Δ mos1Δ ugo1Δ*, isolate #2) were grown to logarithmic growth phase and diluted to an OD at 600 nm of 0.1, then further diluted in 10 fold increments and 4 μl of each dilution spotted onto appropriate medium (2 days of incubation on YEPD, 6 days of incubation on YEPGE at 30°C). YEPGE: non-fermentable media, YEPD: fermentable media.

6.5 Figures

Figure 6.1 Deletion of *mgm1Δ*, but not *fzo1Δ* or *ugo1Δ* completely rescues the growth defect of *dnm1Δ mos1Δ* cells.

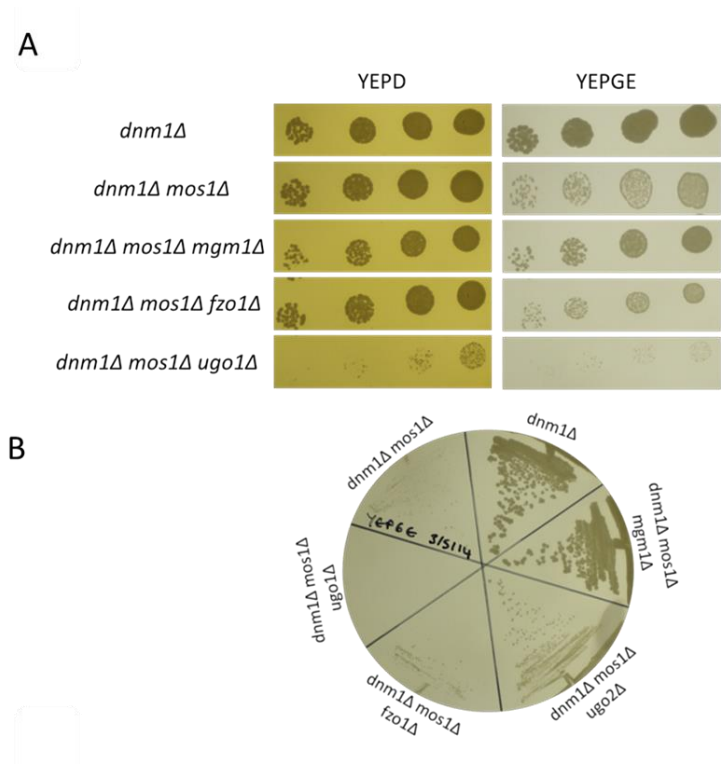


Figure 6.2 *dnm1Δ mos1Δ ugo1Δ* cells have dramatically reduced growth rate on both fermentable and non-fermentable medium.

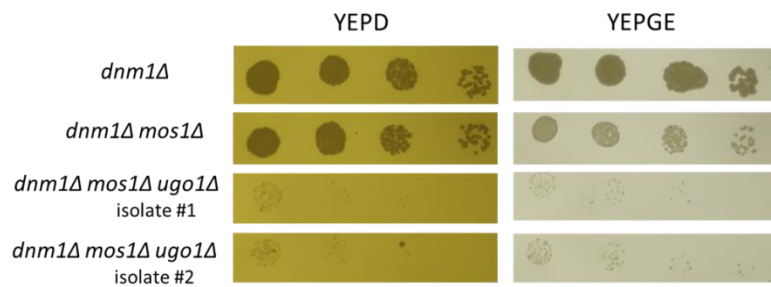


Table 6.1 List of strains used in Chapter 6

Strain	Genotype	Source	Parental strain(s)	Method Used
RJ867	<i>MATa leu2Δ0 met15Δ0 ura3Δ0 his3Δ200 trp1Δ63</i>	75		
RJ868	<i>MATa leu2Δ0 met15Δ0 ura3Δ0 his3Δ200 trp1Δ63</i>	75		
CDD65	<i>MATa his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0 cyh2</i>	Constructed by Dr. C. Dunn		RJ867 mutated to <i>cyh2</i> selected on 10 mg/L CHX after transformation with <i>cyh2</i> PCR product
CDD66	<i>MATa his3Δ200 leu2Δ0 met15Δ0 trp1Δ63 ura3Δ0 cyh2</i>	Constructed by Dr. C. Dunn		RJ868 selected on 10 mg/L CHX after transformation with <i>cyh2</i> PCR product
CDD75	<i>MATa/MATa his3Δ200/his3Δ200 leu2Δ0/leu2Δ0 met15Δ0/met15Δ0 trp1Δ63/trp1Δ63 ura3Δ0/ura3Δ0 cyh2/cyh2</i>	Constructed by Dr. C. Dunn	CDD65, CDD66	CDD65 crossed with CDD66
CDD102	<i>MATa/MATa his3Δ200/his3Δ200 leu2Δ0/leu2Δ0 met15Δ0/met15Δ0 trp1Δ63/trp1Δ63 ura3Δ0/ura3Δ0 cyh2/cyh2 dnm1Δ::URA3/DNM1</i>	This study	CDD75	<i>DNM1</i> disrupted with <i>URA3</i> in CDD75 using primers 38/39 and pRS306 template
CDD115	<i>MATa/MATa his3Δ200/his3Δ200 leu2Δ0/leu2Δ0 met15Δ0/met15Δ0</i>	This study	CDD102	<i>MGM1</i> disrupted with <i>HIS3</i> in CDD102 using primers 40/42 and pRS303 template

	<i>trp1Δ63/trp1Δ63</i> <i>ura3Δ0/ura3Δ0 cyh2/cyh2</i> <i>dnm1Δ::URA3/DNM1</i> <i>mgm1Δ::HIS3/MGM1</i>			
CDD715	<i>MATα/MATα</i> <i>dnm1Δ::LEU2/DNM1</i> <i>mgm1Δ::HIS3/MGM1</i> <i>mos1Δ::kanMX/MOS1</i> <i>cyh2/cyh2</i>	This study	CDD115	<i>MOS1</i> disrupted with <i>kanMX4</i> in CDD115 with primers 552/553 using plasmid b81 (pRS306K)
CDD729	<i>MATα dnm1Δ::LEU2 cyh2</i> <i>mos1Δ::kanMX4</i>	This study	CDD715	Sporulation
CDD732	<i>MATα dnm1Δ::LEU2 cyh2</i>	This study	CDD715	Sporulation
CDD733	<i>MATα dnm1Δ::LEU2 cyh2</i> <i>mos1Δ::kanMX4</i> <i>mgm1Δ::HIS3</i>	This study	CDD715	Sporulation
CDD737	<i>MATα dnm1Δ::LEU2 cyh2</i> <i>mos1Δ::kanMX4</i> <i>ugo2Δ::HIS3</i>	This study	CDD729	<i>UGO2</i> disrupted with <i>HIS3</i> in CDD729 using primers 575/574 and template pRS303
CDD738	<i>MATα dnm1Δ::LEU2 cyh2</i> <i>fzo1Δ::HIS3 mos1Δ::kanMX</i>	This study	CDD729	<i>FZO1</i> disrupted in CDD729 with <i>HIS3</i> using primers 30/31 and template pRS303
CDD742	<i>MATα dnm1Δ::LEU2</i> <i>ugo1Δ::HIS3</i> <i>mos1Δ::kanMX4 cyh2</i>	This study	CDD729	<i>UGO1</i> disrupted in CDD729 with <i>HIS3</i> using primers 571/572 and template pRS303

(Information for the plasmids used as templates in PCR reactions can be obtained from Table 3.2)

Table 6.2 List of oligonucleotides used in Chapter 6

Number	Sequence
30	ATGTCTGAAGGAAAACAACAATTCAAAGACAGCAATAAACAGATTGTACTGAGAGTGCAC
31	CTAATCGATGTCTAAATTTATTTCTTCCACCATCAATTTTCTGTGCGGTATTTACACCG
38	ATGGCTAGTTTAGAAGATCTTATTCCTACTGTCAACAAGCAGATTGTACTGAGAGTGCAC
39	TTACAGAATATTACTAATAAGGGTTGCAGCCTTTTTATAACTGTGCGGTATTTACACCG
40	ATGAATGCGAGCCCAGTACGGCTTTTAATTCTGAGAAGACAGATTGTACTGAGAGTGCAC
42	GGGCTCGAGGTGTCAGTAAATAACAGAG
552	CTACGAGAGGGAATAAACACGGAAAAAGACAAAATATACCAGATTGTACTGAGAGTGCAC
553	GAATATATATAAAGCATCGTCGCTTAAGACTAAACCTTCGCTGTGCGGTATTTACACCG
571	TTTTTGTTTCCAATAGTATAGGTTTAACTCAACCCCCCAAGATTGTACTGAGAGTGCAC
572	CCATGGGCGAACATAAAAAAAAAAATGGGGGACTATCCCAGTCTGTGCGGTATTTACACCG
574	ATACAGACAGTGAAAAGTAATAAATTGCCCAAGTGTGAACAGATTGTACTGAGAGTGCAC
575	TTACAATATTTTGAATGAGAATGTGTCTTTTTTTAAGTGACTGTGCGGTATTTACACCG

