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Mitochondria as integral modulators of cellular signaling

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Mitochondria as integral modulators of cellular signaling

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Department of General and Microbial Biochemistry, Institute of Molecular and Cell Biology, Universit of Tartu, Estonia.

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LIST OF ORIGINAL PUBLICATIONS

The following original publications will be referred to in the text by their Roman numerals:

I Aun A[1], Tamm T[1], Sedman J. (2013). Dysfunctional mitochondria modulate cAMP-PKA signaling and filamentous and invasive growth of *Saccharomyces cerevisiae*. Genetics. 193(2):467–81.
[1]Authors contributed equally to this work

II Reimand J, Aun A, Vilo J, Vaquerizas JM, Sedman J, Luscombe NM. (2012). m:Explorer: multinomial regression models reveal positive and negative regulators of longevity in yeast quiescence. Genome Biol. 13(6):R55.

III Gerhold JM, Aun A, Sedman T, Jõers P, Sedman J. (2010). Strand invasion structures in the inverted repeat of *Candida albicans* mitochondrial DNA reveal a role for homologous recombination in replication. Mol Cell. 39(6):851–61.

My contribution to the articles is as follows:
Ref I Designed and performed the experiments together with Tamm T, participated in the data analysis and drafted the manuscript.
Ref II Designed and performed all the *in vivo* experiments, participated in the analysis of experimental data.
Ref III Performed the PFGE and relative mtDNA copy number experiments, participated in the fork direction experiments and in the data analysis.

ABBREVIATIONS

3-AT	3-amino-1,2,4-triazole
AAA	ATPase associated with a variety of cellular activities
ARS	autonomously replicating sequence
cAMP	cyclic adenosine monophosphate
bp	base pair
CCCP	carbonyl cyanide *m*-chlorophenyl hydrazone
CL	cardiolipin
CLS	chronological life span
DAPI	4′,6-diamidino-2-phenylindole
ERC	extrachromosomal rDNA circle
ETC	electron transport chain
FG	filamentous growth
FRE	filamentous response element
GAAC	general amino acid control
GAP	GTPase activating protein
GEF	guanine nucleotide-exchange factor
GPI	glycosyl-phosphatidyl-inositol
i-AAA	AAA that faces the *i*ntermembrane space
IMM	inner mitochondrial membrane
IMS	intermembrane space
ISC	iron-sulfur cluster
m-AAA	AAA that faces the *m*atrix side
MAPK	mitogen activated protein kinase
MNNG	*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine
MSX	L-methionine sulfoximine
MTC	mitochondrial translation control
NCR	nitrogen catabolite repressed
NGI	nuclear genome instability
OAA	oxaloacetate
OXPHOS	oxidative phosphorylation
OMM	outer mitochondrial membrane
PE	phosphatidylethanolamine
PFGE	pulsed field gel electrophoresis
PG	phosphatidylglycerol
PKA	protein kinase A
RLS	replicative life span
ROS	reactive oxygen species
RTG	retrograde
SCF	Skp1-Cullin-F-box protein
SCM	synthetic complete medium
SLAD	synthetic low-ammonium dextrose
SLIK	SAGA-like

SOD	superoxide dismutase
T7EndoI	T7 endonuclease I
TCA	tricarboxylic acid
TR	transcriptional regulator
TopoI	topoisomerase I
TOR	target of rapamycin
UAS	upstream activating sequence
wt	wild type
YPD	yeast-extract, peptone, dextrose
YPG	yeast-extract, peptone, glycerol

INTRODUCTION

Mitochondria are eukaryotic organelles that are responsible for energy production through oxidative phosphorylation (OXPHOS). They also coordinate cellular metabolism through synthesis and degradation of metabolic intermediates and compartmentalize assembly of heme and iron-sulfur clusters (ISCs). Besides these essential functions, mitochondria are increasingly recognized as important hubs in cellular signaling. On the one hand, mitochondrial function can be modulated by the activities of conserved signaling pathways, such as the cAMP mediated protein kinase A (PKA) (Cannon et al, 1990; Dejean et al, 2002; Russell et al, 1993), target of rapamycin (TOR) (Bonawitz et al, 2007; Pan & Shadel, 2009), and Snf1 signaling (Mayer et al, 2011; Ulery et al, 1994). On the other hand, it is becoming evident that mitochondrial functional state itself can serve as a signal that feeds into and modulates activities of some of the conserved signaling cascades. It has been shown that in *Saccharmyces cerevisiae* (*S. cerevisiae*) the mitochondrial dysfunction can modulate the cAMP-PKA pathway activity in autophagy inducing conditions (Graef & Nunnari, 2011). It has also been demonstrated that in yeast cells with dysfunctional mitochondria the activity of the TOR pathway becomes downregulated (Kawai et al, 2011).

Signaling pathways that can modulate mitochondrial function, including the cAMP-PKA, TOR and Snf1 signaling, have been characterized mostly in the context of sensing and mediating the nutritional signals of the environment to the cell (Zaman et al, 2008). It is therefore apparent that modulation of mitochondrial function is an integrated part of the cellular response to nutritional cues. Additionally, the same signaling pathways have been shown to be involved in the activation of a specific morphological differentiation program that leads to filamentous growth (FG) in starvation conditions (Brückner & Mösch, 2012). There is evidence for mitochondrial function also in this process (Jin et al, 2008; Kang & Jiang, 2005; Lorenz et al, 2000). While respiratory deficient mutants are defective in filament formation, it is not clear by which means mitochondria are contributing to the process. Elucidating the role of mitochondrial function in FG would provide one missing piece in understanding the regulatory events evoked upon nutrient starvation. Possible interactions that occur between functional state of mitochondria and signaling pathways can potentially add to the general knowledge of cellular signaling architecture.

The first part of this thesis provides a literature overview of rearrangements that occur in the *S. cerevisiae* cell upon loss of functional mitochondrial DNA (mtDNA). Further on, a role for mitochondrial dysfunction in modulation of yeast FG and longevity, two processes related to different starvation conditions, are reviewed. The second part of the thesis summarizes experimental work aimed to characterize cellular responses to starvation conditions. The major focus of the studies was to elucidate why mitochondrial dysfunction leads to

impaired FG response (Ref I). The interaction between the functional state of mitochondria and cAMP-PKA signaling will be discussed in the context of different strain backgrounds and possible interference points. Next, a role for several transcriptional regulators (TR) predicted *in silico* to orchestrate yeast quiescence was verified in chronological life span (CLS) experiments (Ref II). Possible mechanisms by which some of these TR may influence yeast CLS will be discussed. The study also includes topological description of *C. albicans* mtDNA (Ref III), the yeast where FG is connected to virulence determinants.

REVIEW OF LITERATURE

Mitochondria are eukaryotic organelles that contain their own genome. Phylogenomic comparisons indicate that mitochondria originate from a single endosymbiontic event from within α – Proteobacteria (Richards & van der Giezen, 2006; Yang et al, 1985) that supposedly lead to compartmentalization and formation of a eukaryotic cell (Koonin, 2010). In most animals and fungi the mitochondrial genome encodes about a dozen proteins essential for assembly of functional electron transport chain (ETC) and ribosomal RNA-s and tRNA-s of mitochondrial translation system (Gray, 2012). Altogether, mitochondria contain roughly 1000 proteins that are encoded by the nuclear genome (Pagliarini et al, 2008; Sickmann et al, 2003). They are transported to mitochondria via diverse protein import machineries that depend on the nature of protein targeting information and suborganellar localization (Chacinska et al, 2009). Although the mitochondrial genome is of monophyletic origin, comparative proteomics indicates rather complex evolutionary history of the mitochondrial proteome. Only ~10% – 15% of yeast mitochondrial proteins originate clearly from α – Proteobacterial lineage (Karlberg et al, 2000; Marcotte et al, 2000). This suggests adjustment and regulation of key mitochondrial processes according to necessity of specific lineages through recruitment of new nuclear genes (Gray, 2012).

On micrometer scale mitochondria are organized into tubular network like structures (Hoffmann & Avers, 1973). Their numbers are regulated through growth and division of pre-existing organelles. In fact, mitochondria undergo constant fusion and fission events that render mitochondrial network highly dynamic. Depending on organism, cell type and functional state mitochondria spend most of the time as either canonical solitary units (in yeast stationary phase cultures) or as an interconnected web (in exponentially growing yeast cells) (Jakobs et al, 2003; Nunnari et al, 1997; Palmer et al, 2011; Rafelski, 2013). Mitochondrial dynamics is also part of mitochondrial quality control system that targets dysfunctional mitochondria to degradation via specialized form of autophagy termed mitophagy (Twig et al, 2008; Twig & Shirihai, 2011).

On nanometer scale the mitochondrial double membrane creates four morphologically distinct compartments: outer mitochondrial membrane (OMM) which can be viewed as a first barrier between cytosol and mitochondria, intermembrane space (IMS) which contains proteins that transport ions, metabolites, lipids and proteins between mitochondrial membranes, inner mitochondrial membrane (IMM) forming the main barrier that separates the mitochondrial matrix from cytosol and which is a platform for ATP production through OXPHOS, and finally the interior of the organelle called mitochondrial matrix, a compartment that contains mtDNA, mitochondrial translation system, enzymes of mtDNA propagation and mitochondrial metabolic pathways. The best characterized metabolic pathway in the mitochondrial matrix is the

tricarboxylic acid (TCA) cycle. The mitochondrial matrix also houses anabolic pathways of heme, ISC, lipid and steroid biosynthesis and catabolic pathways of fatty acid and protein degradation (Osellame et al, 2012; Scheffler, 2001). In addition, mitochondria function as signaling platforms that generate and mediate cellular signals. Examples include activation of the retrograde (RTG) mitochondria-to-nucleus signaling in response to mitochondrial dysfunction in yeast cells (Liu & Butow, 2006) and initiation of NF-κB stress response from OMM in case of viral invasion in mammalian cells (Seth et al, 2005). Keeping in mind the plethora of mitochondrial functions, it is not surprising that mitochondria are integrated into cell fate decision making by playing part in regulation of apoptosis and longevity (Bonawitz & Shadel, 2007; Osellame et al, 2012).

Perturbation of any of the above mentioned aspects of mitochondrial metabolism, morphology, or dynamics can lead to cellular malfunction and contribute to wide range of pathologies including metabolic and degenerative diseases, cancer and aging (Palmer et al, 2011; Wallace, 2010). Since many aspects of mitochondrial metabolism and biogenesis were first examined in the budding yeast *S. cerevisiae,* it provides a valuable tool for detailed mechanistic studies of various mitochondrial functions in the cell. The ability to survive on fermentable carbon sources upon depletion of mtDNA has made this yeast particularly useful in elucidating the interplay between mitochondrial dysfunction and cellular metabolism (Baile & Claypool, 2013; Zdralević et al, 2012).

1. Respiratory deficient cells of S. cerevisiae

1.1. MtDNA of S. cerevisiae

The mitochondrial genome of *S. cerevisiae* has a unit size of 85,8 kbp and is one of the largest among Hemiascomycetes or budding yeasts (Solieri, 2010). It is characterized by the presence of long AT rich noncoding sequences, low gene density and high intron content. Pulsed field gel electrophoresis (PFGE) and electron microscopy studies indicate that in yeasts the mtDNA is composed of complex branched structures, head-to-tail concatemeres of multiple genome units, and to a small extent of circular molecules with single stranded or double stranded DNA tails (Bendich, 1996; Maleszka et al, 1991).

The mitochondrial genome of *S. cerevisiae* encodes 8 genes: one subunit of respiratory Complex III, three subunits of respiratory Complex IV, three subunits of ATP synthase Complex V (Figure 1C), and one ribosomal protein (*VAR1*). It also contains genes essential for the mitochondrial translation system: both 21S and 15S ribosomal RNAs, all 24 tRNAs, and the 9S RNA component of RNase P that is required for pre-tRNA processing (Foury et al, 1998). Depending on the strain, the mitochondrial genome of *S. cerevisiae* contains 7–8 putative replication origins or *ori* sequences (de Zamaroczy et al,

1984; Faugeron-Fonty et al, 1984). Four of the *ori* sequences (*ori1, 2, 3, 5*) hold uninterrupted transcription initiation sites and are thought to be active (Baldacci & Bernardi, 1982; de Zamaroczy et al, 1984; Foury et al, 1998). Since components of respiratory Complexes III, IV and V are encoded by the mitochondrial genome, maintenance of the mtDNA is essential for respiratory competence of the cell.

Being facultative anaerobe, *S. cerevisiae* can produce sufficient amounts of energy equivalents for growth and division via fermentation. Therefore, loss of wild type (wt) mtDNA (rho^{+}) and respiratory function is tolerated by this yeast in the presence of fermentable carbon source. Loss of functional mtDNA leads to emergence of mutants that either lack the mitochondrial genome altogether (rho^{0}) or are retaining noncoding short fragments (rho^{-}). Compared with wt cells the mutants form small so-called *petite* colonies that cannot grow on non-fermentable carbon sources (Contamine & Picard, 2000). The fact that *rho* mutants are relatively easy to identify has been utilized in many genetics studies to track down proteins involved in mtDNA metabolism. According to the *Saccharomyces* Genome Database about 300 nuclear genes encode proteins that influence maintenance and stability of the mitochondrial genome (Cherry et al, 2012). These include not only proteins directly involved in mtDNA transactions (replication, recombination, repair, transcription), but also enzymes and structural components of diverse cellular processes such as fatty acid metabolism, morphology of OMM, transport of molecules, components of OXPHOS complexes and ATP synthesis (Lipinski et al, 2010). The connection between some of these processes and mitochondrial genome stability is not understood.

Despite the plethora of factors known to participate in mtDNA metabolism, the detailed description of *S. cerevisiae* mitochondrial genome replication remains incomplete. The proposed models include transcription primed replication initiation from active *ori* elements (Baldacci & Bernardi, 1982) and initiation of replication by homologous invasion of 3′ single-stranded DNA into double-stranded circular mtDNA molecule that sets off rolling circle mode of elongation (Ling & Shibata, 2002; Maleszka et al, 1991). The picture has gotten more complicated by the finding that some DNA-maintenance enzymes essential for rho^{+} mitochondrial genome metabolism are dispensable in rho^{-} strains. Still, many enzymes directly involved in mtDNA transactions have been characterized. Some of the factors involved in mtDNA metabolism and packaging that are discussed or mentioned in this thesis include the mtDNA polymerase Mip1, deletion of which leads to complete loss of mtDNA (Genga et al, 1986); the mtRNA polymerase Rpo41 (Fangman et al, 1990; Greenleaf et al, 1986) and the helicase Hmi1 (Sedman et al, 2000) that are both required for wt mitochondrial genome stability but not for rho^{-} genome propagation.

mtDNA is organized into compact DNA-protein structures termed nucleoids that are readily visualized *in vivo* with DNA-binding dyes. There are approximately 50–70 nucleoids per diploid (Miyakawa et al, 1984) and

10–40 per haploid cell (Meeusen & Nunnari, 2003). According to the estimated number of 50 copies of the mitochondrial genome per haploid cell (Williamson, 2002), one to five equivalents of mitochondrial genome are organized as protein-DNA complexes in these structures. Compared with matrix proteins that diffuse freely in the organelles interior, the nucleoids movement appears to be restricted (Azpiroz & Butow, 1993; Nunnari et al, 1997). This is consistent with the observations that nucleoids associate with two membrane spanning replisomes (Meeusen & Nunnari, 2003) and their segregation is actively regulated non-stochastic process (Nunnari et al, 1997; Okamoto et al, 1998). Therefore the nucleoids function not only as DNA packaging centers but also as replisomes and segregational units.