BIBLIOGRAPHY

1. Horvath, S. E. & Daum, G. Lipids of mitochondria. *Prog. Lipid Res.* **52**, 590–614 (2013).
2. Lill, R. *et al.* The role of mitochondria in cellular iron-sulfur protein biogenesis and iron metabolism. *Biochimica et Biophysica Acta - Molecular Cell Research* **1823**, 1491–1508 (2012).
3. Saraste, M. Oxidative Phosphorylation at the fin de siècle. *Science (80-.).* **283**, 1488–93 (1999).
4. Gray, M., Burger, G. & Lang, B. F. The origin and early evolution of mitochondria. *Genome Biol.* **2**, reviews1018.1–reviews1018.5 (2001).
5. Lang, B. F., Gray, M. W. & Burger, G. Mitochondrial genome evolution and the origin of eukaryotes. *Annu. Rev. Genet.* **33**, 351–397 (1999).
6. Hauser, D. N. & Hastings, T. G. Mitochondrial dysfunction and oxidative stress in Parkinson's disease and monogenic parkinsonism. *Neurobiology of Disease* **51**, 35–42 (2013).
7. Gu, X. M., Huang, H. C. & Jiang, Z. F. Mitochondrial dysfunction and cellular metabolic deficiency in Alzheimer's disease. *Neuroscience Bulletin* **28**, 631–640 (2012).
8. Payne, B. A. I. I. & Chinnery, P. F. Mitochondrial dysfunction in aging: Much progress but many unresolved questions. *Biochim. Biophys. Acta - Bioenerg.* (**in press**), (2015).
9. Chen, H. & Chan, D. C. Mitochondrial dynamics-fusion, fission, movement, and mitophagy-in neurodegenerative diseases. *Hum. Mol. Genet.* **18**, (2009).
10. Westermann, B. Mitochondrial fusion and fission in cell life and death. *Nat. Rev. Mol. Cell Biol.* **11**, 872–884 (2010).
11. Wallace, D. C., Fan, W. & Procaccio, V. Mitochondrial energetics and therapeutics. *Annu. Rev. Pathol.* **5**, 297–348 (2010).

12. Chen, X. J. & Clark-Walker, G. D. The petite mutation in yeast: 50 years on. *Int. Rev. Cytol.* **194**, 197–237 (1999).
13. Whittaker, P. A. The petite mutation in yeast. *Subcell. Biochem.* **6**, 175–232 (1979).
14. Contamine, V. & Picard, M. Maintenance and integrity of the mitochondrial genome: a plethora of nuclear genes in the budding yeast. *Microbiol. Mol. Biol. Rev.* **64**, 281–315 (2000).
15. Chen, X. J. & Clark-Walker, G. D. Alpha and beta subunits of F1-ATPase are required for survival of petite mutants in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.* **262**, 898–908 (1999).
16. Garipler, G., Mutlu, N., Lack, N. & Dunn, C. D. Deletion of conserved protein phosphatases reverses defects associated with mitochondrial DNA damage in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 1473–8 (2014).
17. Vandecasteele, G., Szabadkai, G. & Rizzuto, R. Mitochondrial calcium homeostasis: mechanisms and molecules. *IUBMB Life* **52**, 213–219 (2001).
18. Ajioka, R. S., Phillips, J. D. & Kushner, J. P. Biosynthesis of heme in mammals. *Biochim. Biophys. Acta - Mol. Cell Res.* **1763**, 723–736 (2006).
19. Osman, C., Voelker, D. R. & Langer, T. Making heads or tails of phospholipids in mitochondria. *J. Cell Biol.* **192**, 7–16 (2011).
20. Gray, M. W. Mitochondrial evolution. *Cold Spring Harb. Perspect. Biol.* **4**, (2012).
21. Adams, K. L., Daley, D. O., Qiu, Y. L., Whelan, J. & Palmer, J. D. Repeated, recent and diverse transfers of a mitochondrial gene to the nucleus in flowering plants. *Nature* **408**, 354–357 (2000).
22. Thorsness, P. E. & Fox, T. D. Nuclear mutations in *Saccharomyces cerevisiae* that affect the escape of DNA from mitochondria to the nucleus. *Genetics* **134**, 21–28 (1993).
23. Wallace, D. C. Mitochondrial DNA mutations in disease and aging. *Environmental and Molecular Mutagenesis* **51**, 440–450 (2010).
24. Foury, F., Roganti, T., Lecrenier, N. & Purnelle, B. The complete sequence of the mitochondrial genome of *Saccharomyces cerevisiae*. *FEBS Lett.* **440**, 325–331 (1998).
25. Vries, S. & Grivell, L. A. Purification and characterization of a rotenone-insensitive NADH: Q6 oxidoreductase from mitochondria of *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **176**, 377–384 (1988).

26. Devenish, R. J., Prescott, M. & Rodgers, A.J. The structure and function of mitochondrial F₁F₀-ATP synthases. *Int Rev Cell Mol Biol.* **267**, 1–58 (2008). doi:10.1016/S1937-6448(08)00601-1
27. Adachi, K. *et al.* Coupling of Rotation and Catalysis in F₁-ATPase Revealed by Single-Molecule Imaging and Manipulation. *Cell* **130**, 309–321 (2007).
28. Campanella, M., Parker, N., Tan, C. H., Hall, A. M. & Duchen, M. R. IF1: setting the pace of the F₁F₀-ATP synthase. *Trends in Biochemical Sciences* **34**, 343–350 (2009).
29. Rooney, J. P. *et al.* PCR based determination of mitochondrial DNA copy number in multiple species. *Methods Mol Biol.* **1241**, 23–38 (2015). doi:10.1007/978-1-4939-1875-1_3
30. Copeland, W. C. The mitochondrial DNA polymerase in health and disease. *Subcell. Biochem.* **50**, 211–222 (2010).
31. Osman, C., Noriega, T. R., Okreglak, V., Fung, J. C. & Walter, P. Integrity of the yeast mitochondrial genome, but not its distribution and inheritance, relies on mitochondrial fission and fusion. *Proc. Natl. Acad. Sci.* **112**, E947–E956 (2015). doi:10.1073/pnas.1501737112
32. Ruiz-Pesini, E. *et al.* An enhanced MITOMAP with a global mtDNA mutational phylogeny. *Nucleic Acids Res.* **35**, (2007).
33. Dianov, G. L. *et al.* Base excision repair in nuclear and mitochondrial DNA. *Prog Nucleic Acid Res Mol Biol* **68**, 285–297 (2001).
34. Tuppen, H. A. L., Blakely, E. L., Turnbull, D. M. & Taylor, R. W. Mitochondrial DNA mutations and human disease. *Biochim. Biophys. Acta* **1797**, 113–128 (2010).
35. Wong, L. J. C. Diagnostic challenges of mitochondrial DNA disorders. *Mitochondrion* **7**, 45–52 (2007).
36. Finsterer, J. Leigh and Leigh-Like Syndrome in Children and Adults. *Pediatric Neurology* **39**, 223–235 (2008).
37. Menezes, M. P. & Ouvrier, R. A. Peripheral neuropathy associated with mitochondrial disease in children. *Dev. Med. Child Neurol.* **54**, 407–414 (2012).
38. Chinnery, P. F. *et al.* Risk of developing a mitochondrial DNA deletion disorder. *Lancet* **364**, 592–596 (2004).
39. El-Hattab, A. W. & Scaglia, F. Mitochondrial DNA Depletion Syndromes: Review and Updates of Genetic Basis, Manifestations, and Therapeutic Options. *Neurotherapeutics* **10**, 186–198 (2013).

40. Saneto, R. P., Cohen, B. H., Copeland, W. C. & Naviaux, R. K. Alpers-huttenlocher syndrome. *Pediatric Neurology* **48**, 167–178 (2013).
41. Harman, D. Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* **11**, 298–300 (1956).
42. Trifunovic, A. *et al.* Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* **429**, 417–423 (2004).
43. Kujoth, G. C. *et al.* Mitochondrial DNA mutations, oxidative stress, and apoptosis in mammalian aging. *Science* (80-.). **309**, 481–4 (2005).
44. Boveris, A. & Navarro, A. Brain mitochondrial dysfunction in aging. *IUBMB Life* **60**, 308–314 (2008).
45. Kraytsberg, Y. *et al.* Mitochondrial DNA deletions are abundant and cause functional impairment in aged human substantia nigra neurons. *Nat. Genet.* **38**, 518–520 (2006).
46. Ye, K., Lu, J., Ma, F., Keinan, A. & Gu, Z. Extensive pathogenicity of mitochondrial heteroplasmy in healthy human individuals. *Proc. Natl. Acad. Sci.* **111**, 10654–10659 (2014). doi:10.1073/pnas.1403521111
47. Parikh, S. *et al.* A modern approach to the treatment of mitochondrial disease. *Current Treatment Options in Neurology* **11**, 414–430 (2009).
48. Pfeiffer, G. *et al.* New treatments for mitochondrial disease-no time to drop our standards. *Nat. Rev. Neurol.* **9**, 474–81 (2013).
49. Mollet, J. *et al.* Prenyldiphosphate synthase, subunit 1 (PDSS1) and OH-benzoate polyprenyltransferase (COQ2) mutations in ubiquinone deficiency and oxidative phosphorylation disorders. *J. Clin. Invest.* **117**, 765–772 (2007).
50. Glover, E. I. *et al.* A randomized trial of coenzyme Q10 in mitochondrial disorders. *Muscle and Nerve* **42**, 739–748 (2010).
51. Koga, Y. *et al.* Effects of L-arginine on the acute phase of strokes in three patients with MELAS. *Neurology* **58**, 827–828 (2002).
52. Tarnopolsky, M. A., Roy, B. D. & MacDonald, U. R. A randomized, controlled trial of creatine monohydrate in patients with mitochondrial cytopathies. *Muscle and Nerve* **20**, 1502–1509 (1997).
53. Shoffner, J. M. *et al.* Spontaneous Kearns-Sayre/chronic external ophthalmoplegia plus syndrome associated with a mitochondrial DNA deletion: a slip-replication model and metabolic therapy. *Proceedings of the National Academy of Sciences of the United States of America* **86**, 7952–7956 (1989).

54. Ephrussi, B. Action de l'acriflavine sur les levures .I. La mutation petite colonie. *Ann. Inst. Pasteur* **76**, 351–367 (1949).
55. Ephrussi, B. Action de l'acriflavine sur les levures. II. Etude genetique du mutant. *Ann. Inst. Pasteur* **76**, 419–450 (1949).
56. Tavlitzki, J. Action de l'acriflavine sur les levures. III. Etude de la croissance des mutants 'petite colonie.'. *Ann. Inst. Pasteur* **76**, 498–509 (1949).
57. Slonimski, P. Action de l'acriflavine sur les levures. IV. Mode d'utilisation du glucose par les mutants petite colonie. *Ann. Inst. Pasteur* **76**, 510–530 (1949).
58. Slonimski, P. & Ephrussi, B. Action de l'acriflavine sur les levures. V. Le systeme des cytochromes des mutants 'petite colonie'. *Ann. Inst. Pasteur* **77**, 47–63 (1949).
59. Ephrussi, B. & Hottinguer, H. Direct Demonstration of the Mutagenic Action of Euflavine on Baker's Yeast. *Nature* **166**, 956–956 (1950).
60. Ephrussi, B. & Hottinguer, E. Cytoplasmic constituents of Heredity. On an unstable cell state in yeast. *Cold Spring Harb. Symp. Quant. Biol.* **16**, 75–85 (1951).
61. Ephrussi, B., de Margerie-Hottinguer, H. & Roman, H. Suppressiveness: A New Factor in the Genetic Determinism of the Synthesis of Respiratory Enzymes in Yeast. *Proc. Natl. Acad. Sci. U. S. A.* **41**, 1065–1071 (1955).
62. Schatz, G., Haslbrunner, E. & Tuppy, H. Deoxyribonucleic acid associated with yeast mitochondria. *Biochem. Biophys. Res. Commun.* **15**, 127–132 (1964).
63. Corneo, G., Moore, C., Sanadi, D. R., Grossman, L. I. & Marmur, J. Mitochondrial DNA in yeast and some mammalian species. *Science* **151**, 687–689 (1966).
64. Mounolou, J. C., Jakob, H. & Slonimski, P. P. Mitochondrial DNA from yeast 'petite' mutants: specific changes in buoyant density corresponding to different cytoplasmic mutations. *Biochem. Biophys. Res. Commun.* **24**, 218–224 (1966).
65. Tewari, K. K., Vötsch, W., Mahler, H. R. & Mackler, B. Biochemical correlates of respiratory deficiency. VI. Mitochondrial DNA. *J. Mol. Biol.* **20**, 453–481 (1966).
66. Goldring, E. S., Grossman, L. I., Krupnick, D., Cryer, D. R. & Marmur, J. The petite mutation in yeast. Loss of mitochondrial deoxyribonucleic acid during induction of petites with ethidium bromide. *J. Mol. Biol.* **52**, 323–335 (1970).
67. Drgon, T., Sabová, L., Nelson, N. & Kolarov, J. ADP/ATP translocator is essential only for anaerobic growth of yeast *Saccharomyces cerevisiae*. *FEBS Lett.* **289**, 159–162 (1991).

68. Kováčová, V., Irmířerová, J. & Kováč, L. Oxidative phosphorylation in yeast. IV. Combination of a nuclear mutation affecting oxidative phosphorylation with cytoplasmic mutation to respiratory deficiency. *Biochim. Biophys. Acta - Bioenerg.* **162**, 157–163 (1968).
69. Kolarov, J., Kolarova, N. & Nelson, N. A third ADP/ATP translocator gene in yeast. *J. Biol. Chem.* **265**, 12711–12716 (1990).
70. Dupont, C. H., Mazat, J. P. & Guerin, B. The role of adenine nucleotide translocation in the energization of the inner membrane of mitochondria isolated from rho + and rho degree strains of *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **132**, 1116–1123 (1985).
71. Campbell, C. L., Tanaka, N., White, K. H. & Thorsness, P. E. Mitochondrial morphological and functional defects in yeast caused by yme1 are suppressed by mutation of a 26S protease subunit homologue. *Mol. Biol. Cell* **5**, 899–905 (1994).
72. Campbell, C. L. & Thorsness, P. E. Escape of mitochondrial DNA to the nucleus in yme1 yeast is mediated by vacuolar-dependent turnover of abnormal mitochondrial compartments. *J. Cell Sci.* **111**, 2455–2464 (1998).
73. Dunn, C. D., Lee, M. S., Spencer, F. A. & Jensen, R. E. A genome wide screen for petite-negative yeast strains yields a new subunit of the i-AAA protease complex. *Mol. Biol. Cell* **17**, 213–226 (2006).
74. Dunn, C. D., Tamura, Y., Sesaki, H. & Jensen, R. E. Mgr3p and Mgr1p are adaptors for the mitochondrial i-AAA protease complex. *Mol. Biol. Cell* **19**, 5387–5397 (2008).
75. Dunn, C. D. & Jensen, R. E. Suppression of a defect in mitochondrial protein import identifies cytosolic proteins required for viability of yeast cells lacking mitochondrial DNA. *Genetics* **165**, 35–45 (2003).
76. Senapin, S., Chen, X. J. & Clark-Walker, G. D. Transcription of TIM9, a new factor required for the petite-positive phenotype of *Saccharomyces cerevisiae*, is defective in spt7 mutants. *Curr. Genet.* **44**, 202–210 (2003).
77. Kerscher, O., Sepuri, N. B. & Jensen, R. E. Tim18p is a new component of the Tim54p-Tim22p translocon in the mitochondrial inner membrane. *Mol. Biol. Cell* **11**, 103–116 (2000).
78. Weber, E. R., Rooks, R. S., Shafer, K. S., Chase, J. W. & Thorsness, P. E. Mutations in the mitochondrial ATP synthase gamma subunit suppress a slow-growth phenotype of yme1 yeast lacking mitochondrial DNA. *Genetics* **140**, 435–442 (1995).

79. Kominsky, D. J., Brownson, M. P., Updike, D. L. & Thorsness, P. E. Genetic and biochemical basis for viability of yeast lacking mitochondrial genomes. *Genetics* **162**, 1595–1604 (2002).
80. Kominsky, D. J. & Thorsness, P. E. Expression of the *Saccharomyces cerevisiae* gene YME1 in the petite-negative yeast *Schizosaccharomyces pombe* converts it to petite-positive. *Genetics* **154**, 147–154 (2000).
81. Clark-Walker, G. D., Hansbro, P. M., Gibson, F. & Chen, X. J. Mutant residues suppressing $\rho 0$ -lethality in *Kluyveromyces lactis* occur at contact sites between subunits of F1-ATPase. *Biochim. Biophys. Acta - Protein Struct. Mol. Enzymol.* **1478**, 125–137 (2000).
82. Lai-Zhang, J. & Mueller, D. M. Complementation of deletion mutants in the genes encoding the F1-ATPase by expression of the corresponding bovine subunits in yeast *S. cerevisiae*. *Eur. J. Biochem.* **267**, 2409–2418 (2000).
83. Smith, C. P. & Thorsness, P. E. Formation of an energized inner membrane in mitochondria with a γ -deficient F1-ATPase. *Eukaryot. Cell* **4**, 2078–2086 (2005).
84. Dean, S., Gould, M. K., Dewar, C. E. & Schnauffer, A. C. Single point mutations in ATP synthase compensate for mitochondrial genome loss in trypanosomes. *Proc. Natl. Acad. Sci. U. S. A.* **110**, 14741–6 (2013).
85. Schnauffer, A., Clark-Walker, G. D., Steinberg, A. G. & Stuart, K. The F1-ATP synthase complex in bloodstream stage trypanosomes has an unusual and essential function. *EMBO J.* **24**, 4029–4040 (2005).
86. King, M. P. & Attardi, G. Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. *Science* **246**, 500–503 (1989).
87. Bayona-Bafaluy, M. P., Müller, S. & Moraes, C. T. Fast adaptive coevolution of nuclear and mitochondrial subunits of ATP synthetase in orangutan. *Mol. Biol. Evol.* **22**, 716–724 (2005).
88. Tiranti, V. *et al.* Mutations of SURF-1 in Leigh disease associated with cytochrome c oxidase deficiency. *Am. J. Hum. Genet.* **63**, 1609–1621 (1998).
89. Inoue, K. *et al.* Isolation of mitochondrial DNA-less mouse cell lines and their application for trapping mouse synaptosomal mitochondrial DNA with deletion mutations. *J. Biol. Chem.* **272**, 15510–15515 (1997).
90. Kukat, A. *et al.* Generation of $\rho 0$ cells utilizing a mitochondrially targeted restriction endonuclease and comparative analyses. *Nucleic Acids Res.* **36**, e44 (2008).