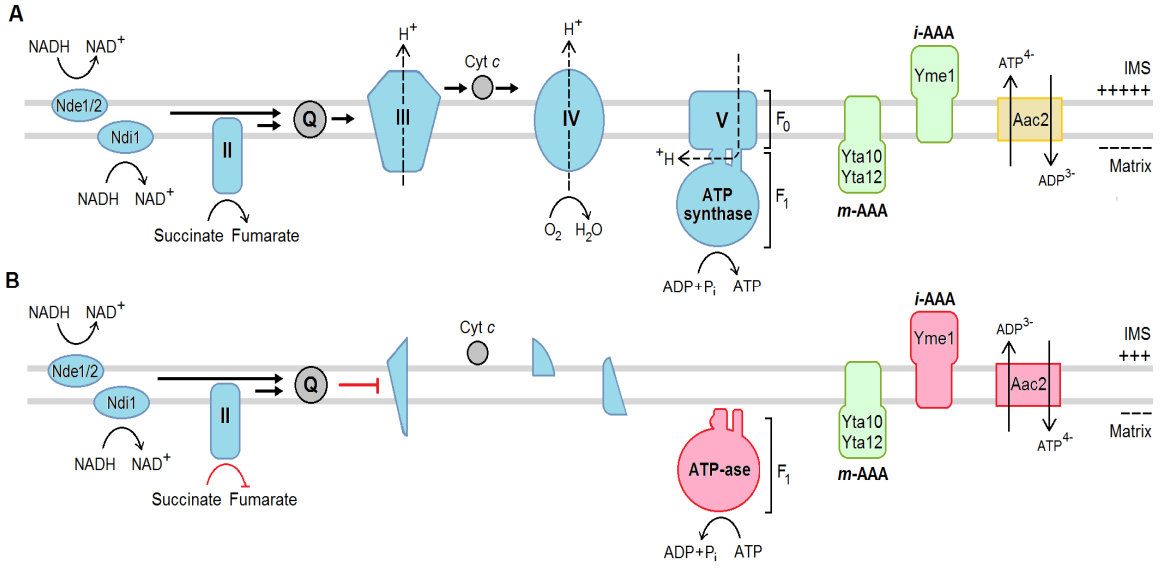
Containing approximately 30 different proteins, the composition of the *S. cerevisiae* nucleoids is surprisingly versatile (Kucej & Butow, 2007). Quite expectedly, enzymes involved in DNA metabolism constitute substantial part of the structure. However, with formaldehyde crosslinking it was shown that nucleoids also contain a number of heat shock proteins and metabolic enzymes seemingly unrelated to DNA maintenance (Chen et al, 2005; Kaufman et al, 2000). Best characterized examples include involvement of Ilv5 (enzyme involved in branched-chain amino acid biosynthesis) and Aco1 (TCA cycle enzyme) in mtDNA metabolism. Upregulation of *ILV5* by Gcn4 pathway in starvation conditions results in increased nucleoid numbers (MacAlpine et al, 2000). Upregulation of *ACO1* by HAP/RTG signaling restores DNA maintenance defects of strains deleted for *ABF2* encoding mtDNA packaging protein (Chen et al, 2005). Based largely on these findings it has been argued that this kind of protein bifunctionality enables coupling of mitochondrial metabolism to mtDNA protection and maintenance (Chen & Butow, 2005; Kucej & Butow, 2007). Indeed, it appears that nucleoids undergo remodeling in response to metabolic cues. Under respiring conditions the mtDNA ratio to Abf2 is increased and nucleoids form more open structure that can presumably facilitate transcription. In fermenting growth mode and upon amino acid starvation, Hsp60 and Ilv5 are recruited, respectively and nucleoids become more tightly packed (Kucej et al, 2008). These examples emphasize that mitochondrial metabolic and DNA maintenance functions are intertwined and can influence each other in complex ways.

1.2. Aberrant respiratory chain of *rho* mutants

As pointed out before, *S. cerevisiae* belongs to microorganisms that are classified as facultative anaerobes. Unlike typical facultative anaerobes, the budding yeast prefers fermentation even if the oxygen and functional ETC are present. In cells growing on glucose about 95% of the sugar becomes catabolized through fermentation (Lagunas, 1981). Therefore, in rich growth

conditions the *rho* mutants and wt cells derive their energy in a similar fashion. However, these two cell types differ from each other in a number of ways.

Loss of functional mtDNA results in aberrant ETC where Complexes III and IV become disorganized due to the absence of essential core subunits. Likewise, the Complex V cannot be assembled into functional ATP synthase (Figure 1). The first complex to be affected is Complex III, ubiquinol-cytochrome *c* oxidoreductase that is composed of three catalytic proteins: cytochrome *b* (Cob), cytochrome c_1 and the Rieske Fe/S protein, and seven nonredox subunits (Smith et al, 2012). Cytochrome *b* is encoded by the mitochondrial genome and forms the hydrophobic core that nucleates complex assembly. In rho^0 strains that lack Cob protein all other subunits of Complex III are either lost or diminished from IMM and functional complexes do not assemble (Zara et al, 2004). Similarly, Complex IV, the cytochrome c oxidase that catalyzes the final transfer of electrons to molecular oxygen, cannot form upon depletion of mtDNA. The catalytic core of the electron transfer is composed of three mitochondrial encoded proteins (Cox1, 2, 3) flanked by nine nuclear-encoded small subunits that are important for assembly, protection and modulation of the core activity (Soto et al, 2012). In *rho* mutants where the core of Complex IV is missing, the remaining subunits do not assemble into tightly bound complex and their binding to IMM is greatly weakened (Ebner et al, 1973). Complex V, the ATP synthase is composed of two sectors: membrane embedded F_0 sector that makes up the proton channel and F_1 sector that is linked to inner membrane and catalyzes synthesis of ATP. In yeast, the F_0 sector is composed of twelve subunits and three components of the inhibitory complex (Devenish et al, 2000). The F_1 sector is composed of five subunits (α, β, γ, δ, ε) where three α and three β subunits form a hexameric ring structure that is connected to the F_0 portion by a central stalk composed of a γ subunit and small δ and ε proteins. Proton translocation through F_0 sector drives rotation of the F_1 γ subunit that causes sequential conformational changes in the three β subunits resulting in ATP synthesis (Boyer, 1997). The mtDNA encodes the F_0 components 6 and 9 that form the proton channel and subunit 8 that has a structural role in the assembly of the sector. It may also take part in conformational changes that occur during enzyme catalysis (Devenish et al, 2000). As a result in rho^0 mutants the functional F_0 sector cannot be brought together. However, the F1 portion that is able to hydrolyze ATP (F_1-ATPase) still exists in fully assembled form in the matrix compartment (Schatz, 1968).



C

Complex II:	Complex III:	Complex IV:	Complex V:	
Sdh1 (*SDH1*)	**Cyt b (*COB*)***	**Cox1 (*COX1*)***	**F_0:**	**F_1:**
Sdh2 (*SDH2*)	**Cyt c (*CYT1*)**	**Cox2 (*COX2*)***	subunit a (*ATP6*)*	subunit α (*ATP1*)
	Rieske Fe/S	Cox3 (*COX3*)*	subunit c (*ATP9*)*	subunit β (*ATP2*)
Sdh3 (*SDH3*)	**protein (*RIP1*)**		subunit 8 (*ATP8*)*	subunit γ (*ATP3*)
Sdh4 (*SDH4*)		Cox4 (*COX4*)	subunit b (*ATP4*)	subunit δ (*ATP16*)
	Cor1 (*COR1*)	Cox5a (*COX5a*)	subunit 5 (*ATP5*)	subunit ε (*ATP15*)
	Cor2 (*COR2*)	Cox5b (*COX5b*)	subunit d (*ATP7*)	
	Qcr7 (*QCR7*)	Cox6 (*COX6*)	subunit h (*ATP14*)	**Inhibitor complex**
	Qcr8 (*QCR8*)	Cox7 (*COX7*)	subunit f (*ATP17*)	**of F_0:**
	Qcr6 (*QCR6*)	Cox8 (*COX8*)	subunit j/i (*ATP18*)	Inh1 (*INH1*)
	Qcr9 (*QCR9*)	Cox7a (*COX9*)	subunit e (*ATP21*)	Stf1 (*STF1*)
	Qcr10 (*QCR10*)	Cox9 (*COX12*)	subunit g (*ATP20*)	Stf2 (*STF2*)
		Cox10 (*COX13*)	subunit k (*ATP19*)	

Figure 1: Schematic representation of the ETC of *S. cerevisiae* wt cells and *rho* mutants. **A** – Function of Complex I which is not present in the budding yeast is replaced by three NADH dehydrogenases: Ndi1 and Nde1/2 that transfer electrons from NADH generated in matrix and cytosol, respectively to ubiquinone without concomitant proton pumping. Proton pumping through Complexes III and IV generates membrane potential ($\Delta\Psi_M$) required for ATP synthesis and mitochondrial biogenesis. Metalloprotease complexes facing the matrix side *m*-AAA (composed of Yme10 and Yme12 proteins) and the intermembrane side *i*-AAA (composed of Yme1) are required for assembly of respiratory complexes and turnover of misfolded or unassembled proteins. Major adenine nucleotide carrier is encoded by *AAC2*. **B** – In *rho* mutants the electron transfer and ATP synthesis become disrupted due to the absence of essential core subunits of Complexes III, IV and V. Proteins and complexes depicted in red become essential in *rho* cells. The generation and maintenance of membrane potential becomes dependent on Aac2 translocase, F_1 ATPase and Yme1 activities. **C** – Protein composition and respective genes of respiratory Complexes II, III, IV and V. Subunits encoded by the mitochondrial genome are denoted by asterisks; components of catalytic core involved in electron transfer are written in bold. Q – ubiquinone, Cyt *c* – cytochrome *c*, F_0 – F_0 sector of the ATP synthase, F_1 – F_1 sector of the ATP synthase, IMS – intermembrane space.

Consequently the loss of functional mtDNA leads to a stop in electron flow through ETC, proton pumping by ETC, synthesis of ATP through OXPHOS, and accumulation of nuclear encoded components of respiratory complexes (Figure 1B). Also the TCA cycle comes to a stop due to the inability of Complex II to transfer electrons to ubiquinone that becomes trapped in reduced ubiquinol (QH_2) state. Since degradation of non-assembled or damaged proteins at the IMM is essential for mitochondrial homeostasis (Baker et al, 2011) *rho*^{0} cells must likely take measures to maintain that balance.

The degradation of non-assembled or damaged proteins at IMM is carried out by two conserved AAA (ATPase associated with a variety of cellular activities) metalloproteases: *m*-AAA composed of Yta10 and Yta12 that faces the *m*atrix side and *i*-AAA composed of Yme1 that faces the *i*ntermembrane space (Arlt et al, 1996; Leonhard et al, 1996) (Figure 1A). Interestingly both proteases also show chaperon like activity. The *m*-AAA protease affects assembly of ETC complexes and ATP synthase (Arlt et al, 1998; Paul & Tzagoloff, 1995). Yme1 has been shown to bind and prevent aggregation of structurally unrelated proteins *in vitro* and deletion of *YME1* leads to increased aggregation of various IMS proteins *in vivo* (Leonhard et al, 1999; Schreiner et al, 2012). In addition to mitochondrial and nuclear encoded components of ETC, the *m*- and *i*-AAA proteases are also able to degrade various model substrates (Leonhard et al, 2000; Leonhard et al, 1996). The substrate specificity of the proteases is therefore rather degenerate and mostly dependent on the folding state of the target protein (Gerdes et al, 2012). Although studies of *m*- and *i*-AAA function have been performed in cells with functional mitochondrial genome, the general concept that they form an essential proteolytic system holds also true in *rho* mutants where the turnover of nuclear encoded unassembled proteins of IMM has to be kept under control. This is supported by the fact that Yme1 becomes essential for *rho* cell viability (Chapter 1.3. in Review of Literature) (Thorsness et al, 1993).

1.3. Genes and processes essential for *rho* cell viability

Functional ETC drives proton pumping across the IMM and generation of the membrane potential $\Delta\Psi_M$ that is required to fuel the ATP synthase. However, the $\Delta\Psi_M$ is also essential for protein import into mitochondria (Gasser et al, 1982; Schleyer et al, 1982) and consequently for mitochondrial biogenesis and cell viability. In *rho* mutants the $\Delta\Psi_M$ cannot be generated neither through proton pumping or reversible proton translocation through the F_1F_0-ATP synthase at the expense of ATP hydrolysis. Instead a third mechanism is implemented and a number of genes involved in IMM homeostasis that are non-essential in wt cell become indispensable. Deletion of those genes leads to petite-negative phenotype i.e. inability to grow without functional mtDNA (Chen & Clark-Walker, 2000).

ADP/ATP carrier Aac2 and F_1-ATPase activity

The generation of $\Delta\Psi_M$ in *rho* mutants is established by the major ADP/ATP translocator Aac2 that becomes essential in *rho* cells (Kovácová et al, 1968; Subík et al, 1972). Aac2 mediated electrogenic exchange of ATP (with a negative charge of –4, ATP^{-4}) into and ADP (with a negative charge of –3, ADP^{3-}) out of the matrix generates electric potential sufficient for cell viability but lower compared with wt mitochondria (Dupont et al, 1985) (Figure 1B). Also, *rho* mutants become dependent on the F_1-ATPase activity since disruption of genes encoding α, β, γ and δ subunits results in lethality or extremely slow growth (Chen & Clark-Walker, 1999; Giraud & Velours, 1997; Weber et al, 1995). In turn, mutations that increase the F_1-ATPase activity are able to overcome the petite negative phenotype of subunit γ deficiency (Smith & Thorsness, 2005). It has been proposed that high ATP hydrolysis rate is required to keep matrix ADP^{3-} concentration at level that supports efficient ADP^{3-}/ATP^{4-} exchange and maintenance of the $\Delta\Psi_M$ (Giraud & Velours, 1997).

i-AAA protease complex

As mentioned above, the integral IMM protease Yme1 becomes essential in *rho* cells (Thorsness et al, 1993). Likewise, deletion of *MGR1* and *MGR3* genes that encode substrate adaptors of the Yme1, leads to petite-negative phenotype (Dunn et al, 2006; Dunn et al, 2008). Although *i*-AAA complex was suggested to play an essential role in proteolytic turnover of unassembled IMM proteins that accumulate in the absence of functional mitochondrial genome (Dunn et al, 2006), the Yme1 may also have a more specific function in maintaining *rho* cell viability. Specifically, it has been proposed that the *i*-AAA protease may regulate the F_1-ATPase activity. Mutations in α and γ subunits that increase F_1-ATPase activity and elevate $\Delta\Psi_M$ suppress the slow growth phenotype of *rho* mutant *yme1Δ* cells (Kominsky et al, 2002; Weber et al, 1995). Based on these findings it was hypothesized that Yme1 may be responsible for degradation of F1-ATPase inhibitor, contributing thereby to $\Delta\Psi_M$ maintenance (Kominsky et al, 2002).