91. Chen, W. W. *et al.* Inhibition of ATP1F1 ameliorates severe mitochondrial respiratory chain dysfunction in mammalian cells. *Cell Rep.* **7**, 27–34 (2014).
92. Goffeau, A. *et al.* Life with 6000 genes. *Science* **274**, 546, 563–567 (1996).
93. Foury, F. & Kucej, M. Yeast mitochondrial biogenesis: A model system for humans? *Current Opinion in Chemical Biology* **6**, 106–111 (2002).
94. Smith, A. M. *et al.* Competitive genomic screens of barcoded yeast libraries. *J. Vis. Exp.* **54**, 2864 (2011). doi:10.3791/2864
95. Joyce, A. R. & Palsson, B. Ø. The model organism as a system: integrating ‘omics’ data sets. *Nat. Rev. Mol. Cell Biol.* **7**, 198–210 (2006).
96. Hurt, E. & Vanloon, A. How proteins find mitochondria and intramitochondrial compartments. *Trends in Biochemical Sciences* **11**, 204–207 (1986).
97. Tzagoloff, A. & Myers, A. M. Genetics of Mitochondrial Biogenesis. *Annu. Rev. Biochem.* **55**, 249–285 (1986).
98. Barrientos, A. Yeast Models of Human Mitochondrial Diseases. *IUBMB Life* **55**, 83–95 (2008).
99. Lasserre, J.-P. *et al.* Yeast as a system for modeling mitochondrial disease mechanisms and discovering therapies. *Dis. Model. Mech.* **8**, 509–526 (2015).
100. Gancedo, J. M. Yeast carbon catabolite repression. *Microbiol. Mol. Biol. Rev.* **62**, 334–361 (1998).
101. Altmann, K., Dürr, M. & Westermann, B. *Saccharomyces cerevisiae* as a model organism to study mitochondrial biology: general considerations and basic procedures. *Methods Mol. Biol.* **372**, 81–90 (2007).
102. Merz, S. & Westermann, B. Genome-wide deletion mutant analysis reveals genes required for respiratory growth, mitochondrial genome maintenance and mitochondrial protein synthesis in *Saccharomyces cerevisiae*. *Genome Biol.* **10**, R95 (2009).
103. Dimmer, K. S. *et al.* Genetic Basis of Mitochondrial Function and Morphology in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **13**, 847–853 (2002).
104. Newlon, C. S., Ludescher, R. D. & Walter, S. K. Production of petites by cell cycle mutants of *Saccharomyces cerevisiae* defective in DNA synthesis. *MGG Mol. Gen. Genet.* **169**, 189–194 (1979).
105. Devin, A. B. *et al.* The start gene CDC28 and the genetic stability of yeast. *Yeast* **6**, 231–243 (1990).

106. Liu, Z. & Butow, R. A. Mitochondrial retrograde signaling. *Annu. Rev. Genet.* **40**, 159–185 (2006).
107. Butow, R. A. & Avadhani, N. G. Mitochondrial Signaling The Retrograde Response. *Molecular Cell* **14**, 1–15 (2004).
108. Liu, Z. & Butow, R. A. A transcriptional switch in the expression of yeast tricarboxylic acid cycle genes in response to a reduction or loss of respiratory function. *Mol. Cell. Biol.* **19**, 6720–6728 (1999).
109. Epstein, C. B. *et al.* Genome-wide responses to mitochondrial dysfunction. *Mol. Biol. Cell* **12**, 297–308 (2001).
110. Newcomb, L. L., Diderich, J. A., Slattery, M. G. & Heideman, W. Glucose regulation of *Saccharomyces cerevisiae* cell cycle genes. *Eukaryot. Cell* **2**, 143–149 (2003).
111. Ohlmeier, S., Kastaniotis, A. J., Hiltunen, J. K. & Bergmann, U. The Yeast Mitochondrial Proteome, a Study of Fermentative and Respiratory Growth. *J. Biol. Chem.* **279**, 3956–3979 (2004).
112. Conrad, M. *et al.* Nutrient sensing and signaling in the yeast *Saccharomyces cerevisiae*. *FEMS Microbiol. Rev.* **38**, 254–299 (2014).
113. Zaman, S., Lippman, S. I., Zhao, X. & Broach, J. R. How *Saccharomyces* responds to nutrients. *Annu. Rev. Genet.* **42**, 27–81 (2008).
114. Xue, Y., Battle, M. & Hirsch, J. P. GPR1 encodes a putative G protein-coupled receptor that associates with the Gpa2p galpha subunit and functions in a ras-independent pathway. *EMBO Journal* **17**, 1996–2007 (1998).
115. Kübler, E., Mösch, H. U., Rupp, S. & Lisanti, M. P. Gpa2p, a G-protein α -subunit, regulates growth and pseudohyphal development in *Saccharomyces cerevisiae* via a cAMP-dependent mechanism. *J. Biol. Chem.* **272**, 20321–20323 (1997).
116. Ozcan, S. & Johnston, M. Three different regulatory mechanisms enable yeast hexose transporter (HXT) genes to be induced by different levels of glucose. *Mol. Cell. Biol.* **15**, 1564–1572 (1995).
117. Rudoni, S., Colombo, S., Coccetti, P. & Martegani, E. Role of guanine nucleotides in the regulation of the Ras/cAMP pathway in *Saccharomyces cerevisiae*. *Biochim. Biophys. Acta - Mol. Cell Res.* **1538**, 181–189 (2001).
118. Nakafuku, M. *et al.* Isolation of a second yeast *Saccharomyces cerevisiae* gene (GPA2) coding for guanine nucleotide-binding regulatory protein: studies on its structure and possible functions. *Proc. Natl. Acad. Sci. U. S. A.* **85**, 1374–1378 (1988).

119. Kraakman, L. *et al.* A *Saccharomyces cerevisiae* G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. *Mol. Microbiol.* **32**, 1002–1012 (1999).
120. Versele, M., De Winde, J. H. & Thevelein, J. M. A novel regulator of G protein signalling in yeast, Rgs2, downregulates glucose-activation of the cAMP pathway through direct inhibition of Gpa2. *EMBO J.* **18**, 5577–5591 (1999).
121. Ptacek, J. *et al.* Global analysis of protein phosphorylation in yeast. *Nature* **438**, 679–684 (2005).
122. Robertson, L. S. & Fink, G. R. The three yeast A kinases have specific signaling functions in pseudohyphal growth. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 13783–13787 (1998).
123. Robertson, L. S., Causton, H. C., Young, R. A. & Fink, G. R. The yeast A kinases differentially regulate iron uptake and respiratory function. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 5984–5988 (2000).
124. Tudisca, V. *et al.* Differential localization to cytoplasm, nucleus or P-bodies of yeast PKA subunits under different growth conditions. *Eur. J. Cell Biol.* **89**, 339–348 (2010).
125. Chevtzoff, C., Vallortigara, J., Avéret, N., Rigoulet, M. & Devin, A. The yeast cAMP protein kinase Tpk3p is involved in the regulation of mitochondrial enzymatic content during growth. *Biochim. Biophys. Acta - Bioenerg.* **1706**, 117–125 (2005).
126. Garrett, S. & Broach, J. Loss of Ras activity in *Saccharomyces cerevisiae* is suppressed by disruptions of a new kinase gene, YAKI, whose product may act downstream of the cAMP-dependent protein kinase. *Genes Dev.* **3**, 1336–1348 (1989).
127. Wang, Y. *et al.* Ras and Gpa2 mediate one branch of a redundant glucose signaling pathway in yeast. *PLoS Biol.* **2**, (2004).
128. Griffioen, G. *et al.* Nucleocytoplasmic distribution of budding yeast protein kinase A regulatory subunit Bcy1 requires Zds1 and is regulated by Yak1-dependent phosphorylation of its targeting domain. *Mol. Cell. Biol.* **21**, 511–523 (2001).
129. Benard, G. *et al.* Mitochondrial bioenergetics and structural network organization. *J. Cell Sci.* **120**, 838–848 (2007).
130. Alto, N. M., Soderling, J. & Scott, J. D. Rab32 is an A-kinase anchoring protein and participates in mitochondrial dynamics. *J. Cell Biol.* **158**, 659–668 (2002).

131. Huang, L. J., Durick, K., Weiner, J. A., Chun, J. & Taylor, S. S. D-AKAP2, a novel protein kinase A anchoring protein with a putative RGS domain. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 11184–11189 (1997).
132. Dagda, R. K. & Das Banerjee, T. Role of protein kinase A in regulating mitochondrial function and neuronal development: implications to neurodegenerative diseases. *Rev. Neurosci.* **26**, 359–70 (2015).
133. Dagda, R. K. *et al.* Mitochondrially localized PKA reverses mitochondrial pathology and dysfunction in a cellular model of Parkinson's disease. *Cell Death and Differentiation* **18**, 1914–1923 (2011).
134. Feliciello, A., Gottesman, M. E. & Avvedimento, E. V. cAMP-PKA signaling to the mitochondria: Protein scaffolds, mRNA and phosphatases. *Cellular Signalling* **17**, 279–287 (2005).
135. Demlow, C. M. & Fox, T. D. Activity of Mitochondrially Synthesized Reporter Proteins Is Lower Than That of Imported Proteins and Is Increased by Lowering cAMP in Glucose-Grown *Saccharomyces cerevisiae* Cells. *Genetics* **165**, 961–974 (2003).
136. Aun, A., Tamm, T. & Sedman, J. Dysfunctional mitochondria modulate cAMP-PKA signaling and filamentous and invasive growth of *saccharomyces cerevisiae*. *Genetics* **193**, 467–481 (2013).
137. Graef, M. & Nunnari, J. Mitochondria regulate autophagy by conserved signalling pathways. *EMBO J.* **30**, 2101–2114 (2011).
138. McBride, H. M., Neuspiel, M. & Wasiak, S. Mitochondria: More Than Just a Powerhouse. *Current Biology* **16** (2006).
139. Leadsham, J. E. & Gourlay, C. W. cAMP/PKA signaling balances respiratory activity with mitochondria dependent apoptosis via transcriptional regulation. *BMC Cell Biol.* **11**, 92 (2010).
140. Sudo, T. *et al.* Potent effects of novel anti-platelet aggregatory cilostamide analogues on recombinant cyclic nucleotide phosphodiesterase isozyme activity. *Biochem. Pharmacol.* **59**, 347–356 (2000).
141. Zuo, L., Li, Q., Sun, B., Xu, Z. & Ge, Z. Cilostazol promotes mitochondrial biogenesis in human umbilical vein endothelial cells through activating the expression of PGC-1 α . *Biochem. Biophys. Res. Commun.* **433**, 52–7 (2013).
142. Lin, M. T., Simon, D. K., Ahn, C. H., Kim, L. M. & Beal, M. F. High aggregate burden of somatic mtDNA point mutations in aging and Alzheimer's disease brain. *Hum. Mol. Genet.* **11**, 133–145 (2002).

143. Zhu, X. *et al.* Mitochondrial abnormalities and oxidative imbalance in Alzheimer disease. *J. Alzheimers. Dis.* **9**, 147–153 (2006).
144. Sheng, B. *et al.* Impaired mitochondrial biogenesis contributes to mitochondrial dysfunction in Alzheimer's disease. *J. Neurochem.* **120**, 419–429 (2012).
145. Diffley, J. F. & Stillman, B. A close relative of the nuclear, chromosomal high-mobility group protein HMG1 in yeast mitochondria. *Proc. Natl. Acad. Sci. U. S. A.* **88**, 7864–7868 (1991).
146. Parisi, M. A., Xu, B. & Clayton, D. A. A human mitochondrial transcriptional activator can functionally replace a yeast mitochondrial HMG-box protein both in vivo and in vitro. *Mol. Cell. Biol.* **13**, 1951–1961 (1993).
147. Loewith, R. & Hall, M. N. Target of rapamycin (TOR) in nutrient signaling and growth control. *Genetics* **189**, 1177–1201 (2011).
148. Toda, T., Cameron, S., Sass, P. & Wigler, M. SCH9, a gene of *Saccharomyces cerevisiae* that encodes a protein distinct from, but functionally and structurally related to, cAMP-dependent protein kinase catalytic subunits. *Genes Dev.* **2**, 517–527 (1988).
149. Roosen, J. *et al.* PKA and Sch9 control a molecular switch important for the proper adaptation to nutrient availability. *Mol. Microbiol.* **55**, 862–880 (2005).
150. Zhang, A., Shen, Y., Gao, W. & Dong, J. Role of Sch9 in regulating Ras-cAMP signal pathway in *Saccharomyces cerevisiae*. *FEBS Lett.* **585**, 3026–3032 (2011).
151. Carlson, M., Osmond, B. C. & Botstein, D. Mutants of yeast defective in sucrose utilization. *Genetics* **98**, 25–40 (1981).
152. Wilson, W. A., Hawley, S. A. & Hardie, D. G. Glucose repression/derepression in budding yeast: SNF1 protein kinase is activated by phosphorylation under derepressing conditions, and this correlates with a high AMP:ATP ratio. *Curr. Biol.* **6**, 1426–1434 (1996).
153. Estruch, F., Treitel, M. A., Yang, X. & Carlson, M. N-terminal mutations modulate yeast SNF1 protein kinase function. *Genetics* **132**, 639–650 (1992).
154. Hong, S. P. & Carlson, M. Regulation of Snf1 protein kinase in response to environmental stress. *J. Biol. Chem.* **282**, 16838–16845 (2007).
155. Dihazi, H., Kessler, R. & Eschrich, K. Glucose-induced stimulation of the Ras-cAMP pathway in yeast leads to multiple phosphorylations and activation of 6-phosphofructo-2-kinase. *Biochemistry* **42**, 6275–6282 (2003).

156. Portela, P., Howell, S., Moreno, S. & Rossi, S. In vivo and in vitro phosphorylation of two isoforms of yeast pyruvate kinase by protein kinase A. *J. Biol. Chem.* **277**, 30477–30487 (2002).
157. Reinders, A., Bürckert, N., Boller, T., Wiemken, A. & De Virgilio, C. *Saccharomyces cerevisiae* cAMP-dependent protein kinase controls entry into stationary phase through the Rim15p protein kinase. *Genes Dev.* **12**, 2943–2955 (1998).
158. Wanke, V. *et al.* Caffeine extends yeast lifespan by targeting TORC1. *Mol. Microbiol.* **69**, 277–285 (2008).
159. Pedruzzi, I. *et al.* TOR and PKA Signaling Pathways Converge on the Protein Kinase Rim15 to Control Entry into G0. *Mol. Cell* **12**, 1607–1613 (2003).
160. Zhang, N., Wu, J. & Oliver, S. G. Gis1 is required for transcriptional reprogramming of carbon metabolism and the stress response during Transition into stationary phase in yeast. *Microbiology* **155**, 1690–1698 (2009).
161. Martínez-Pastor, M. T. *et al.* The *Saccharomyces cerevisiae* zinc finger proteins Msn2p and Msn4p are required for transcriptional induction through the stress response element (STRE). *EMBO J.* **15**, 2227–2235 (1996).
162. Görner, W. *et al.* Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes Dev.* **12**, 586–97 (1998).
163. Masoro, E. J. Subfield history: caloric restriction, slowing aging, and extending life. *Sci. Aging Knowledge Environ.* **2003**, RE2 (2003).
164. Wei, M. *et al.* Life span extension by calorie restriction depends on Rim15 and transcription factors downstream of Ras/PKA, Tor, and Sch9. *PLoS Genet.* **4**, 0139–0149 (2008).
165. Fabrizio, P., Pozza, F., Pletcher, S. D., Gendron, C. M. & Longo, V. D. Regulation of longevity and stress resistance by Sch9 in yeast. *Science* **292**, 288–290 (2001).
166. Kaeberlein, M. *et al.* Regulation of yeast replicative life span by TOR and Sch9 in response to nutrients. *Science* **310**, 1193–1196 (2005).
167. Swinnen, E., Ghillebert, R., Wilms, T. & Winderickx, J. Molecular mechanisms linking the evolutionary conserved TORC1-Sch9 nutrient signalling branch to lifespan regulation in *Saccharomyces cerevisiae*. *FEMS Yeast Res.* **14**, 17–32 (2014).
168. Verghese, J., Abrams, J., Wang, Y. & Morano, K. a. Biology of the heat shock response and protein chaperones: budding yeast (*Saccharomyces cerevisiae*) as a model system. *Microbiol. Mol. Biol. Rev.* **76**, 115–58 (2012).

169. Ferguson, S. B. *et al.* Protein kinase A regulates constitutive expression of small heat-shock genes in an Msn2/4p-independent and Hsf1p-dependent manner in *Saccharomyces cerevisiae*. *Genetics* **169**, 1203–1214 (2005).
170. Treger, J. M., Magee, T. R. & McEntee, K. Functional analysis of the stress response element and its role in the multistress response of *Saccharomyces cerevisiae*. *Biochem. Biophys. Res. Commun.* **243**, 13–19 (1998).
171. Wiederrecht, G., Seto, D. & Parker, C. S. Isolation of the gene encoding the *S. cerevisiae* heat shock transcription factor. *Cell* **54**, 841–53 (1988).
172. Sorger, P. K. & Pelham, H. R. Yeast heat shock factor is an essential DNA-binding protein that exhibits temperature-dependent phosphorylation. *Cell* **54**, 855–864 (1988).
173. Li, N. *et al.* Effects of heat stress on yeast heat shock factor-promoter binding in vivo. *Acta Biochim. Biophys. Sin. (Shanghai)*. **38**, 356–362 (2006).
174. Lee, P. *et al.* Rim15-dependent activation of Hsf1 and Msn2/4 transcription factors by direct phosphorylation in *Saccharomyces cerevisiae*. *FEBS Lett.* **587**, 3648–3655 (2013).
175. Lee, P., Cho, B. R., Joo, H. S. & Hahn, J. S. Yeast Yak1 kinase, a bridge between PKA and stress-responsive transcription factors, Hsf1 and Msn2/Msn4. *Mol. Microbiol.* **70**, 882–895 (2008).
176. Warner, J. R. The economics of ribosome biosynthesis in yeast. *Trends in Biochemical Sciences* **24**, 437–440 (1999).
177. Huber, A. *et al.* Sch9 regulates ribosome biogenesis via Stb3, Dot6 and Tod6 and the histone deacetylase complex RPD3L. *EMBO J.* **30**, 3052–3064 (2011).
178. Lippman, S. I. & Broach, J. R. Protein kinase A and TORC1 activate genes for ribosomal biogenesis by inactivating repressors encoded by Dot6 and its homolog Tod6. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 19928–19933 (2009).
179. Lempiainen, H. & Shore, D. Growth control and ribosome biogenesis. *Curr Opin Cell Biol* **21**, 855–863 (2009).
180. Jorgensen, P. *et al.* A dynamic transcriptional network communicates growth potential to ribosome synthesis and critical cell size. *Genes Dev.* **18**, 2491–2505 (2004).
181. Oficjalska-Pham, D. *et al.* General Repression of RNA Polymerase III Transcription Is Triggered by Protein Phosphatase Type 2A-Mediated Dephosphorylation of Maf1. *Mol. Cell* **22**, 623–632 (2006).