Insertion of proteins into the IMM

Besides the above mentioned proteins that play a role in $\Delta\Psi_M$ generation, components of the TIM22 translocase pathway (*TOM70*, *TIM18*, *TIM54*, conditional allele of *TIM10*) that are required for the insertion of proteins into the IMM, have also been shown to become essential in *rho* mutants (Dunn & Jensen, 2003). The petite-negative phenotype of defective TIM22 complex was shown to be suppressed by overexpression of several cytosolic proteins. These suppressors could be divided into two categories: chaperones and proteins that may bind and facilitate precursor protein import (Ssb1, Cct6, Icy1) and protein that appears to decrease the rate of protein synthesis (Tip41). Also, decreasing

the cellular translation with cycloheximide suppressed the growth defect of a *rho*$^{+}$ *tim18Δ* cells (Dunn & Jensen, 2003). These results suggest that decreased $\Delta\Psi_M$ in *rho* mutants magnifies the import inefficiency of an incomplete TIM22 complex. It was proposed that this may lead to accumulation and aggregation of mitochondrial precursor proteins in the cytosol leading to cellular toxicity. This toxicity in turn can be alleviated by overproduction of chaperones or by downregulation of the cellular protein synthesis (Dunn & Jensen, 2003).

Synthesis of mitochondrial phospholipids

Another process that becomes essential in *rho* mutants is the synthesis of mitochondrial phospholipids including phosphatidylglycerol (PG), cardiolipin (CL), and phosphatidylethanolamine (PE) (Figure 2). It has been shown that deletion of *TAM41* that encodes for mitochondrial CDP-diacylglycerol synthase leads to inability of yeast cells to grow on EtBr containing medium that depletes cells of mtDNA (Gallas et al, 2006; Tamura et al, 2013). Although similar results were earlier obtained with strains deleted for *PGS1* that cannot catalyze the first committed and rate-limiting step of CL biosynthesis (Chang et al, 1998; Janitor & Subík, 1993), it was later shown that the *pgs1Δ* cells are not "true petite-negatives" (Zhong et al, 2005). Instead they display defects of cell wall synthesis and their inability to grow on EtBr medium can be restored in the presence of osmotic stabilizer sorbitol (Zhong et al, 2005). However, no such rescue effect was seen for *tam41Δ* strains (Gallas et al, 2006). Therefore the proposed role for CL and other membrane phospholipids in the maintenance of *rho* cell viability that has before been discussed for *pgs1Δ* strains (Chen & Clark-Walker, 2000; Contamine & Picard, 2000) is still worth to be considered in the context of *tam41Δ* cells with extensions of some of the CL and PG function(s) to other lipids synthesized downstream of Tam41.

The non-bilayer-forming CL is the characteristic phospholipid of IMM that stabilizes the respiratory supercomplexes and enhances the efficiency of energy production (Claypool, 2009). CL has been shown to play a part in modulation of the ADP/ATP carrier (AAC) activity. Assays with reconstituted Aac2 demonstrate that virtually no nucleotide exchange can be detected when AAC is purified from *crd1Δ* cells (Hoffmann et al, 1994; Jiang et al, 2000). Although the absence of CL does not result in petite-negative phenotype, the concomitant increase in CL precursor, PG and the non-bilayer-forming lipid PE have been suggested to substitute for CL function in this situation (Chang et al, 1998; Zhong et al, 2004). It has been proposed that in mitochondria of *rho* mutants the enhancement of Aac2 activity by CL (and/or PG and PE) may be one critical factor to maintain $\Delta\Psi_M$ above a certain threshold (Chen & Clark-Walker, 2000; Contamine & Picard, 2000). The results from *rho*$^{+}$ *tam41Δ* mutants support the role for membrane phospholipids in AAC metabolism as the AAC assembly becomes defective in these cells (Kutik et al, 2008).

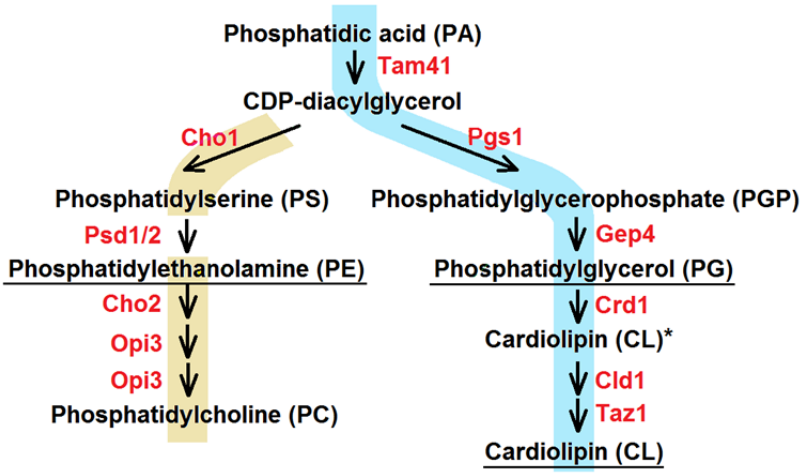


Figure 2. Cardiolipin and phosphatidylcholine synthesis pathways in *S. cerevisiae*. Enzymes are written in red. (CL)* indicates cardiolipin precursor. Blue indicates reactions that take place in mitochondria, beige indicates reactions that occur in endoplasmic reticulum (ER). Glycerolipids that are discussed in text are underlined. Pathway composition and subcellular localization of enzymes according to (Chen & Clark-Walker, 2000; Henry et al, 2012).

Cells lacking *TAM41* also show alterations in assembly of protein translocase complexes (TIM23 and TIM22) and accumulation of mitochondrial pre-proteins (Kutik et al, 2008; Tamura et al, 2013). Therefore, the petite-negative phenotype of *tam41Δ* cells may stem not only from impaired Aac2 activity but also from inefficient protein import into mitochondria. The latter may lead to cytosolic toxicity as suggested by Dunn and Jensen (2003) in the context of TIM22 deficiency in *rho* mutants. Alternatively, the impaired protein import machinery could lead to defects in mitochondrial biogenesis or deterioration of some essential IMM or matrix functions. Recently it was demonstrated that the CL deficient *rho*$^{+}$ cells are perturbed in ISC biogenesis (Patil et al, 2013). Deletion of *CRD1* led to upregulation of iron regulon genes, elevated mitochondrial iron levels, sensitivity to reactive oxygen species (ROS) and iron supplementation – all hallmarks of defective ISC assembly. It was proposed that the most likely explanation for this perturbation is the alteration of mitochondrial membranes and protein complexes that drive the mitochondrial protein import (Patil et al, 2013). The decreased $\Delta\Psi_M$ of *rho* mutants in combination with assembly defects of protein import machineries due to changed membrane phospholipid balance may therefore result in altered matrix function(s) such as the ISC biosynthesis that is essential for cell viability. Whether the impaired functions of Aac2 and protein translocation machineries in *tam41Δ* cells are additively contributing to development of petite-negative phenotype is yet to be determined.

Nuclear genome instability of rho mutants

Although increased nuclear genome instability (NGI) that occurs upon loss of mtDNA does not lead to lethality, it is one characteristic of *rho* mutants worth considering in this chapter. Early reports demonstrated that *rho* mutants display 2–3 fold increase of spontaneous mutations in their nuclear genome (Flury et al, 1976; Von Borstel et al, 1971). Subsequent scrutiny led to the proposal that this mutagenic nature is mediated through nuclear error-prone translesion DNA synthesis (Rasmussen et al, 2003). This suggestion was based on the finding that deletion of DNA polymerase zeta complex subunits *REV1, 3* and *7* that play a part in fixation of most of the spontaneous mutations occurring in the genome, leads to suppression of mutagenesis in rho^0 strain (Rasmussen et al, 2003).

Recently, much higher NGI in *rho* mutants has been demonstrated. When the chromosomal breakage or chromosomal loss (instead of point mutations) was measured, 10–30 fold increase in NGI was observed (Dirick et al, 2014; Veatch et al, 2009). Therefore, it appears that most of the nuclear DNA damage that occurs in *rho* cells results from DNA breakage. It was shown by Gottschling and co-workers that the main parameter that influences the NGI in rho^0 cells is not the loss of respiratory capacity but a decreased $\Delta\Psi_M$ (Veatch et al, 2009). It was also observed that the NGI was paralleled by increase in cellular iron content and a specific transcriptional signature both being characteristic of cells with defective ISC biosynthesis. Also, repression of *NAR1* gene function that mediates packaging of ISC into non-mitochondrial proteins led to increased NGI (Veatch et al, 2009). Previously, it has been shown that iron incorporation into the cytosolic ISC containig protein Leu1 is considerably decreased upon CCCP uncoupling causing depletion of $\Delta\Psi_M$ (Kispal et al, 1999) and the enzymatic activities of mitochondrial ISC containing aconitase and succinate dehydrogenase are reduced up to 4-fold in rho^0 cells (Kaut et al, 2000). Taking into account these reports it was suggested by Gottschling and co-workers that overall iron metabolism becomes compromised in *rho* mutants due to reduced $\Delta\Psi_M$ (Veatch et al, 2009). This in turn was proposed to lead to decreased concentration of mature ISC containing proteins involved in DNA repair pathways (Veatch et al, 2009). Interestingly, recent findings have shown that ISC clusters are also bound by nuclear replicative polymerases being important in polymerase complex stabilization (Netz et al, 2012). Altogether it is becoming increasingly clear that both replication and repair of nuclear DNA depend tightly on cellular iron metabolism. The mitochondrial and cytosolic factors that influence ISC synthesis are therefore recognized as important regulators influencing both of these processes (Waisertreiger et al, 2012). This emphasizes possible functional link between mitochondrial dysfunction, impaired ISC biosynthesis and NGI.

The NGI of *rho* mutants has also been shown to be influenced by metabolic and environmental conditions (Dirick et al, 2014). Increased pH of the growth medium that has been shown to improve growth properties of rho^0 cells

(Garipler & Dunn, 2013) also led to decreased NGI in *rho*^{0} cells (Dirick et al, 2014). This effect was thought to take place through increased $\Delta\Psi_M$ of *rho* mutants that may lead to improved ISC metabolism. However, some of the environmental factors, like elevated levels of glucose or ethanol were suggested to negatively influence NGI through metabolic byproduct dependent DNA damage (Dirick et al, 2014). Whether and which metabolic byproducts could influence the genome stability in *rho* mutants is yet to be determined.

Altogether, the viability and well-being of *rho* mutants becomes dependent on a number of factors that are involved in the maintenance of $\Delta\Psi_M$, IMM composition and protein import. It seems that these aspects of IMM homeostasis have lost some of the robustness in *rho*^{0} cells and become more fragile to disturbances. This in turn could potentially affect processes occurring not only at the IMM but also in the mitochondrial matrix, the cytosol or the nucleus.

2. RTG signaling pathway

2.1. Rearranged nuclear gene expression of *rho* mutants

In *rho*^{0} cells considerable changes in nuclear gene expression take place to cope with the loss of respiratory chain activity and concomitant stop in the TCA cycle. In general, petite cells show increased expression of genes that function in mitochondrial biogenesis, iron regulation, TCA cycle, peroxisomal metabolism, nutrient uptake, stress response and drug resistance (Devaux et al, 2002; Epstein et al, 2001; Hallstrom & Moye-Rowley, 2000; Hughes et al, 2000; Traven et al, 2001). Several communication routes from mitochondria to nucleus have been described. For one, the intergenomic signaling that is triggered in the absence of mtDNA but not upon respiratory deficiency in the presence of functional mitochondrial genome leads to downregulation of nuclear genes encoding components of respiratory complexes (Dagsgaard et al, 2001; Woo et al, 2009). This suggests that the intergenomic signaling coordinates expression of mitochondria and nuclear encoded genes and decreases futile attempts to assemble respiratory complexes in the absence of mtDNA (Woo et al, 2009). Second, the RTG pathway is activated in response to the lack of respiration and stop of the TCA cycle to reconfigure cellular metabolism (Liu & Butow, 2006). Since a number of genes induced in *rho* mutants do not show dependence neither on RTG nor intergenomic signaling, other yet undescribed pathways between two genomes may exist (Epstein et al, 2001; Woo et al, 2009).

2.2. RTG pathway in metabolic reconfiguration

The communication route from mitochondria to nucleus that is most thoroughly studied to date, both functionally and component-wise, is the RTG signaling pathway. It was first proposed that the retrograde response is evoked as an adaptation to reduced mitochondrial function and stop of the TCA cycle to reconfigure cellular metabolism and compensate for production of biosynthetic intermediates e.g. glutamate that serves as the nitrogen donor in biosynthetic reactions (Liu & Butow, 1999; Liu & Butow, 2006; Small et al, 1995) (Figure 3).

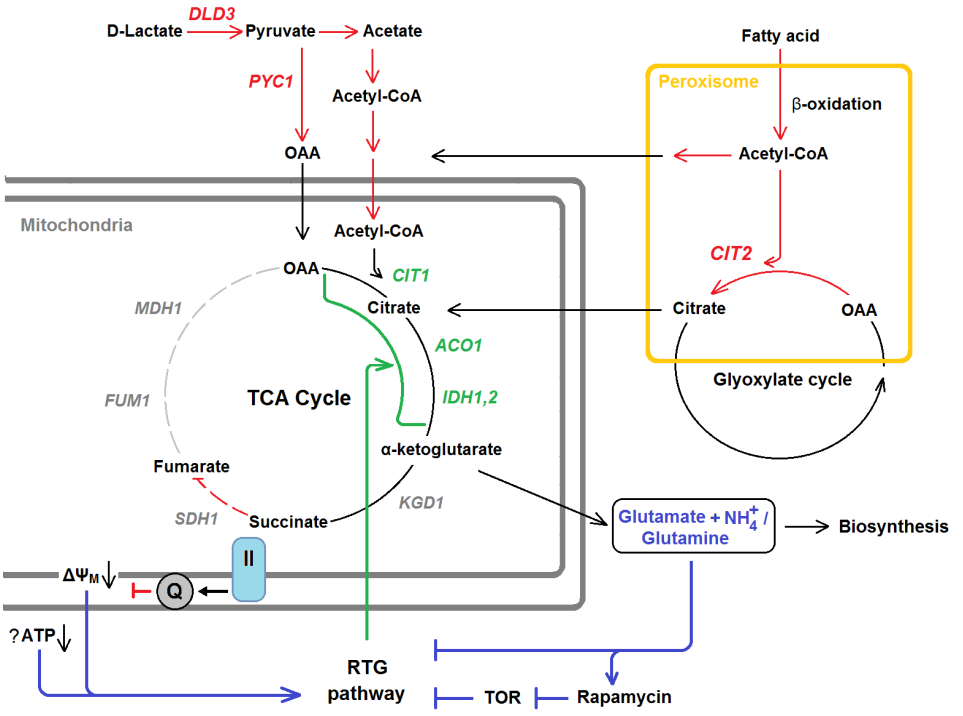


Figure 3. Metabolic adaptation in cells with dysfunctional mitochondria. Genes that become directly regulated by the RTG pathway are shown in green (switch from HAP control to RTG) or red (become upregulated), TCA genes that become repressed in *rho*^{0} mutants are shown in gray. Red arrows indicate metabolic pathways that are induced in *rho*^{0} cells according to transcriptional profiling or direct measurements of gene expression (Chelstowska et al, 1999; Epstein et al, 2001). Red dotted line indicates stop in the TCA cycle in *rho*^{0} mutants due to compromise at the level of succinate dehydrogenase (*SDH1*). Potential signals and pathways that activate or repress the RTG pathway are shown with blue arrows and T-bars, respectively. Modified from (Liu & Butow, 2006).