182. Moir, R. D. *et al.* Protein kinase A regulates RNA polymerase III transcription through the nuclear localization of Maf1. *Proc Natl Acad Sci U S A* **103**, 15044–15049 (2006).
183. Fox, T. D. Mitochondrial protein synthesis, import, and assembly. *Genetics* **192**, 1203–1234 (2012).
184. Schmidt, O. *et al.* Regulation of mitochondrial protein import by cytosolic kinases. *Cell* **144**, 227–239 (2011).
185. Rao, S. *et al.* Biogenesis of the preprotein translocase of the outer mitochondrial membrane: protein kinase A phosphorylates the precursor of Tom40 and impairs its import. *Molecular Biology of the Cell* **23**, 1618–1627 (2012).
186. Gerbeth, C. *et al.* Glucose-induced regulation of protein import receptor tom22 by cytosolic and mitochondria-bound kinases. *Cell Metab.* **18**, 578–587 (2013).
187. Becker, T., Vögtle, F.-N., Stojanovski, D. & Meisinger, C. Sorting and assembly of mitochondrial outer membrane proteins. *Biochim. Biophys. Acta* **1777**, 557–63 (2008).
188. Chacinska, A., Koehler, C. M., Milenkovic, D., Lithgow, T. & Pfanner, N. Importing Mitochondrial Proteins: Machineries and Mechanisms. *Cell* **138**, 628–644 (2009).
189. Toda, T., Cameron, S., Sass, P., Zoller, M. & Wigler, M. Three different genes in *S. cerevisiae* encode the catalytic subunits of the cAMP-dependent protein kinase. *Cell* **50**, 277–287 (1987).
190. Livas, D., Almering, M. J. H., Daran, J.-M., Pronk, J. T. & Gancedo, J. M. Transcriptional responses to glucose in *Saccharomyces cerevisiae* strains lacking a functional protein kinase A. *BMC Genomics* **12**, 405 (2011).
191. Dirick, L. *et al.* Metabolic and Environmental Conditions Determine Nuclear Genomic Instability in Budding Yeast Lacking Mitochondrial DNA. *G3: Genes|Genomes|Genetics* **4**, 411–423 (2013).
192. Veatch, J. R., McMurray, M. A., Nelson, Z. W. & Gottschling, D. E. Mitochondrial Dysfunction Leads to Nuclear Genome Instability via an Iron-Sulfur Cluster Defect. *Cell* **137**, 1247–1258 (2009).
193. Puig, S., Askeland, E. & Thiele, D. J. Coordinated remodeling of cellular metabolism during iron deficiency through targeted mRNA degradation. *Cell* **120**, 99–110 (2005).

194. Parikh, V. S., Morgan, M. M., Scott, R., Clements, L. S. & Butow, R. A. The mitochondrial genotype can influence nuclear gene expression in yeast. *Science* **235**, 576–580 (1987).
195. Miceli, M. V., Jiang, J. C., Tiwari, A., Rodriguez-Quiñones, J. F. & Jazwinski, S. M. Loss of mitochondrial membrane potential triggers the retrograde response extending yeast replicative lifespan. *Front. Genet.* **2**, (2012).
196. Cherry, J. M. *et al.* Saccharomyces Genome Database: The genomics resource of budding yeast. *Nucleic Acids Res.* **40**, (2012).
197. Jazayeri, M. *et al.* Inducible expression of a dominant negative DNA polymerase- γ depletes mitochondrial DNA and produces a rho0 phenotype. *J. Biol. Chem.* **278**, 9823–9830 (2003).
198. Schleyer, M., Schmidt, B. & Neupert, W. Requirement of a membrane potential for the posttranslational transfer of proteins into mitochondria. *Eur. J. Biochem.* **125**, 109–116 (1982).
199. Garipler, G. & Dunn, C. D. Defects associated with mitochondrial DNA damage can be mitigated by increased vacuolar pH in *Saccharomyces cerevisiae*. *Genetics* **194**, 285–290 (2013).
200. Breslow, D. K. *et al.* A comprehensive strategy enabling high-resolution functional analysis of the yeast genome. *Nat. Methods* **5**, 711–718 (2008).
201. Hoppins, S. *et al.* A mitochondrial-focused genetic interaction map reveals a scaffold-like complex required for inner membrane organization in mitochondria. *J. Cell Biol.* **195**, 323–340 (2011).
202. Huh, W.-K. *et al.* Global analysis of protein localization in budding yeast. *Nature* **425**, 686–691 (2003).
203. Cohen, Y. & Schuldiner, M. Advanced methods for high-throughput microscopy screening of genetically modified yeast libraries. *Methods Mol. Biol.* **781**, 127–159 (2011).
204. Brix, J., Dietmeier, K. & Pfanner, N. Differential recognition of preproteins by the purified cytosolic domains of the mitochondrial import receptors Tom20, Tom22, and Tom70. *J. Biol. Chem.* **272**, 20730–20735 (1997).
205. Söllner, T., Pfaller, R., Griffiths, G., Pfanner, N. & Neupert, W. A mitochondrial import receptor for the ADP/ATP carrier. *Cell* **62**, 107–115 (1990).
206. Young, J. C., Hoogenraad, N. J. & Hartl, F. U. Molecular chaperones Hsp90 and Hsp70 deliver preproteins to the mitochondrial import receptor Tom70. *Cell* **112**, 41–50 (2003).

207. Wegele, H., Müller, L. & Buchner, J. Hsp70 and Hsp90--a relay team for protein folding. *Rev. Physiol. Biochem. Pharmacol.* **151**, 1–44 (2004).
208. Endo, T. & Yamano, K. Transport of proteins across or into the mitochondrial outer membrane. *Biochim. Biophys. Acta* **1803**, 706–714 (2010).
209. Schmidt, O., Pfanner, N. & Meisinger, C. Mitochondrial protein import: from proteomics to functional mechanisms. *Nat. Rev. Mol. Cell Biol.* **11**, 655–667 (2010).
210. Hahn, J. S. & Thiele, D. J. Activation of the *Saccharomyces cerevisiae* Heat Shock Transcription Factor under Glucose Starvation Conditions by Snf1 Protein Kinase. *J. Biol. Chem.* **279**, 5169–5176 (2004).
211. Yamamoto, A., Mizukami, Y. & Sakurai, H. Identification of a novel class of target genes and a novel type of binding sequence of heat shock transcription factor in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **280**, 11911–11919 (2005).
212. Amorós, M. & Estruch, F. Hsf1p and Msn2/4p cooperate in the expression of *Saccharomyces cerevisiae* genes HSP26 and HSP104 in a gene- and stress type-dependent manner. *Mol. Microbiol.* **39**, 1523–1532 (2001).
213. Halladay, J. T. & Craig, E. A. A heat shock transcription factor with reduced activity suppresses a yeast HSP70 mutant. *Mol. Cell. Biol.* **15**, 4890–4897 (1995).
214. Pedruzzi, I. *Saccharomyces cerevisiae* Ras/cAMP pathway controls post-diauxic shift element-dependent transcription through the zinc finger protein Gis1. *EMBO J.* **19**, 2569–2579 (2000).
215. Garipler, G. Uncovering new approaches for treatment of mitochondrial disease using budding yeast and fruit flies. *Unpubl. MSc. Thesis, Koc Univ.* (2013).
216. Huber, A. *et al.* Characterization of the rapamycin-sensitive phosphoproteome reveals that Sch9 is a central coordinator of protein synthesis. *Genes Dev.* **23**, 1929–1943 (2009).
217. Klein, C. & Struhl, K. Protein kinase A mediates growth-regulated expression of yeast ribosomal protein genes by modulating RAP1 transcriptional activity. *Mol. Cell. Biol.* **14**, 1920–1928 (1994).
218. Bandyopadhyay, S. *et al.* Rewiring of genetic networks in response to DNA damage. *Science* **330**, 1385–1389 (2010).
219. Deminoff, S. J. & Santangelo, G. M. Rap1p requires Gcr1p and Gcr2p homodimers to activate ribosomal protein and glycolytic genes, respectively. *Genetics* **158**, 133–143 (2001).

- 220. Hedbacker, K., Townley, R. & Carlson, M. Cyclic AMP-dependent protein kinase regulates the subcellular localization of Snf1-Sip1 protein kinase. *Mol Cell Biol* **24**, 1836–1843 (2004).
- 221. Kong, M., Ditsworth, D., Lindsten, T. & Thompson, C. B. $\alpha 4$ Is an Essential Regulator of PP2A Phosphatase Activity. *Mol. Cell* **36**, 51–60 (2009).
- 222. Jacinto, E., Guo, B., Arndt, K. T., Schmelzle, T. & Hall, M. N. TIP41 interacts with TAP42 and negatively regulates the TOR signaling pathway. *Mol. Cell* **8**, 1017–1026 (2001).
- 223. Decker, C. J. & Parker, R. P-bodies and stress granules: possible roles in the control of translation and mRNA degradation. *Cold Spring Harbor perspectives in biology* **4**, (2012).
- 224. Cougot, N., Babajko, S. & Séraphin, B. Cytoplasmic foci are sites of mRNA decay in human cells. *J. Cell Biol.* **165**, 31–40 (2004).
- 225. Huang, L. *et al.* Mitochondria associate with P-bodies and modulate microRNA-mediated RNA interference. *J. Biol. Chem.* **286**, 24219–24220 (2011).
- 226. Ramachandran, V., Shah, K. H. & Herman, P. K. The cAMP-Dependent Protein Kinase Signaling Pathway Is a Key Regulator of P Body Foci Formation. *Mol. Cell* **43**, 973–981 (2011).
- 227. Mitchell, S. F., Jain, S., She, M. & Parker, R. Global analysis of yeast mRNPs. *Nat. Struct. Mol. Biol.* **20**, 127–33 (2013).
- 228. Tadauchi, T., Inada, T., Matsumoto, K. & Irie, K. Posttranscriptional regulation of HO expression by the Mkt1-Pbp1 complex. *Mol. Cell. Biol.* **24**, 3670–3681 (2004).
- 229. Dimitrov, L. N., Brem, R. B., Kruglyak, L. & Gottschling, D. E. Polymorphisms in multiple genes contribute to the spontaneous mitochondrial genome instability of *Saccharomyces cerevisiae* S288C strains. *Genetics* **183**, 365–383 (2009).
- 230. Brachmann, C. B. *et al.* Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14**, 115–132 (1998).
- 231. Avrahami-Moyal, L., Braun, S. & Engelberg, D. Overexpression of PDE2 or SSD1-V in *Saccharomyces cerevisiae* W303-1A strain renders it ethanol-tolerant. *FEMS Yeast Res.* **12**, 447–455 (2012).
- 232. Torgersen, K. M. *et al.* Selective activation of cAMP-dependent protein kinase type I inhibits rat natural killer cell cytotoxicity. *J. Biol. Chem.* **272**, 5495–5500 (1997).

233. Bodenmiller, B. *et al.* Phosphoproteomic analysis reveals interconnected system-wide responses to perturbations of kinases and phosphatases in yeast. *Sci. Signal.* **3** (2010).
234. Lockshon, D. *et al.* A role for recombination junctions in the segregation of mitochondrial DNA in yeast. *Cell* **81**, 947–955 (1995).
235. Tiraboschi, P., Hansen, L. A., Thal, L. J. & Corey-Bloom, J. The importance of neuritic plaques and tangles to the development and evolution of AD. *Neurology* **62**, 1984–1989 (2004).
236. Olanow, C. W. & McNaught, K. S. P. Ubiquitin-proteasome system and Parkinson's disease. *Movement Disorders* **21**, 1806–1823 (2006).
237. Marbella, L. E., Cho, H. S. & Spence, M. M. Observing the translocation of a mitochondria-penetrating peptide with solid-state NMR. *Biochim. Biophys. Acta - Biomembr.* **1828**, 1674–1682 (2013).
238. Geissler, A. *et al.* Membrane potential-driven protein import into mitochondria. The sorting sequence of cytochrome b(2) modulates the $\Delta\psi$ -dependence of translocation of the matrix-targeting sequence. *Mol. Biol. Cell* **11**, 3977–3991 (2000).
239. Yun, C. W., Bauler, M., Moore, R. E., Klebba, P. E. & Philpott, C. C. The Role of the FRE Family of Plasma Membrane Reductases in the Uptake of Siderophore-Iron in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **276**, 10218–10223 (2001).
240. Askwith, C. *et al.* The FET3 gene of *S. cerevisiae* encodes a multicopper oxidase required for ferrous iron uptake. *Cell* **76**, 403–410 (1994).
241. Stearman, R., Yuan, D. S., Yamaguchi-Iwai, Y., Klausner, R. D. & Dancis, A. A permease-oxidase complex involved in high-affinity iron uptake in yeast. *Science* **271**, 1552–1557 (1996).
242. Heymann, P., Ernst, J. F. & Winkelmann, G. Identification and substrate specificity of a ferrichrome-type siderophore transporter (Arn1p) in *Saccharomyces cerevisiae*. *FEMS Microbiol. Lett.* **186**, 221–227 (2000).
243. Yuan, D. S. *et al.* The Menkes/Wilson disease gene homologue in yeast provides copper to a ceruloplasmin-like oxidase required for iron uptake. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 2632–2636 (1995).
244. Kaut, A., Lange, H., Diekert, K., Kispal, G. & Lill, R. Isa1p is a component of the mitochondrial machinery for maturation of cellular iron-sulfur proteins and requires conserved cysteine residues for function. *J. Biol. Chem.* **275**, 15955–15961 (2000).

245. Kispal, G., Csere, P., Prohl, C. & Lill, R. The mitochondrial proteins Atm1p and Nfs1p are essential for biogenesis of cytosolic Fe/S proteins. *EMBO J.* **18**, 3981–3989 (1999).
246. Azuma, K., Ohtsuka, H., Mita, S., Murakami, H. & Aiba, H. Identification and characterization of an Ecl1-family gene in *Saccharomyces cerevisiae*. *Biosci. Biotechnol. Biochem.* **73**, 2787–2789 (2009).
247. Imazu, H. & Sakurai, H. *Saccharomyces cerevisiae* heat shock transcription factor regulates cell wall remodeling in response to heat shock. *Eukaryot. Cell* **4**, 1050–1056 (2005).
248. Sickmann, A. *et al.* The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 13207–13212 (2003).
249. Shi, Y., Mosser, D. D. & Morimoto, R. I. Molecular chaperones as HSF1-specific transcriptional repressors. *Genes Dev.* **12**, 654–666 (1998).
250. Morishita, T. *et al.* Requirement of *Saccharomyces cerevisiae* Ras for completion of mitosis. *Science* **270**, 1213–1215 (1995).
251. Mösch, H. U., Roberts, R. L. & Fink, G. R. Ras2 signals via the Cdc42/Ste20/mitogen-activated protein kinase module to induce filamentous growth in *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 5352–5356 (1996).
252. Werner-Washburne, M., Braun, E., Johnston, G. C. & Singer, R. A. Stationary phase in the yeast *Saccharomyces cerevisiae*. *Microbiol. Mol. Biol. Rev.* **57**, 383–401 (1993).
253. Howard, S. C., Chang, Y. W., Budovskaya, Y. V. & Herman, P. K. The RAS/PKA signaling pathway of *Saccharomyces cerevisiae* exhibits a functional interaction with the sin4p complex of the RNA polymerase II holoenzyme. *Genetics* **159**, 77–89 (2001).
254. Türkel, S. & Bisson, L. F. Transcription of the HXT4 gene is regulated by Gcr1p and Gcr2p in the yeast *S. cerevisiae*. *Yeast* **15**, 1045–1057 (1999).
255. Dingley, S., Chapman, K. A. & Falk, M. J. Fluorescence-activated cell sorting analysis of mitochondrial content, membrane potential, and matrix oxidant burden in human lymphoblastoid cell lines. *Methods Mol. Biol.* **837**, 231–239 (2012).
256. Aon, M. A., Cortassa, S., Lemar, K. M., Hayes, A. J. & Lloyd, D. Single and cell population respiratory oscillations in yeast: A 2-photon scanning laser microscopy study. *FEBS Lett.* **581**, 8–14 (2007).
257. Amberg, D. C., Burke, D. J. & Strathern, J. N. *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual, 2005 Edition. A Cold Spring Harbor*

- Laboratory Course Manual* (2005). at <<http://www.amazon.com/Methods-Yeast-Genetics-Spring-Laboratory/dp/0879697288>>
258. Sikorski, R. S. & Hieter, P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**, 19–27 (1989).
 259. Orlova, M., Barrett, L. & Kuchin, S. Detection of endogenous Snfl and its activation state: Application to *Saccharomyces* and *Candida* species. *Yeast* **25**, 745–754 (2008).
 260. Goecks, J., Nekrutenko, A. & Taylor, J. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol.* **11**, R86 (2010).
 261. Trapnell, C. *et al.* Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* **7**, 562–78 (2012).
 262. Sesaki, H. & Jensen, R. E. Division versus fusion: Dnm1p and Fzo1p antagonistically regulate mitochondrial shape. *J. Cell Biol.* **147**, 699–706 (1999).
 263. Hoppins, S., Lackner, L. & Nunnari, J. The machines that divide and fuse mitochondria. *Annu. Rev. Biochem.* **76**, 751–780 (2007).
 264. Bereiter-Hahn, J. Behavior of mitochondria in the living cell. *Int. Rev. Cytol.* **122**, 1–63 (1990).
 265. Bereiter-Hahn, J. & Vöth, M. Dynamics of mitochondria in living cells: shape changes, dislocations, fusion, and fission of mitochondria. *Microsc. Res. Tech.* **27**, 198–219 (1994).
 266. Chen, H., McCaffery, J. M. & Chan, D. C. Mitochondrial Fusion Protects against Neurodegeneration in the Cerebellum. *Cell* **130**, 548–562 (2007).
 267. Chan, D. C. Fusion and Fission: Interlinked Processes Critical for Mitochondrial Health. *Annual Review of Genetics* **46**, 120830114430006 (2011).
 268. Hermann, G. J. *et al.* Mitochondrial fusion in yeast requires the transmembrane GTPase Fzo1p. *J. Cell Biol.* **143**, 359–373 (1998).
 269. Rapaport, D., Brunner, M., Neupert, W. & Westermann, B. Fzo1p is a mitochondrial outer membrane protein essential for the biogenesis of functional mitochondria in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **273**, 20150–20155 (1998).
 270. Sesaki, H. & Jensen, R. E. UGO1 encodes an outer membrane protein required for mitochondrial fusion. *J. Cell Biol.* **152**, 1123–1134 (2001).