In *rho* mutants the TCA cycle comes to a halt due to compromised succinate dehydrogenase (Complex II) activity and reactions that convert succinate to oxaloacetate (OAA) cease to operate. As a result the transcriptional regulation of the first three steps of the TCA cycle (enzymes encoded by *CIT1, ACO1, IDH1,2*) switches from Hap2-5 control to RTG pathway to maintain sufficient synthesis of α-ketoglutarate, a precursor of glutamate (Liu & Butow, 1999). Genome wide transcriptional profiling of *rho* mutants indicates that the supply of citrate, acetyl-CoA, and OAA become replenished by upregulation of genes involved in fatty acid β-oxidation and glyoxylate cycle in peroxisomes (e.g. *CIT2*), genes involved in acetyl-CoA synthesis and transport to mitochondria, and induction of anapleurotic reaction of OAA synthesis from pyruvate (by upregulation of pyruvate carboxylase *PYC1*) (Epstein et al, 2001; Liu & Butow, 2006). In consequence the peroxisomal, cytoplasmic and mitochondrial metabolic fluxes become reconfigured to support the synthesis of adequate levels of α-ketoglutarate, glutamate and glutamine.

The prototypical target gene of the RTG pathway is the peroxisomal isoform of citrate synthase, *CIT2* that becomes induced up to 30 fold in cells with dysfunctional mitochondria on raffinose medium (Liao et al, 1991). The maximal induction of *CIT2* mRNA can be observed in rho^0 cells, whereas somewhat smaller increase takes place in wt cells where the respiratory chain is inhibited with antimycin A or the genes encoding TCA cycle enzymes Cit1 or Mdh1 are deleted (Chelstowska & Butow, 1995; Liao et al, 1991). Importantly the basal expression level and magnitude of *CIT2* mRNA induction upon mitochondrial dysfunction depend on growth conditions and yeast strain background (Dilova & Powers, 2006; Kirchman et al, 1999; Liao et al, 1991). Therefore, the mere absence of mtDNA is not sufficient nor exclusively required to increase the *CIT2* expression. It appears that inputs from nutritional status and/or strain specific differences also play a role in this process (Chapter 2.4. in Review of Literature).

2.3. RTG pathway components

The RTG pathway positive (Rtg1-3, Grr1) and negative (Mks1, Lst8, Bmh1/2) regulators (Figure 4) were first identified with genetic screens for mutants that showed decreased *CIT2* promotor-driven reporter gene expression or bypassed the requirement for pathway positive regulator Rtg2 for reporter activity (Jia et al, 1997; Liao & Butow, 1993; Liu et al, 2001; Liu et al, 2003; Liu et al, 2005; Sekito et al, 2002).

The sensor proximal to mitochondrial dysfunction is Rtg2, a cytoplasmic protein with N-terminal ATP binding motif that shares some sequence similarity with bacterial polyphosphatases (Liao & Butow, 1993). According to genetic and transactivation studies the Rtg2 acts upstream of transcription factors Rtg1 and Rtg3 (Liao & Butow, 1993; Rothermel et al, 1995; Rothermel

et al, 1997) and is required for pathway activation by sequestering negative regulator Mks1 (Liu et al, 2003). In addition, it has been shown that Rtg2 protein can be purified from yeast whole-cell extracts as a component of SAGA-like (SLIK) histone acetyltransferase-coactivator complex. This complex behaves as transcriptional stimulator and binds to *CIT2* promoter. Therefore, the Rtg2 seems to have an auxiliary role in positive regulation of the pathway as the facilitator of target gene transcription (Pray-Grant et al, 2002).

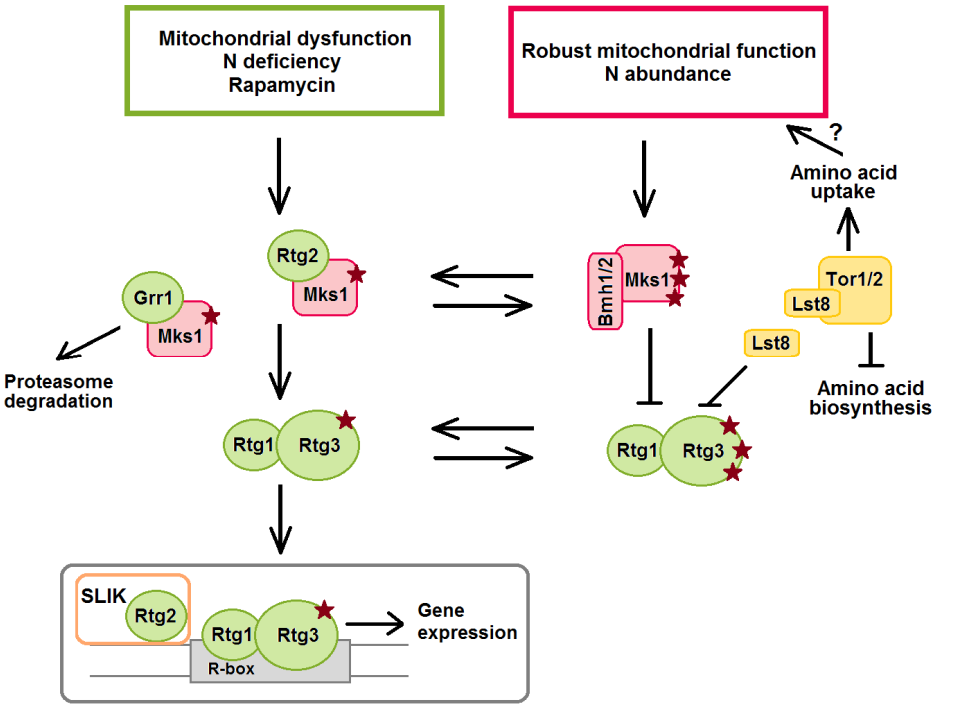


Figure 4. RTG signaling pathway. Positive regulators of the RTG signaling are shown in green, negative in red, TOR pathway components in beige. Stars indicate phosphorylation. A signal from dysfunctional mitochondria and/or metabolic intermediates is transduced to Rtg2 and/or Mks1. Mks1 becomes hypophosphorylated and sequestered by Rtg2, followed by hypophosphorylation of the Rtg3 and translocation of the Rtg1-Rtg3 to the nucleus that leads to transcription of target genes. When the pathway is inactive, e.g. in cells with robust mitochondrial function in rich growth condition the negative regulator of the pathway, Mks1, is released from Rtg2 inhibition, becomes hyperphosphorylated and forms a complex with Bmh1/2 proteins. Rtg3 becomes hyperphosphorylated and Rtg1-Rtg3 are retained in the cytoplasm. Lst8 acts as a negative regulator of the pathway both upstream and downstream of Rtg2, possibly as a component of the TOR complex. Grr1 functions to polyubiquinate and target free Mks1 to degradation.

Rtg1 and Rtg3 are basic helix-loop-helix leucine zipper (bHLH-Zip) transcription factors that bind as heterodimers to R-box (GGTCAC) sequence in *CIT2* and *DLD3* promoters and activate transcription through two transactivating domains of Rtg3 (Chelstowska et al, 1999; Jia et al, 1997; Rothermel et al, 1995; Rothermel et al, 1997). When the RTG pathway is inactive, the Rtg1-Rtg3 proteins are located in the cytoplasm and Rtg3 is hyperphosphorylated (Komeili et al, 2000; Sekito et al, 2000). Upon pathway activation, Rtg3 is dephosphorylated and Rtg1-Rtg3 localize to the nucleus (Dilova & Powers, 2006; Komeili et al, 2000; Sekito et al, 2000). However, the Rtg3 phosphorylation state does not correlate strictly with *CIT2* expression, since longer periods of rapamycin treatment that otherwise activates the pathway causes hyperphosphorylation of the Rtg3 while the *CIT2* expression remains high (Dilova & Powers, 2006). Deletion of *RTG1* and *RTG3* results in glutamate auxotrophy of rho^0 mutants and wt cells with repressed mitochondrial function (wt cells grown on glucose medium), indicating a role for the RTG pathway in cellular glutamate (nitrogen) homeostasis (Jia et al, 1997; Liao & Butow, 1993; Liu & Butow, 1999).

The changes in Rtg1-Rtg3 localization are paralleled by dynamic interaction between Rtg2 and Mks1 (Dilova et al, 2002; Liu et al, 2003; Sekito et al, 2002; Tate et al, 2002). Mks1 is a cytoplasmic phosphoprotein and the phosphorylation pattern of the Mks1 has been shown to change in RTG pathway inducing conditions (Dilova et al, 2002; Sekito et al, 2002). Hypophosphorylation of Mks1 correlates with the association with Rtg2 and significant *CIT2* gene expression (Dilova et al, 2004; Liu et al, 2003; Sekito et al, 2002). Therefore, a central role for Mks1 in the integration of pathway activating signals has been proposed (Dilova et al, 2004). When the RTG pathway is inactive, the Mks1 becomes hyperphosphorylated, forms a complex with two redundant 14-3-3 proteins Bmh1/2 and the transcription factors Rtg1-Rtg3 are retained in the cytoplasm (Liu et al, 2003). Free Mks1 that is not complexed with either Rtg2 or Bmh1/2 becomes polyubiquitinated and targeted for degradation by Grr1, a component of the SCF ubiquitin-ligase complex. It has been proposed that keeping the unbound Mks1 level low contributes to a tight on-off control of the pathway (Liu et al, 2005).

Another negative regulator of the RTG signaling is Lst8, an essential protein that forms a complex with Tor1 and Tor2 proteins and provides a link between RTG and TOR signaling (Chen & Kaiser, 2003; Liu et al, 2001). Different mutant alleles of Lst8 regulate *CIT2* expression and rescue glutamate auxotrophy of the cell acting both upstream and downstream of Rtg2 (Liu et al, 2001). This suggests a dual regulation of the RTG signaling by Lst8. It also supports the finding that RTG dependent gene expression is one effector of the TOR signaling (Shamji et al, 2000).

2.4. Activation of the RTG pathway

The actual signals that trigger the RTG response have not been firmly established despite extensive description of RTG pathway components after the discovery that mitochondrial dysfunction elicits changes in nuclear gene expression. It has been shown that there is an inverse relationship between $\Delta\Psi_M$ and RTG pathway activity. By monitoring *CIT2* induction and Rtg3-GFP localization, it was shown that deletion of *COX4* from wt cells leads to both decreased $\Delta\Psi_M$ and activation of the RTG pathway (Miceli et al, 2011). RTG signaling could be downregulated in *rho* cells with *ATP1-111* mutation that generates higher $\Delta\Psi_M$ due to increased F_1-ATPase activity (Francis et al, 2007; Miceli et al, 2011). However, the mechanism by which decreased $\Delta\Psi_M$ triggers the RTG response remains to be resolved. One candidate molecule of RTG signaling induction is ATP. In cellular lysates, ATP disrupts the interaction between Rtg2 and Mks1 within a narrow range of physiological concentrations (Zhang et al, 2013). Since the integrity of the Rtg2 ATP-binding domain is required for Mks1 binding, it is attractive to speculate that the Rtg2 may sense the intracellular ATP levels (Liu et al, 2003; Zhang et al, 2013). However, direct evidence for such a mechanism is missing.

Although first studied in *rho*^{0} background, the RTG pathway can also be activated in wt cells that are grown on the repressive carbon source glucose (Liu & Butow, 1999), in cells that are shifted to certain nitrogen media (Tate et al, 2002), or treated with growth inhibitor rapamycin that inactivates the TOR pathway and mimics nitrogen starvation (Komeili et al, 2000). The RTG dependent genes are efficiently repressed in *rho* mutants by the addition of glutamate to the growth medium, indicating that a negative feedback loop from balanced nitrogen metabolism downregulates the pathway (Liu & Butow, 1999). Therefore a more general role for the RTG response in sensing and regulating cellular nitrogen levels seems to emerge and signal(s) deriving from the metabolic state of the cell are likely to feed into the pathway.

It has been proposed that either NH_4^+ (as positive regulator) or glutamine (as negative regulator) behave as molecules that modulate RTG pathway activity. For one, in some yeast strains *CIT2* induction can be observed in wt cells that are grown on nitrogen sources that are degraded to ammonia (urea, allantoin, NH_4^+, glutamine) but not on glutamate or proline that is degraded to glutamate (Tate & Cooper, 2003; Tate et al, 2002). Also, *CIT2* induction by rapamycin requires that NH_4^+ or amino acids that can be degraded to NH_4^+ (arginine, glutamine) are present in the growth medium, suggesting that inhibition of the RTG pathway by TOR signaling is relieved only when ammonia is present (Dilova et al, 2004). However, since deletion of the transcriptional regulator *URE2* that leads to increased intracellular level of NH_4^+ does not influence *CIT2* expression significantly, the sole increase in NH_4^+ is not sufficient to trigger RTG signaling (Dilova et al, 2004). Moreover, since proline (which is degraded to glutamate) can elicit RTG signaling with and/or without rapamycin in some

strain backgrounds (Tate & Cooper, 2003), the role for NH_4^+ in RTG pathway regulation remains ambiguous.

The treatment of cells with the glutamate analogue MSX that inhibits glutamine synthesis and deletion of glutamine synthetase *GLN1* leads to increased *CIT2* expression, suggesting that glutamine (and not glutamate) starvation acts as a potent RTG pathway inducer (Crespo et al, 2002; Dilova et al, 2004). Since relative intracellular levels of α-ketoglutarate, glutamate, ammonia and glutamine are influenced by their inter-conversion, it is difficult to separate regulatory roles that each of these molecules or their relative ratios play in RTG signaling.

In addition, histidine starvation brought about by 3-AT, a competitive inhibitor of His3, can induce nuclear localization of Rtg3-GFP and expression of *CIT2* (Giannattasio et al, 2005). This induction is independent of glutamate repression, general amino acid control pathway (GAAC), and Gln3 that activates nitrogen catabolite repressed (NCR) genes. The mechanism by which histidine starvation elicits the RTG pathway remains to be resolved (Giannattasio et al, 2005).

3. Mitochondrial dysfunction in filamentous and invasive growth of *S. cerevisiae*

3.1. Induction of filamentous and invasive growth

Depending on specific nutritional conditions, the metabolism and morphology of *S. cerevisiae* is programmed to achieve the most optimal response (Figure 5). In rich media, the ovoid yeast cells undergo rapid mitotic growth until one or more nutrient(s) become limiting. Starvation for a single nutrient leads to growth arrest and entrance into stationary phase, or quiescence. Upon severe starvation when one essential nutrient (nitrogen, phosphate or sulfur) and glucose become depleted but the non-fermentable carbon source is still present, the diploid yeast cells initiate sporulation. When the nitrogen or carbon source drop to levels that limit rapid growth but are not yet completely depleted, both haploid and diploid cells can switch to FG (Zaman et al, 2008).