271. Wong, E. D. *et al.* The dynamin-related GTPase, Mgm1p, is intermembrane space protein required for maintenance of fusion competent mitochondria. *J. Cell Biol.* **151**, 341–352 (2000).
272. Meeusen, S., McCaffery, J. M. & Nunnari, J. Mitochondrial fusion intermediates revealed in vitro. *Science* **305**, 1747–1752 (2004).
273. Meeusen, S. *et al.* Mitochondrial Inner-Membrane Fusion and Crista Maintenance Requires the Dynamin-Related GTPase Mgm1. *Cell* **127**, 383–395 (2006).
274. Züchner, S. *et al.* Mutations in the mitochondrial GTPase mitofusin 2 cause Charcot-Marie-Tooth neuropathy type 2A. *Nat. Genet.* **36**, 449–451 (2004).
275. Delettre, C. *et al.* Nuclear gene OPA1, encoding a mitochondrial dynamin-related protein, is mutated in dominant optic atrophy. *Nat. Genet.* **26**, 207–210 (2000).
276. Abrams, A. J. *et al.* Mutations in SLC25A46, encoding a UGO1-like protein, cause an optic atrophy spectrum disorder. *Nat. Genet.* (2015). doi:10.1038/ng.3354
277. Praefcke, G. J. K. & McMahon, H. T. The dynamin superfamily: universal membrane tubulation and fission molecules? *Nat. Rev. Mol. Cell Biol.* **5**, 133–147 (2004).
278. Zhang, Y., Chan, N. C., Ngo, H. B., Gristick, H. & Chan, D. C. Crystal structure of mitochondrial fission complex reveals scaffolding function for Mitochondrial division 1 (Mdv1) coiled coil. *J. Biol. Chem.* **287**, 9855–9861 (2012).
279. Tieu, Q., Okreglak, V., Naylor, K. & Nunnari, J. The WD repeat protein, Mdv1p, functions as a molecular adaptor by interacting with Dnm1p and Fis1p during mitochondrial fission. *J. Cell Biol.* **158**, 445–452 (2002).
280. Cervený, K. L. & Jensen, R. E. The WD-repeats of Net2p interact with Dnm1p and Fis1p to regulate division of mitochondria. *Mol. Biol. Cell* **14**, 4126–4139 (2003).
281. Griffin, E. E., Graumann, J. & Chan, D. C. The WD40 protein Caf4p is a component of the mitochondrial fission machinery and recruits Dnm1p to mitochondria. *J. Cell Biol.* **170**, 237–248 (2005).
282. Fekkes, P., Shepard, K. A. & Yaffe, M. P. Gag3p, an outer membrane protein required for fission of mitochondrial tubules. *J. Cell Biol.* **151**, 333–340 (2000).
283. Mutlu, N., Garipler, G., Akdoğan, E. & Dunn, C. D. Activation of the Pleiotropic Drug Resistance Pathway Can Promote Mitochondrial DNA Retention by Fusion-Defective Mitochondria in *Saccharomyces cerevisiae*. *G3 (Bethesda)*. **4**, 1247–1258 (2014).

284. Lackner, L. L., Ping, H., Graef, M., Murley, A. & Nunnari, J. Endoplasmic reticulum-associated mitochondria-cortex tether functions in the distribution and inheritance of mitochondria. *Proc. Natl. Acad. Sci. U. S. A.* **110**, E458–67 (2013).
285. Hammermeister, M., Schödel, K. & Westermann, B. Mdm36 is a mitochondrial fission-promoting protein in *Saccharomyces cerevisiae*. *Mol. Biol. Cell* **21**, 2443–2452 (2010).
286. Hallstrom, T. C. & Moye-Rowley, W. S. Multiple signals from dysfunctional mitochondria activate the pleiotropic drug resistance pathway in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **275**, 37347–37356 (2000).
287. Kolaczowski, M., Kolaczowska, A., Gaigg, B., Schneiter, R. & Moye-Rowley, W. S. Differential regulation of ceramide synthase components LAC1 and LAG1 in *Saccharomyces cerevisiae*. *Eukaryot. Cell* **3**, 880–892 (2004).
288. Soeiro, M. N. C. *et al.* Novel amidines and analogues as promising agents against intracellular parasites: a systematic review. *Parasitology* **140**, 1–23 (2013). doi:10.1017/S0031182013000292
289. Feldman, C. Pneumonia associated with HIV infection. *Curr. Opin. Infect. Dis.* **18**, 165–170 (2005).
290. Bujdakova, H., Kuchta, T., Sidoova, E. & Gvozdjakova, A. Anti-Candida activity of four antifungal benzothiazoles. *FEMS Microbiol. Lett.* **112**, 329–334 (1993).
291. Rowe, T. C., Weissig, V. & Lawrence, J. W. Mitochondrial DNA metabolism targeting drugs. *Advanced Drug Delivery Reviews* **49**, 175–187 (2001).
292. Croft, S. L. & Brazil, R. P. Effect of pentamidine isethionate on the ultrastructure and morphology of *Leishmania mexicana amazonensis* in vitro. *Ann. Trop. Med. Parasitol.* **76**, 37–43 (1982).
293. Basselin, M., Lawrence, F. & Robert-Gero, M. Altered transport properties of pentamidine-resistant *Leishmania donovani* and *L. amazonensis* promastigotes. *Parasitology Research* **83**, 413–418 (1997).
294. Lanteri, C. A., Tidwell, R. R. & Meshnick, S. R. The mitochondrion is a site of trypanocidal action of the aromatic diamidine DB75 in bloodstream forms of *Trypanosoma brucei*. *Antimicrob. Agents Chemother.* **52**, 875–882 (2008).
295. Zhang, Y., Bell, A., Perlman, P. S. & Leibowitz, M. J. Pentamidine inhibits mitochondrial intron splicing and translation in *Saccharomyces cerevisiae*. *RNA* **6**, 937–951 (2000).
296. Briceland, L. L. & Bailie, G. R. Pentamidine-associated nephrotoxicity and hyperkalemia in patients with AIDS. *DICP* **25**, 1171–1174 (1991).

297. Ludewig, G., Williams, J. M., Li, Y. & Staben, C. Effects of pentamidine isethionate on *Saccharomyces cerevisiae*. *Antimicrob. Agents Chemother.* **38**, 1123–1128 (1994).
298. Hatfield, C., Kasarskis, A. & Staben, C. Pentamidine sensitivity and resistance in *Saccharomyces cerevisiae* as a model for pentamidine effects on *Pneumocystis carinii*. *J. Protozool.* **38**, 70S–71S (1991).
299. Örs, Ş. T., Akdoğan, E. & Dunn, C. D. Mutation of the mitochondrial large ribosomal RNA can provide pentamidine resistance to *Saccharomyces cerevisiae*. *Mitochondrion* **18**, 7–11 (2014).
300. Zakharov, I. A. & Yarovoy, B. P. Cytofusion as a new tool in studying the cytoplasmic heredity in yeast. *Mol. Cell. Biochem.* **14**, 15–18 (1977).
301. Liu, Y., Tidwell, R. R. & Leibowitz, M. J. Inhibition of in vitro splicing of a group I intron of *Pneumocystis carinii*. *J. Eukaryot. Microbiol.* **41**, 31–38 (1994).
302. Milette, K. E. & Leibowitz, M. J. Pentamidine inhibition of group I intron splicing in *Candida albicans* correlates with growth inhibition. *Antimicrob. Agents Chemother.* **44**, 958–966 (2000).
303. Von der Malsburg, K. *et al.* Dual Role of Mitofilin in Mitochondrial Membrane Organization and Protein Biogenesis. *Dev. Cell* **21**, 694–707 (2011).
304. Herrmann, J. M. MINOS Is Plus: A Mitofilin Complex for Mitochondrial Membrane Contacts. *Developmental Cell* **21**, 599–600 (2011).
305. Pfanner, N. *et al.* Uniform nomenclature for the mitochondrial contact site and cristae organizing system. *Journal of Cell Biology* **204**, 1083–1086 (2014).
306. Itoh, K., Tamura, Y., Iijima, M. & Sesaki, H. Effects of Fcj1-Mos1 and mitochondrial division on aggregation of mitochondrial DNA nucleoids and organelle morphology. *Mol. Biol. Cell* **24**, 1842–51 (2013).
307. Sesaki, H., Southard, S. M., Hobbs, A. E. A. & Jensen, R. E. Cells lacking Pcp1p/Ugo2p, a rhomboid-like protease required for Mgm1p processing, lose mtDNA and mitochondrial structure in a Dnm1p-dependent manner, but remain competent for mitochondrial fusion. *Biochem. Biophys. Res. Commun.* **308**, 276–283 (2003).
308. Hoppins, S., Horner, J., Song, C., McCaffery, J. M. & Nunnari, J. Mitochondrial outer and inner membrane fusion requires a modified carrier protein. *J. Cell Biol.* **184**, 569–581 (2009).
309. Sesaki, H. & Jensen, R. E. Ugo1p links the Fzo1p and Mgm1p GTPases for mitochondrial fusion. *J. Biol. Chem.* **279**, 28298–28303 (2004).

310. Coonrod, E. M., Karren, M. A. & Shaw, J. M. Ugo1p is a multipass transmembrane protein with a single carrier domain required for mitochondrial fusion. *Traffic* **8**, 500–511 (2007).
311. Korner, C. *et al.* The C-terminal domain of Fcj1 is required for formation of crista junctions and interacts with the TOB/SAM complex in mitochondria. *Molecular Biology of the Cell* **23**, 2143–2155 (2012).
312. Ott, C. *et al.* Sam50 Functions in Mitochondrial Intermembrane Space Bridging and Biogenesis of Respiratory Complexes. *Molecular and Cellular Biology* **32**, 1173–1188 (2012).
313. Friedman JR, Mourier A, Yamada J, McCaffery JM, N. J. MICOS coordinates with respiratory complexes and lipids to establish mitochondrial inner membrane architecture. *Elife* (2015).
314. Westermann, B. Mitochondrial membrane fusion. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1641**, 195–202 (2003).

VITA

Emel Akdoğan was born in Ankara, Turkey on March 11, 1990. She received her B.Sc. Degree in Molecular Biology and Genetics from Middle East Technical University, Ankara, in 2013. From September 2013 to August 2015, she worked as a teaching and research assistant at Koç University, Istanbul, Turkey. Her M.S. study is titled as “Reduced cAMP-dependent protein kinase (PKA) signaling can increase the fitness of *Saccharomyces cerevisiae* cells lacking mtDNA”.