Extensive studies of FG in budding yeast started more than two decades ago when it was shown that the ∑1278 diploid strain can initiate FG on nitrogen-poor media (Gimeno et al, 1992). During the switch to FG considerable changes in cellular gene expression, metabolism and morphology take place. As a result, the otherwise ovoid yeast cells elongate, switch to unipolar budding pattern and remain physically attached due to increase in cell wall adherence (Gimeno et al, 1992; Lo & Dranginis, 1998). FG is stimulated on nitrogen-poor media even further with aromatic alcohols (Chen & Fink, 2006) and is strongly enhanced in nitrogen-rich media when glucose is replaced with sucrose (Van de Velde & Thevelein, 2008). The morphological readjustments that occur upon these

specific nutritional conditions contribute to formation of pseudohyphae that spread from the colony and are able to penetrate the growth substrate. The latter is termed invasive growth. Haploid yeast cells undergo FG on media containing short chain alcohols (Dickinson, 1996; Lorenz et al, 2000) and can switch to invasive growth upon glucose depletion (Cullen & Sprague, 2000; Roberts & Fink, 1994). On semisolid medium the yeast colony undergoes remarkable expansion that leads to mat (or biofilm) formation (Reynolds & Fink, 2001). The switch to filamentous and invasive growth is thought to facilitate foraging for nutrients and/or escaping the unfavorable growth environments.

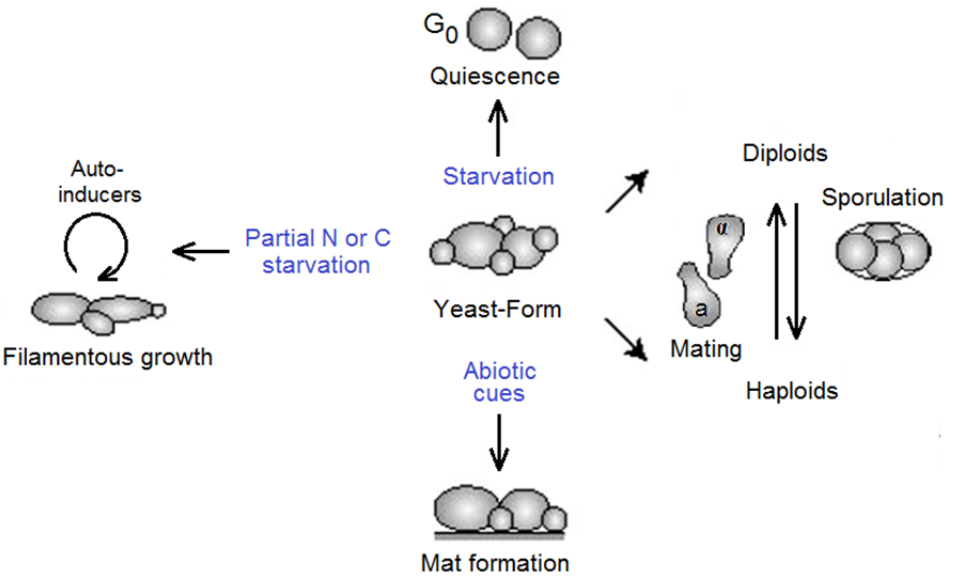


Figure 5: Response of *S. cerevisiae* cells to different environmental conditions. Both haploid and diploid cells can enter G0 quiescent state upon nutrient (carbon, nitrogen, phosphate, or sulfur) starvation, switch to FG when nutrients (nitrogen or carbon) become scarce but are not yet depleted, or initiate biofilm formation upon certain abiotic cues. Secreted alcohols act as auto-inducers of FG. Haploid cells can mate with opposite mating type cells to form diploids that in turn can undergo sporulation. Sporulation occurs in specific nutritional conditions that have to meet three criteria: absence of essential growth nutrient (such as nitrogen, phosphate or sulfur) that leads to arrest in G1, the absence of glucose, and the presence of non-fermentable carbon source. Picture modified from (Cullen & Sprague, 2012), conditions that induce specific responses are reviewed in (Zaman et al, 2008).

In opportunistic commensal fungi the switch to FG has been connected to pathogenicity (Lengeler et al, 2000; Sudbery, 2011). *Candida albicans* (*C. albicans*), the common member of skin and mucosal flora can grow vegetatively in at least three morphogenetic forms. In addition to yeast and pseudohyphal growth, *C. albicans* can also acquire hyphal growth mode where cells form long filaments with no constrictions at the junctions of neighboring cells. The morphological plasticity, especially the switch between yeast and hyphal growth modes, has been shown to be important virulence determinant of that opportunistic fungus (Sudbery, 2011). Signaling pathways that regulate switch to filamentous and invasive growth as well as some of the invasion characteristics of filamentous cells are conserved between *C. albicans* and *S. cerevisiae*. Therefore the FG has remained under extensive study in *S. cerevisiae* as it is a non-pathogenic microorganism with more easily tractable genetics.

Common laboratory strains of *S. cerevisiae* have lost the ability to undergo morphological differentiation to FG mode. This has probably happened due to selection of strains for the ease of manipulation during laboratory cultivation (Liu et al, 1996). Therefore, in *S. cerevisiae* the FG is studied in strains with certain genetic backgrounds, typically ∑1278 or SK1.

3.2. Regulation of filamentous and invasive growth

To understand the mechanism of FG regulation, much effort has been focused on how different nutrient sensing signal transduction pathways are coordinated and signals between them integrated to achieve specific response (Cullen & Sprague, 2012). The genome wide transcriptional profiling (Prinz et al, 2004) and recent screen of ∑1278b deletion mutant collection (Ryan et al, 2012) indicate that ~10–15% of the genes in the yeast genome have a potential role in FG, invasion and biofilm formation. Although all these responses are regulated by slightly different stimuli and require somewhat distinct sets of genes to show a full phenotype, these responses also share a number of overlapping regulatory and effector proteins (~ 60 genes) that make up the core components of differentiation program (Ryan et al, 2012). Despite the fact that hundreds of genes become regulated upon FG, invasion and biofilm formation and many of them are required for all three responses to occur, the canonical target *FLO11/MUC1* has been exploited most often as solitary transcriptional and translational readout of all three processes.

Flo11 is a cell surface glycoprotein that mediates adhesion to other cells and abiotic surfaces and is therefore required for invasive and filamentous growth and biofilm formation (Lo & Dranginis, 1996; Lo & Dranginis, 1998; Reynolds & Fink, 2001). Flo11 is attached to the cell wall by its glycosyl-phosphatidyl-inositol (GPI) anchor and is thought to mediate adhesive interactions by increasing the cell surface hydrophobicity (Brückner & Mösch, 2012; Douglas

et al, 2007; Reynolds & Fink, 2001). Most of the total Flo11 is shed from the cell surface, a process that presumably allows dynamic regulation of cell adherence properties and formation of an extracellular matrix-like material (Karunanithi et al, 2010). The *FLO11* promoter is one of the largest in the budding yeast genome, spanning more than 3kb. It contains many upstream activating sequences (UAS) and repression elements (Lo & Dranginis, 1996; Rupp et al, 1999) and is regulated by more than twenty transcriptional regulators and chromatin remodelling factors that are therefore all linked to FG regulation (Brückner & Mösch, 2012). Two most extensively studied signaling cascades that are essential for *FLO11* expression and filamentous and invasive growth are the FG specific MAPK and the cAMP activated PKA pathways (Figure 6).

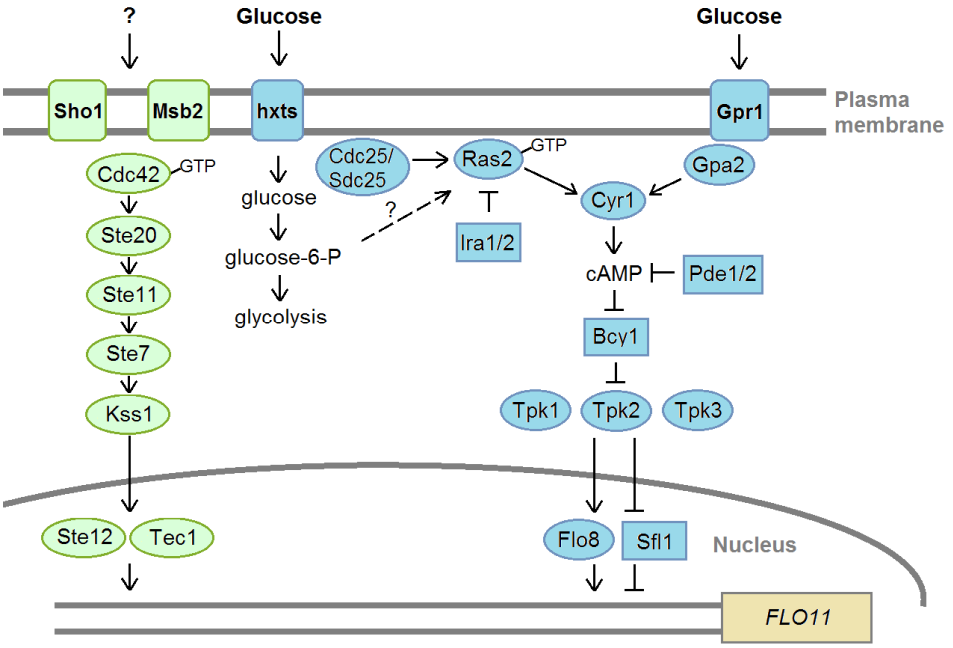


Figure 6. The FG specific MAPK and cAMP-PKA pathways converge on the promoter of *FLO11* to regulate filamentous and invasive growth. The components of FG MAPK are depicted in green and the components of cAMP-PKA signaling are depicted in blue. Arrows indicate positive regulation, T-bars indicate inhibition. Dashed line represents interaction which may not be direct. Ovals indicate positive, rectangles negative regulators of the pathway. Transcription factors are positioned to the nuclear compartment.

The FG specific MAPK pathway

Briefly, the core of FG specific MAPK cascade is composed of Ste20, Ste11, and Ste7 kinases and the pathway-specific MAPK Kss1 (Liu et al, 1993; Roberts & Fink, 1994) (Figure 6, depicted in green). The pathway activation depends on the transmembrane osmosensor Sho1 and the mucin family member Msb2 that form a complex with active GTP-bound Cdc42 (Cullen et al, 2004; Cullen et al, 2000). Cdc42 binds to Ste20 and this interaction is required for localization of the Ste20 to the plasma membrane and induction of FG (Leberer et al, 1997; Peter et al, 1996). Subsequent signaling through the Ste20→Ste11→Ste7→Kss1 kinase cascade leads to activation of the transcription factors Tec1 and Ste12, followed by their cooperative binding to the filamentous response elements (FREs) in target gene promoters (Madhani & Fink, 1997). Among other targets, FREs are found in the promoters of *FLO11, MSB2* and *TEC1* (Lo & Dranginis, 1998; Madhani & Fink, 1997). Induction of the latter two genes indicates positive feedback regulation of the pathway activity.

The cAMP-PKA pathway

The cAMP activated PKA pathway is a critical sensor of glucose availability in eukaryotic cells (Figure 6, depicted in blue). Activation of the cAMP-PKA signaling and components of the pathway have been extensively summarized in many reviews of nutritional connection to yeast growth (Broach, 2012; Cullen & Sprague, 2012; Santangelo, 2006; Thevelein & de Winde, 1999; Zaman et al, 2008). In essence, the pathway coordinates cell cycle progression and cell mass accumulation in response to glucose through stimulation of glycolysis and ribosome biogenesis, and through repression of genes that are involved in stress response, glyconeogenesis and metabolism of storage carbohydrates (Thevelein & de Winde, 1999; Zaman et al, 2008). The cAMP-PKA pathway is activated when extra- and/or intracellular signals stimulate the adenylate cyclase Cyr1 (Casperson et al, 1985; Kataoka et al, 1985). This is followed by a rapid but transient increase in the intracellular cAMP level (van der Plaat, 1974). cAMP binds to the PKA regulatory subunit Bcy1, dissociating thereby a tetrameric complex that consists of two inhibitory Bcy1 and two catalytic PKA subunits (Toda et al, 1987a; Toda et al, 1987b). The catalytic subunits are encoded by three closely related genes, *TPK1*, *TPK2* and *TPK3*, that are redundant for viability (Toda et al, 1987b). The cAMP level is determined by the balance between Cyr1 mediated synthesis and phosphodiesterase Pde1 and Pde2 mediated hydrolysis. Pde1 is a low affinity phosphodiesterase that downregulates cAMP peaks evoked by pathway activation (Ma et al, 1999; Nikawa et al, 1987). Pde2 is a high affinity phosphodiesterase that appears to control the basal cAMP level in the cell (Ma et al, 1999; Sass et al, 1986).

In yeast cells, two stimuli trigger the PKA pathway activation: intracellular acidification (Thevelein et al, 1987a) and addition of glucose to derepressed

cells (Thevelein et al, 1987b; van der Plaat, 1974). These stimuli activate Cyr1 either through the GTP-binding Ras1 and Ras2 proteins (Toda et al, 1985) or the Gpr1-Gpa2 receptor system (Colombo et al, 1998; Kraakman et al, 1999; Kübler et al, 1997). It has been shown that intracellular acidification works through Ras activation (Colombo et al, 1998). Glucose activation of cAMP synthesis requires both the Gpr1-Gpa2 receptor system and Ras proteins (Colombo et al, 1998). First, extracellular glucose has to be detected by the Gpr1-Gpa2 receptor system. Second, intracellular glucose must be phosphorylated by hexose kinases and Ras proteins must be activated (Rolland et al, 2000). The molecular mechanisms of Ras activation upon intracellular acidification and glucose signaling are not well understood (Vandamme et al, 2012).

The Ras1 and Ras2 proteins in yeast are highly homologous and their activity is essential for viability (Kataoka et al, 1984). While single deletion of *RAS1* or *RAS2* is tolerated, the double deletion of these genes is lethal (Kataoka et al, 1984). Ras proteins become activated by binding GTP. The GDP/GTP exchange is stimulated by the guanine nucleotide-exchange factors (GEFs) Cdc25 and Sdc25 that act as positive regulators of Ras signaling. The intrinsic GTPase activity of Ras proteins is enhanced by GTPase activating proteins (GAPs) Ira1 and Ira2, that in turn act as negative regulators of Ras activity (Thevelein & de Winde, 1999). Ras proteins undergo extensive post-translational modifications, including palmitoylation and farnesylation (Bhattacharya et al, 1995). Palmitoylation is required for localization of Ras2 to the plasma membrane and induction of transient cAMP peak by glucose. This modification, however, is not essential for cell viability. Farnesylation on the other hand is required for essential function of the Ras2 protein (Bhattacharya et al, 1995). Recent localization studies indicate that Ras2 is also associated with ER and mitochondrial membranes (Belotti et al, 2012; Wang & Deschenes, 2006) and can localize to nuclear compartment (Broggi et al, 2013a). Moreover, since the regulators of Ras activity, Cdc25 and Ira2, and the Ras target Cyr1 were shown to localize to ER and in case of Ira2 also to mitochondrial membranes, these localizations are probably of functional importance (Belotti et al, 2012). Therefore, the compartmentalization of Ras proteins in yeast may play a regulatory role, a situation resembling compartmented cAMP signaling in higher eukaryotes (Beavo & Brunton, 2002).

Constitutive expression of Ras2 or Gpa2 leads to at least three fold change in expression level of approximately 20% of the genes in the yeast genome (Wang et al, 2004). Although most of these genes were shown to be redundantly regulated by other pathway(s), Ras2 and Gpa2 were demonstrated to signal exclusively through PKA. The analysis of protein phosphorylation patterns has identified 364 direct targets for PKA (Ptacek et al, 2005). While the three TPKs are redundant for viability (Toda et al, 1987b), the overlapping set of phosphorylated target proteins is surprisingly small. Only 8 proteins are recognized by all three Tpks (Ptacek et al, 2005). The vast majority of

substrates are recognized by only one of the Tpks, indicating unique modulatory roles for each PKA variant in the cell. In the context of FG, only Tpk2 has been shown to be directly required for diploid pseudohyphal differentiation, while Tpk1 and Tpk3 show inhibitory effects (Pan & Heitman, 1999; Robertson & Fink, 1998).

3.3. Mitochondrial function and filamentous or invasive growth

Screens to find novel modulators of FG have identified several non-overlapping sets of mitochondrial proteins to be involved in the process (Jin et al, 2008; Kang & Jiang, 2005; Lorenz et al, 2000). As summarized in Table 1, deletion of genes involved in various mitochondrial functions such as mitochondrial translation, mitochondrial genome maintenance, components of ETC and mitochondrial fusion machinery can lead to reduced invasive growth and/or filament formation. It has also been shown that the mitochondrial mass is increased upon isoamyl alcohol induced filamentation (Kern et al, 2004). It was proposed that this would prepare the cells for efficient degradation of non-fermentable carbon sources – if encountered during foraging (Kern et al, 2004). However, the role of mitochondrial function in regulating morphological differentiation remains largely unclear. Studies that have addressed the mitochondrial involvement in this process have reported that respiratory deficient diploid and haploid strains are completely defective in filament formation (Jin et al, 2008; Kang & Jiang, 2005). It has been suggested that activation of the RTG pathway in *rho*^{0} cells is a significant component in the inhibition of FG (Jin et al, 2008). This conclusion was based on the finding that deletion of the pathway positive regulator *RTG2* from either wt or *rho*^{0} cells resulted in slightly enhanced filament formation (Jin et al, 2008). Somewhat contradictory results however show that deletion of *RTG2* and another positive regulator of the RTG pathway, *RTG1,* in *rho*$^{+}$ cells blocks haploid invasion (Chavel et al, 2010). The latter results suggest that the RTG signaling plays a positive role in development of invasive growth. Altogether, our understanding of mitochondrial (dys)function and RTG signaling in modulation of filamentous and invasive growth has remained rather incomplete and lacks mechanistic insights. In the present study mitochondrial involvement in haploid filamentation and invasive growth is addressed in more detail.

Table 1. Mitochondrial proteins involved in filamentous and/or invasive growth as identified by genome-wide screens.

Protein	Molecular function	Respiratory growth	Haploid invasion	Haploid FG (1% butanol)	Diploid pseudohyphal growth	Reference
Mitochondrial translation						
Mrp21	Mitochondrial ribosomal protein of the small subunit	absent	+/–	–/+	–	(Lorenz et al, 2000)
Msm1	Mitochondrial methionyl-tRNA synthetase	absent	–	–	–	(Lorenz et al, 2000)
Ifm1	Mitochondrial translation initiation factor 2	absent	–	–	ND	(Jin et al, 2008)
Mef2	Mitochondrial translation elongation factor	absent	–	–	ND	(Jin et al, 2008)
Mst1	Mitochondrial threonyl-tRNA synthetase	absent	–	–	ND	(Jin et al, 2008)
Msw1	Mitochondrial tryptophanyl-tRNA synthetase	absent	–	–	ND	(Jin et al, 2008)
Suv3	ATP-dependent RNA helicase, component of mtRNA degradasome	absent	–	–	ND	(Jin et al, 2008)
Fmp38	Required for mitochondrial ribosome small subunit biogenesis	absent	–	–/+	ND	(Jin et al, 2008)
Mitochondrial genome maintenance						
Hmi1	Mitochondrial 3´-5´ directional DNA helicase	absent	+/–	–/+	+	(Lorenz et al, 2000)
Ilm1	Unknown function, deletion leads to increased loss of mtDNA	decreased rate	ND	+	–	(Kang & Jiang, 2005)
Mdm10	Subunit of ERMES and SAM complex	absent	–	–/+	ND	(Jin et al, 2008)
Mgm101	Mitochondrial nucleoid component, DNA binding, propagation, repair	absent	–	–/+	ND	(Jin et al, 2008)
Mgr1	Subunit of mitochondrial i-AAA protease supercomplex	petite-negative	–	–	ND	(Jin et al, 2008)
Mhr1	Recombinase in mitochondrial homologous recombination	absent	+	–/+	ND	(Jin et al, 2008)
Mmf1	Required for transamination of isoleucine	absent	–	–/+	ND	(Jin et al, 2008)

Protein	Molecular function	Respiratory growth	Haploid invasion	Haploid FG (1% butanol)	Diploid pseudohyphal growth	Reference
Mmm1	OMM, required for normal morphology, mtDNA stability	absent	–	–	ND	(Jin et al, 2008)
Rim1	Single-stranded DNA-binding protein	absent	–	–	ND	(Jin et al, 2008)
Rpo41	Mitochondrial RNA polymerase	absent	–	–/+	ND	(Jin et al, 2008)
Components of ETC						
Atp14	Subunit of the F0 sector of mitochondrial ATP synthase	absent	–	–	ND	(Jin et al, 2008)
Fmc1	Required for assembly and stability of F1 sector of mitochondrial F1F0 ATP synthase at high temperature	absent	–	–	ND	(Jin et al, 2008)
Qcr9	Subunit of Complex III	absent	–	–/+	ND	(Jin et al, 2008)
Coa1	IMM, required for Complex IV assembly	absent	ND	–/+	ND	(Jin et al, 2008)
Fmp25	IMM, required for assembly of Complex III	absent	–	–/+	ND	(Jin et al, 2008)
Other mitochondrial functions						
Ugo1	Component of the OMM fusion machinery	absent	ND	–	ND	(Kang & Jiang, 2005)
Mdm30	Required for normal mitochondrial fusion	absent	–	–/+	ND	(Jin et al, 2008)
Mdj1	Mitochondria synthesized protein folding	absent	–	–/+	ND	(Jin et al, 2008)
***Rho* mutants generated with ethidium bromide**						
rho^{-}		absent	ND	ND	–	(Kang & Jiang, 2005)
rho^{0}		absent	– (1% butanol medium)	–	ND	(Jin et al, 2008)

ND – not determined; – phenotype missing; –/+ decreased phenotype

3.4. Modulation of mitochondrial function by pathways that regulate FG

As summarized in previous Chapter 3.3, the available data concerning the role of mitochondria in FG is relatively scarce. However, some of the nutrient sensing pathways, including Snf1, TOR and cAMP-PKA signaling, that have been shown to regulate *FLO11* expression and filamentation also modulate the functional state of mitochondria. Snf1 signaling regulates the cellular response to glucose depletion through activation of metabolism of non-fermentable carbon source and mitochondrial functions (Broach, 2012). The pathway has also been shown to be required for haploid invasion, *FLO11* expression and diploid pseudohyphal differentiation (Cullen & Sprague, 2000; Kuchin et al, 2002). Therefore, Snf1 signaling is a positive regulator of both mitochondrial metabolic functions and FG, suggesting that upregulation of mitochondrial metabolic pathways may occur in cells undergoing morphological differentiation.

The TOR pathway that controls cellular growth in response to the quality and amount of nitrogen (Broach, 2012) has been shown to inhibit mitochondrial gene expression, OXPHOS complex density and respiration, as all these processes become upregulated upon TOR inactivation by deletion of *TOR1* (Bonawitz et al, 2007; Pan & Shadel, 2009). Although the interactions between TOR signaling and *FLO11* expression are not clear (Broach, 2012), the TOR pathway has been shown to be a positive regulator of diploid filamentation (Cutler et al, 2001). Hence, it seems that while the TOR activity is required for diploid FG, it could potentially restrict the elevation of mitochondrial respiratory function.

One of the major positive regulators studied in the context of filamentous and invasive growth is the cAMP-PKA signaling that was outlined in Chapter 3.2 in Review of Literature. The PKA pathway has also been long associated with regulation of mitochondrial functions exerting opposing effects on mitochondrial activities depending on specific growth conditions.

Downregulation of mitochondrial functions by the cAMP-PKA pathway

In general, in yeast cells downregulation of the PKA pathway is required for entry into diauxic shift and for growth on non-fermentable carbon sources. This is exemplified by the fact that upregulation of the PKA signaling by mutating or deleting the PKA regulatory subunit *BCY1* leads to inability of cells to grow on non-fermentable carbon source (Cannon et al, 1990). It has also been shown that intracellular cAMP levels drop sharply as the cells approach diauxic shift. The cells that carry *CYR1* deletion in combination with mutations in *PDE1* and *PDE2* genes and are thus defective in cAMP generation and degradation, are unable to utilize non-fermentable carbon sources when kept in growth medium with high cAMP concentration (Russell et al, 1993). In addition, the PKA

isoform Tpk2 has been shown to repress genes involved in high-affinity iron uptake and suggested to inhibit thereby mitochondrial respiratory function (Robertson et al, 2000). All these results imply that constitutively active PKA hampers the switch from fermentative to respiratory growth. More recent findings that persistently high levels of cAMP decrease respiratory rate of yeast cells support this conclusion (Leadsham & Gourlay, 2010). This effect was mediated through Tpk2 and Tpk3 activities, as strains deleted for both of these kinases failed to downregulate O_2 consumption. In addition, the *TPK3* gene was shown to be required for downregulation of genes involved in mitochondrial ETC. Also, upon cAMP-PKA pathway activation by deleting *PDE2,* the development of abnormal fragmented and swollen mitochondria was observed. This phenotype could be reverted by deletion of *TPK3*. These results indicate that the hyperactive PKA pathway has deleterious effects on mitochondrial remodeling and that this effect is largely mediated by specific Tpk3 activity (Leadsham & Gourlay, 2010).

The elevated level of Tpk3 was also correlated with increased ROS production (Leadsham & Gourlay, 2010). It has been shown previously that $RAS2^{val19}$ mutation that renders Ras2 constitutively active leads to increased ROS production (Hlavatá et al, 2003; Hlavatá et al, 2008). However, ROS generation in the $RAS2^{val19}$ mutant was shown to be PKA independent (Hlavatá et al, 2003). Therefore it seems that the Ras2-cAMP-PKA pathway may influence mitochondrial function and ROS generation at both Ras2 and PKA (specifically Tpk3) levels. Recent finding by Leadsham et al (2013) offer some insight into how the Ras2 could regulate cellular ROS independently of PKA. They showed that in cells with dysfunctional mitochondria, the Ras2 localizes to mitochondrial membranes independent of PKA and initiates ROS production from ER resident NADPH oxidase Yno1 (Leadsham et al, 2013). The relative contribution of Ras2, PKA, mitochondrial and ER compartments in generation of cellular ROS awaits more precise determination.

Upregulation of mitochondrial function by cAMP-PKA pathway

In contrast to the results described above, it has been shown that in specific conditions, when yeast cells are grown on the respiratory substrate lactate, the PKA can play a positive role in the regulation of mitochondrial respiratory function (Dejean et al, 2002). In this study, activation of the PKA pathway by expression of $Ras2^{val19}$ protein or deletion of *IRA1/IRA2* or *BCY1* genes resulted in increased mitochondrial enzyme content and oxygen consumption as opposed to the situation in glucose medium (Leadsham & Gourlay, 2010). This suggests that the PKA pathway may also play a positive role in controlling biogenesis of OXPHOS complexes under certain conditions (Dejean et al, 2002). Following studies have shown that on respiratory substrate the Tpk3 kinase is specifically involved in the upregulation of mitochondrial enzyme content and respiratory capacity before transition to stationary phase (Chevtzoff et al, 2005). It has also

been demonstrated that deletion of *TPK3* leads to increased ROS production (Chevtzoff et al, 2010). This correlated with decreased activity of the transcription factor complex *HAP2/3/4/5* involved in mitochondrial biogenesis. Based on these findings it was proposed that elevation in ROS level may signal to the nucleus to downregulate mitochondrial biogenesis.

Altogether, it seems that the cAMP-PKA pathway may exert opposing effects on mitochondrial biogenesis, respiratory capacity and ROS production depending on specific growth conditions or yeast strain backgrounds. Since the Snf1 and TOR signaling can also exert somewhat opposing effects on mitochondrial metabolic functions, it is difficult to conclude whether and which mitochondrial functions are up- or downregulated in FG inducing conditions.

4. Mitochondrial dysfunction in yeast longevity

According to the "Free Radical Theory of Aging" proposed almost 60 years ago by Denham Harman, the production of intracellular ROS that leads to accumulation of damaged macromolecules is the major contributor to cellular decline and life span (reviewed in Hwang et al, 2012). Vast research done since has confirmed that changes in mitochondrial metabolism, ROS generation, and oxidative stress resistance modulate the life span of different model organisms. However, the high cellular oxidative damage or manipulation of antioxidant gene expression do not always correlate with expected effects on longevity (Long et al, 2014). Furthermore, low levels of ROS generated by mitochondria have been implicated in induction of cytoprotection through stress-response hormesis, i.e. increased resistance upon following exposure to the stressor due to upregulated stress response pathways (Gems & Partridge, 2008). For yeasts some aspects of mitochondrial (dys)function in connection with longevity are described below.

Yeast life span is influenced by nearly 100 genes and various nutritional and environmental factors that operate through several distinct pathways. In *S. cerevisiae* two models have been established to study longevity. Replicative life span (RLS) is measured by the number of daughter cells a mother cell can produce before entering senescence and describes the aging of mitotically active cells. Chronological life span (CLS) is measured as the length of time a stationary-phase cell retains the capacity to re-enter mitotic growth (stays viable) and is a model of post-mitotic cell aging. Both overlapping and distinct regulatory mechanisms that determine RLS and CLS have been described (Longo et al, 2012).

4.1. Mitochondrial function in RLS

Individual yeast cell can divide a limited number of times, usually around 25 (Longo et al, 2012). During the course of life span the mother cell accumulates cellular damage, e.g. oxidatively damaged proteins, mitochondria with more oxidizing redox potential, higher ROS levels and decreased $\Delta\Psi_M$, and extrachromosomal rDNA circles (ERCs) that are all restrained from daughter cells (Aguilaniu et al, 2003; Lai et al, 2002; McFaline-Figueroa et al, 2011; Sinclair & Guarente, 1997). Also, morphologically mitochondria become fragmented in aging mother cells, while daughter cells retain the capacity to regenerate tubular mitochondrial network (Lam et al, 2011). This asymmetrical inheritance assures that the daughter cell is born young. At the same time it leads to the senescence of a mother cell and eventual death characterized by apoptotic markers (Laun et al, 2001). Since the subject of aging and longevity is under extensive study, the literature regarding the regulation of yeast RLS is fairly extensive. Below I am giving a brief overview of the most studied pathways regulating RLS with a focus on mitochondrial role in the process (Figure 7).

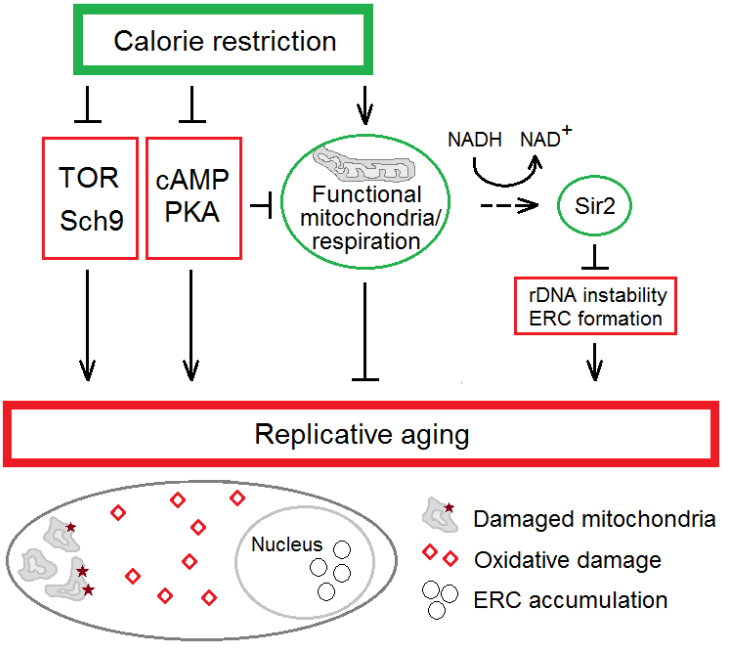


Figure 7. Major pathways and processes modulating yeast RLS. TOR, cAMP-PKA signaling pathways and instability of rDNA/ accumulation of ERCs in aging mother cells have been shown to negatively affect RLS. Sir2 activity and the mitochondrial respiratory and metabolic functions have been associated with increased RLS. Characteristic features of aging cell include damaged and fragmented mitochondria, increase in oxidatively damaged macromolecules and accumulation of ERCs in the nucleus. Modified from (Longo et al, 2012).

One of the most extensively studied modulators of longevity is calorie restriction (CR) that causes life span extension in a wide range of eukaryotic model organisms, including the budding yeast (Longo et al, 2012).

Nutrient sensing TOR and cAMP-PKA pathways have been shown to mediate the effect of CR on yeast RLS. Inactivation of either the TOR (Kaeberlein et al, 2005) or attenuation of the cAMP-PKA signaling (Fabrizio et al, 2004b; Lin et al, 2000) leads to increased RLS. Since CR fails to further extend the life span of these mutants, it has been concluded that the TOR and cAMP-PKA pathways play a central role in mediating the effects of CR on RLS (Kaeberlein et al, 2005; Lin et al, 2000). Importantly, in the context of RLS, the cAMP-PKA pathway does not act through downstream targets Msn2 and Msn4 (Fabrizio et al, 2004b; Lin et al, 2000) that activate expression of stress resistance genes (Martínez-Pastor et al, 1996; Schmitt & McEntee, 1996). Instead, attenuation of cAMP-PKA signaling has been shown to decrease rDNA recombination and accumulation of ERCs (Lin et al, 2000).

The accumulation of ERCs in the nucleolus of the aging mother cell is a type of damage that influences specifically yeast RLS and not CLS (Sinclair & Guarente, 1997). rDNA loci in yeast consist of directly repeated arrays of 100–200 copies of four rRNA genes along with a rARS (rDNA replication origin). Intra-chromatin recombination events lead to excision of rDNA copies from the chromosome and since they contain an ARS element they are stably maintained as plasmid-like circular DNA molecules (Ganley & Kobayashi, 2013). Both, accumulation of ERCs (Sinclair & Guarente, 1997) and instability of rDNA loci (Ganley et al, 2009; Ganley & Kobayashi, 2013) have been proposed to contribute to cellular senescence. The effect of ERC accumulation on RLS has been suggested to stem from sequestration of components of the replication or transcription machinery that finally leads to impaired cell division (Sinclair & Guarente, 1997). The rDNA stability has been proposed to influence RLS through altered gene expression of rDNA that leads to decreased quality of ribosomes (Ganley et al, 2009) or through the rDNA (and genomic) instability that lengthens the cell cycle leading finally to cessation of cell division (Ganley & Kobayashi, 2013).

One of the central regulators of rDNA stability and ERC formation is the NAD-dependent histone deacetylase Sir2 (Imai et al, 2000) that is presumably the most studied yeast aging gene. Sir2 is involved in chromatin silencing and suppression of recombination at the rDNA locus (Gottlieb & Esposito, 1989). Deletion of *SIR2* leads to reduced RLS and increased ERC formation. At the same time, increasing the dose of Sir2 has been shown to result in extension of RLS (Kaeberlein et al, 1999). Recent genetic mapping of natural life span variation has identified *SIR2* and rDNA as the chromosomal loci with the largest effects on longevity, stressing their central role in regulation of RLS (Stumpferl et al, 2012).

Another major effector of RLS is the metabolic shift toward respiration. It has been shown that abrogation of functional ETC by *CYT1* or *ATP2* deletion

leads to failure of CR to increase life span (Lin et al, 2002; Sharma et al, 2011). At the same time increasing the respiratory activity of the cell by overexpression of *HAP4* was shown to be sufficient to extend RLS even in standard growth conditions (Lin et al, 2002). Therefore, it appears that functional ETC and increased respiration play a positive role in determination of RLS. A possible mechanistic interaction between mitochondrial function and life span was suggested by the finding that active respiration correlated with Sir2 regulated rDNA silencing as the *MET15* marker which was integrated into a rDNA locus became inactivated with *HAP4* overexpression in a Sir2-dependent manner (Lin et al, 2002). It has been shown that the Sir2 activity is inhibited by increased NADH concentrations (Lin et al, 2004). Therefore, it was proposed that the CR extends RLS by enhancing mitochondrial respiration that leads to NADH oxidation and Sir2 activation (Lin et al, 2004). These results imply that the functional ETC plays a positive role in RLS extension through regulation of Sir2 activity. Also, they may connect cAMP-PKA pathway that regulates mitochondrial function (Chapter 3.4 in Review of Literature) to Sir2 regulation (Figure 7).

rho^0 mutants and Rtg2 in RLS

The mitochondrial function in connection to RLS is not without controversy. Although the respiratory activity of the cells correlates with increased RLS in CR conditions (Lin et al, 2002), the mitochondrial dysfunction has also been associated with anti-aging effects in some instances (Figure 8).

It has been shown that depending on genetic background of the strain, the loss of mtDNA can either have no effect on longevity or result in both decreased or increased RLS (Kirchman et al, 1999). In a yeast strain where the RTG response has been shown to be robust, a positive correlation between RLS, decreased $\Delta\Psi_M$ and RTG pathway activity has been reported (Miceli et al, 2011). The *rho* mutants with longer life span also showed higher amounts of ERCs compared with the wt strain (Miceli et al, 2011). ERC levels have been shown to be negatively regulated by the RTG pathway component Rtg2 in both wt cells and *rho* mutants (Borghouts et al, 2004). Although in most cases the ERC accumulation correlates negatively with RLS, the petite cells with active RTG signaling seem to regulate both RLS and ERC accumulation in a positive manner.

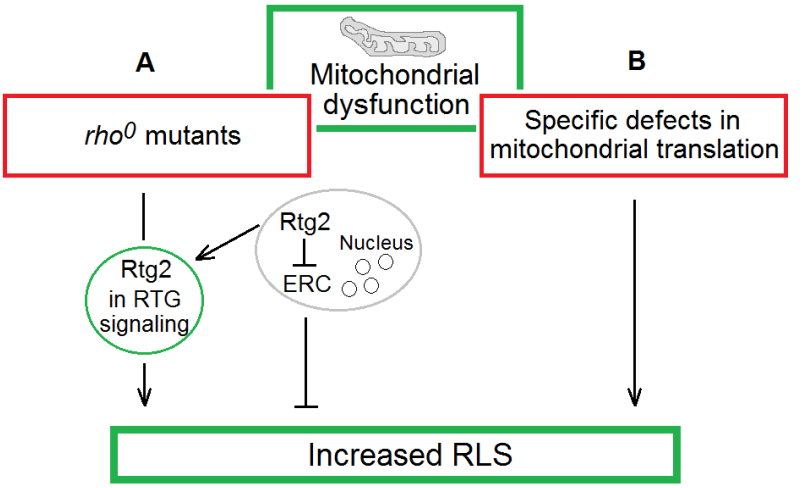


Figure 8. Mitochondrial dysfunction in connection with increased RLS. (A) In some genetic backgrounds, the loss of mtDNA results in increased RLS. This effect is mediated through activation of RTG signaling pathway and involves Rtg2 that appears to affect two distinct processes. First, Rtg2 is involved in the suppression of ERC accumulation in the nucleus through unknown mechanism. The second process regulated by Rtg2 is the activation of RTG signaling, which mobilizes Rtg2 from the first pool and leads to increased RLS by separate, but also undescribed mechanism. (B) Deletion of specific components that are involved in mitochondrial translation leads to increased RLS. These components have been shown to genetically interact with cAMP-PKA and TOR pathways but the mechanism by which the defective mitochondrial translation affects life span is not known.

Therefore, it has been proposed that the Rtg2 protein plays a dual role in yeast RLS regulation (Borghouts et al, 2004) (Figure 8A). First, Rtg2 is involved in suppression of ERC formation when the pathway is inactive or operates at low levels. Second, activation of the RTG pathway mobilizes Rtg2 and modulates processes that overrule the negative effects of high ERC levels. As a result, in *rho* cells Rtg2 becomes withdrawn from the pool that inhibits ERC accumulation (Borghouts et al, 2004; Miceli et al, 2011). As mentioned in Chapter 2.3. in Review of Literature, Rtg2 has been shown to be a component of the SLIK deacetylase complex (Pray-Grant et al, 2002). Rtg2 has also been shown to suppress trinucleotide repeat expansion independently of Rtg1 and Rtg3 (Bhattacharyya et al, 2002). Whether the role played by Rtg2 is similar in ERC and trinucleotide repeat suppression and whether the Rtg2 function in SLIK is related to these functions is yet to be determined.

Mitochondrial translation in RLS

A number of mutants that are defective in mitochondrial translation have been shown to influence yeast RLS (Figure 8B). Deletion of mitochondrial translation control (MTC) components leads to increased RLS independent of the respiratory activity of the cell, ROS generation and oxidative damage (Caballero et al, 2011). Detailed analysis of *SOV1* deletion mutant encoding for one of the MTC components revealed a connection between MTC, downregulated cAMP-PKA signaling and enhanced Sir2-dependent genome silencing, suggesting a possible link between these processes. Since the global absence of mitochondrial translation achieved by deletion of *IMG2* or *IFM1* that encode for the mitochondrial ribosomal protein of the large subunit and mitochondrial translation initiation factor 2, respectively was not sufficient to extend RLS, a more specific role for Sov1/MTC pathway in life span control awaits determination (Caballero et al, 2011; Chen, 2011). Also, deletion of *AFO1* that encodes for a protein of the large mitochondrial ribosomal subunit has been shown to lead to markedly increased RLS (Heeren et al, 2009). The life span extension of *afo1Δ* cells was not accompanied by activation of the RTG pathway despite the respiratory deficiency of the mutant. Also, the *afo1Δ* mutants accumulated ERCs and deletion of *FOB1* that blocks ERC accumulation did not lead to further increase in RLS, indicating that the mechanism of life span extension in these cells is independent of rDNA instability. Instead, a genetic interaction with the TOR pathway was shown, as deletion of *TOR1* and one of the TOR targets, *SFP1* from *afo1Δ* cells curtailed the life span (Heeren et al, 2009).

Recently, it was demonstrated that deletion of the *m*-AAA protease component *YTA10 (AFG3)* leads to extension of the RLS (Delaney et al, 2013a). In addition to the role in correct assembly of ETC complexes (Arlt et al, 1998), Yta10 has also been shown to proteolytically process MrpL32, a mitochondrial ribosomal protein of the large subunit. This processing is required for assembly of mitochondrial ribosome particles and deletion of *YTA10* leads to impaired mitochondrial translation (Nolden et al, 2005). *yta10Δ* cells showed respiratory deficiency, extension of the RLS and reduced cytoplasmic translation as measured by polysome analysis (Delaney et al, 2013a). Therefore, it was speculated that deletion of *YTA10* that leads to the failure to properly assemble mitochondrial ribosomes or other mitochondrial complexes, induces a signal that inhibits cytoplasmic mRNA translation and increases RLS. The signal generated by the *yta10Δ* cells was shown to be independent of the presence of mtDNA. In the same study, the rho^0 mutants and deletion mutants of the ETC and TCA cycle components showed RLS comparable to wt strain, indicating again that there is some specificity with respect to exact type of mitochondrial (translation) dysfunction that affects RLS (Delaney et al, 2013a).

4.2. Mitochondrial function in CLS

CLS is determined by the time yeast cell maintains viability after depletion of one or more essential nutrients. CLS is usually measured after exhaustion of glucose from the growth medium when cells have passed the diauxic shift (switch to mitochondrial respiration), completed last mitotic divisions on non-fermentable carbon sources and entered the stationary phase (De Virgilio, 2012; Longo et al, 2012). Upon entry into stationary phase, the yeast cell culture can be divided into two sub-populations based on different buoyant densities: the lighter fraction containing a heterogeneous population of mostly non-quiescent cells and the denser faction consisting of quiescent cells (Allen et al, 2006). These two fractions are morphologically and physiologically distinct and show different patterns of gene expression (Allen et al, 2006; Aragon et al, 2008). The quiescent cells are unbudded and enter the mitotic cell cycle synchronously when re-feed, contain significant concentrations of glycogen, display elevated stress resistance, reduced levels of apoptosis-related markers, and retain viability for an extended period of time compared with non-quiescent cells. In contrast, cells in the non-quiescent fraction are a mixture of budded and unbudded cells, contain only trace amounts of glycogen, display more markers of apoptosis (e.g. elevated ROS accumulation, DNA fragmentation and loss of membrane integrity) and their reproductive capacity rapidly declines upon entry into mitotic cell cycle (Allen et al, 2006). Although the stationary phase cultures are a mixture of both types of cells, the term quiescence has been often used as a synonym for stationary phase and most CLS studies have not taken into account the heterogeneity of the stationary phase cultures (Allen et al, 2006; De Virgilio, 2012). Nevertheless, the studies of aging factors that modulate CLS of stationary phase cells have identified the basic pathways and components of the "true" quiescence program. In this thesis the term quiescence is used only when the data is obtained from the specific quiescent cell fraction and the chronological aging/ CLS reflects studies performed on stationary phase cultures.

The chronologically aged yeast cells accumulate oxidative damage similarly to old mother cells in RLS experiments (Reverter-Branchat et al, 2004) and eventually die exhibiting markers of apoptosis (Fabrizio et al, 2004a; Herker et al, 2004). This probably reflects the changes occurring in the non-quiescent cell fraction of the aged cell culture as described by Allen et al (2006).

As with RLS, major regulatory pathways that promote chronological aging are TOR and cAMP-PKA signaling (Fabrizio et al, 2003; Fabrizio et al, 2001; Powers et al, 2006) (Figure 9). Among other effectors, these pathways repress the downstream target Rim15 that positively regulates transcription factors Gis1 and Msn2/4 that in turn activate stress response and cellular protection (Pedruzzi et al, 2000; Pedruzzi et al, 2003; Wei et al, 2008). CR has also been shown to increase CLS (Fabrizio & Longo, 2003) through both TOR and PKA signaling (Wei et al, 2008). However, besides regulating Msn2, Msn4 and Gis1 activity, the CR also operates through as yet unidentified mechanisms (Wei et

al, 2008). In addition, mitochondrial respiratory function appears to regulate CLS both independently and downstream of CR and TOR signaling (Longo et al, 2012).

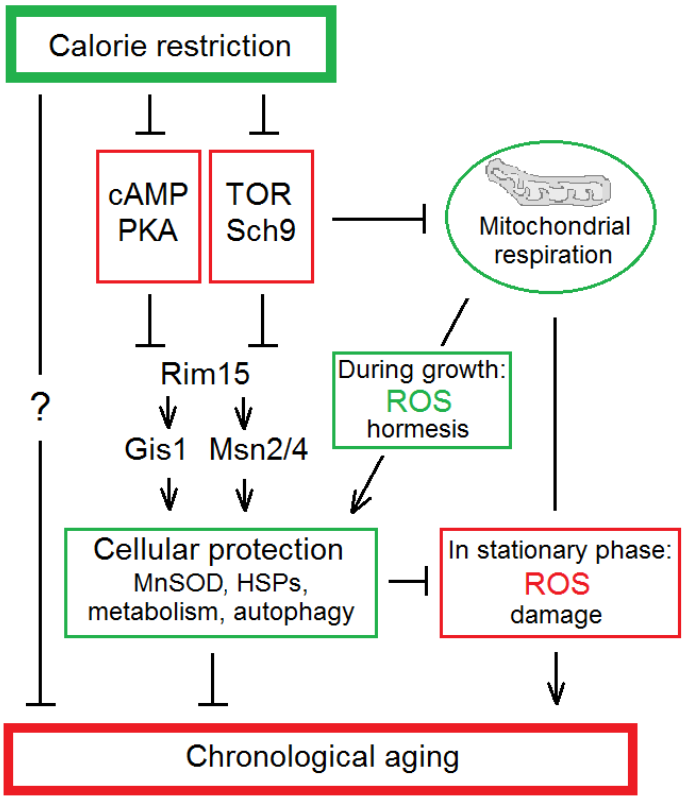


Figure 9. Major regulatory pathways of CLS. Calorie restriction is a potent regulator of CLS, acting through cAMP-PKA and TOR signaling. Both pathways converge on the downstream effector Rim15 that mediates activation of Gis1 and Msn2, Msn4 transcription factors involved in activation of cellular stress response. The CR also operates through as yet unidentified mechanism(s) to modulate chronological aging. Mitochondrial respiratory activity correlates with increased CLS. It has been proposed that during growth the mitochondria generate hormetic ROS that contribute to increased stress resistance in stationary phase. Increased ROS generation during stationary phase has an opposite effect. Modified from (Longo et al, 2012).

Mitochondrial respiratory function in CLS

A strong positive correlation between mitochondrial respiration and longevity in CLS studies has been demonstrated. It has been shown that the cells in the quiescent fraction of stationary phase show increased abundance of proteins that localize to mitochondria and are involved in mitochondrial respiratory function (Davidson et al, 2011). In addition, pregrowth of yeast cells in a non-fermentable carbon source increases maximal CLS and retention of a full replicative potential when the cells reenter cell cycle (Piper et al, 2006). Shifting cells to non-fermentable carbon source altogether result in dramatic extension of CLS (Smith et al, 2007). Likewise, CR leads to metabolic shift from fermentation to aerobic respiration (Oliveira et al, 2008) and TOR inhibition is paralleled by increased mitochondrial translation and respiratory activity in glucose medium where these processes are in general largely repressed (Bonawitz et al, 2007).

At the same time petite cells or mutants with inhibited respiration show dramatic decrease in CLS compared with wt cells (Aerts et al, 2009; Bonawitz et al, 2007; Bonawitz et al, 2006; Ocampo et al, 2012). Apparently a certain beneficial threshold of respiration exists in context of longevity, as respiratory rates below 40% of wt level led to a dramatic decrease in CLS (Ocampo et al, 2012). However, enhanced respiration without accompanying metabolic reconfiguration is not sufficient to extend CLS. *HAP4* overexpression that leads to upregulation of mitochondrial function and biogenesis but does not induce genes involved in metabolism of energy reserves (Lascaris et al, 2003), does not increase CLS (Ocampo et al, 2012). By contrast, CR and inactivation of the TOR pathway that in addition to mitochondrial function also regulate metabolic remodeling, including accumulation of storage carbohydrates (François & Parrou, 2001), results in life span extension. Therefore it was proposed that certain respiratory thresholds are coupled to upregulation of stress resistance and metabolism of energy reserves (Ocampo et al, 2012). In support of this hypothesis it has been shown that addition of trehalose, a storage carbohydrate that is used as energy source during the stationary phase, to exponentially growing *rho*^{0} cells led to CLS extension beyond that observed in wt cells (Ocampo et al, 2012). Of importance is the notion that beneficial effects of both respiration and trehalose addition manifest in CLS only when implemented during exponential growth. Therefore the metabolic adaptation that leads to CLS extension must occur before cells enter stationary phase (Ocampo et al, 2012).

Mitochondria generated ROS in CLS

A second role for mitochondrial function in life span regulation is the generation of adaptive ROS during active growth that results in hormetic response during stationary phase. It has been shown that the CR extends yeast CLS by inducing H_2O_2 generation that enhances SOD (superoxide dismutase)

activity, leading in turn to reduced levels of oxidatively damaged macromolecules in stationary phase (Mesquita et al, 2010). However, inactivation of catalases that also results in higher H_2O_2 levels and increased SOD activity results in CLS extension despite higher rates of oxidative damage. These results establish that CR induced ROS promotes yeast longevity even when the oxidative damage to macromolecules is increased (Mesquita et al, 2010).

A link between downregulated TOR signaling and generation of hormetic ROS has also been demonstrated. In *tor1Δ* strain the CLS extension correlates with coupled respiration, increased membrane potential and ROS production during active growth phase (Pan et al, 2011). Also, treatment of wt cells with menadione that enhances mtROS or inhibition of TORC1 with rapamycin during active growth increases CLS. At the same time, ROS induction during stationary phase has no significant effect on life span extension (Pan et al, 2011). Based on these findings it was proposed that increased mtROS during active growth serves as an adaptive signal by which reduced TOR activity modulates yeast life span. This is similar to beneficial effects of cellular respiration or trehalose addition that affect CLS only when implemented during growth (Ocampo et al, 2012). Altogether, it was proposed that mtROS generated during growth results in an adaptive stress response that leads to reduced $\Delta\Psi_M$ and ROS in stationary phase and ultimately CLS extension (Pan et al, 2011). However, since the treatment of cells with antimycin A that inhibits respiration and promotes ROS generation leads to reduced CLS, the negative effect of low respiratory rate seems to dominate over the ROS generated hormetic signal (Ocampo et al, 2012).

The aforementioned studies suggest that mitochondrial respiratory function must be regulated according to a certain pattern to achieve maximal effect on CLS. During active growth, high respiration rate and increased $\Delta\Psi_M$ contribute to ROS generation and metabolic reconfiguration that supports increased stress resistance and accumulation of energy reserves that in turn promote longevity during stationary phase. When the cells exhaust external nutrient pools and enter stationary phase, they switch to low respiratory rates and maintain low $\Delta\Psi_M$ that are characteristic of stationary phase cells in CR conditions and *tor1Δ* mutants, respectively (Ocampo et al, 2012; Pan et al, 2011). Accordingly, it has been shown that $\Delta\Psi_M$ is reduced in a subpopulation of live cells during CLS experiments and low $\Delta\Psi_M$ in stationary phase predicts subsequent long RLS when the cells re-enter cell cycle (Delaney et al, 2013b). Altogether, a critical role for timing and preserving the functional state of mitochondria in life span extension is evident.

Recently first downstream targets of mtROS in the context of longevity were revealed (Schroeder et al, 2013). By identifying genes commonly induced by menadione and rapamycin treatment (that both increase mtROS) and searching for transcriptional regulators that coordinate the corresponding gene expression response, a histone demethylase Rph1 was identified as possible effector of ROS response. Subsequent analysis of Rph1 regulators suggested a mechanism

by which mtROS activates DNA damage response independently of nuclear DNA damage, resulting in inactivation of Rph1. This in turn leads to methylation of histone 3 at subtelomeric regions and Sir3 dependent telomere silencing that contributes to CLS extension. These results connect mtROS hormesis to DNA damage signaling and epigenetic telomere silencing in CLS regulation (Schroeder et al, 2013).

How the mitochondrial function is regulated to generate hormetic ROS and which reactive oxygen species behave as signal transducers is not entirely clear. Work done in G.S. Shadel's laboratory indicates a positive role for superoxide radicals in signaling in *tor1Δ* cells since overexpression of mitochondrial superoxide dismutase *SOD2* curtails the CLS extension of these strains (Pan et al, 2011). A protective role for O_2^- in stress response is supported by the finding that respiratory mutants defective in superoxide radical generation (Complex III-V mutants and *rho*^{0} cells) are sensitive to H_2O_2 induced oxidative stress. In addition, overexpression of *SOD1* and *SOD2* that reduce O_2^- level leads to increased sensitivity upon H_2O_2 treatment (Thorpe et al, 2013). The latter work stresses the concentration-dependent role for O_2^- in signaling as increasing the O_2^- concentration with menadione leads to growth inhibition. However, as mentioned above, H_2O_2 has also been indicated as protective molecule in CLS extension (Mesquita et al, 2010). This result contradicts previous studies by suggesting a mechanism where decreasing and not increasing the superoxide anion levels through increased H_2O_2 concentration and SOD activity has CLS extending effect. Again, the beneficial concentrations of H_2O_2 are probably in a relatively narrow range since further increase in the H_2O_2 leads to apoptosis and when the concentrations are increased even further, to cell lysis (Madeo et al, 1999). Since Shadel and co-workers showed that the beneficial increase in O_2^- is observed only during active growth phase (Pan et al, 2011), the roles for O_2^- and H_2O_2 in signaling can still be integrated. Possibly, the timing of interplay between levels of these molecules during cell growth determines the final outcome and effect on CLS.

Whether the functional state of mitochondria, ROS generation and life span extension are causal or simply correlated, has not been conclusively demonstrated. It was recently reported that reduced COX (Complex IV) activity due to aberrant expression of *COX4* correlates with high levels of cellular ROS, however, mitochondria purified from these mutants have reduced capability to produce H_2O_2 (Leadsham et al, 2013). Instead, it was shown that activated RAS localizes to mitochondria with reduced $\Delta\Psi_M$ and this correlates with decreased turnover of the ER-localized NADPH oxidase Yno1. Yno1, which produces superoxide radicals by NADPH oxidation, was shown to produce majority of the ROS in *COX4* deficient cells (Leadsham et al, 2013). These results emphasize the role of mitochondria as signaling platforms that can regulate ROS generation indirectly and provide another dimension for the organelles function, perhaps also in the context of CLS.

RESULTS AND DISCUSSION

1. Objectives of the study

The integrity and coordinated regulation of specific mitochondrial functions is essential to achieve optimal cellular adaptation to diverse nutritional conditions. Dysregulation or dysfunctional state of the organelle can lead to loss of viability, decreased fitness, and/or inability to activate appropriate differentiation programs. However, the dysfunctional state of mitochondria has also been connected to increased viability in some experimental conditions. Altogether, mitochondria appear to influence cellular fitness through metabolic and signaling activities. The present study aimed at the characterization of cellular responses to starvation conditions when mitochondrial function is perturbed. Mitochondrial involvement in yeast filamentous and invasive growth was analyzed. Several transcription factors that regulate mitochondrial metabolism were investigated in CLS experiments. The study also includes topological description of *C. albicans* mtDNA, an opportunistic commensal where the adhesion and FG contribute to the pathogenicity.

Main objectives of the present study:

1. To determine how the dysfunctional state of mitochondria interferes with filamentous and invasive growth and to investigate the role of RTG signaling in these responses.
2. To monitor the activities of FG MAPK and cAMP-PKA pathways in *rho*^{0} mutants and to determine the effect of the genetic background of a specific strain on cAMP-PKA signaling.
3. To validate the prediction power of computational method m:Explorer in prediction of yeast longevity regulators.
4. To describe the mtDNA topology of petite-negative yeast *C. albicans*.

2. Respiratory dysfunction interferes with FG signaling (Ref I)

Large-scale studies and genetic screens indicate that various genes encoding proteins that are essential for mitochondrial respiratory function are also required for filamentous and invasive growth (Jin et al, 2008; Kang & Jiang, 2005; Lorenz et al, 2000; Ryan et al, 2012). These factors include proteins involved in mtDNA transactions, mitochondrial translation, mitochondrial morphology maintenance and components of ETC (Table 1). Ethidium bromide generated *rho*$^{-}$ mutants show defective diploid pseudohyphal growth (Kang & Jiang, 2005) and *rho*^{0} mutants obtained the same way cannot undergo 1%-butanol induced haploid filamentous and invasive growth (Jin et al, 2008). Based on these studies it can generally be concluded that mitochondrial respiratory function plays a role in the FG. However, it is unclear by which mechanism respiratory deficiency influences this process.

To characterize the role of mitochondrial function in haploid filamentous and invasive growth in detail, *rho*^{0} and *rho*$^{-}$ mutants were generated in ∑1278b background (Ref. I). Two methods were employed to generate the mutants. First, ethidium bromide mutagenesis was performed to induce the loss of functional mtDNA from wt ∑1278b cells. Second, by deletion of *MIP1* and *RPO41* genes *rho*^{0} and *rho*$^{-}$ strains were generated, respectively. *MIP1* encodes for the mtDNA polymerase and it is essential for mitochondrial genome maintenance (Genga et al, 1986). *RPO41* encodes for the mtRNA polymerase and deletion of the gene results in formation of both *rho*^{0} and *rho*$^{-}$ mutants (Fangman et al, 1990; Greenleaf et al, 1986). The respiratory deficiency of all the constructed mutant strains was verified by the absence of growth on non-fermentable carbon source glycerol. The loss or presence of restructured mtDNA was verified by DAPI staining. *rpo41Δ* mutants selected for further studies were confirmed to be *rho*$^{-}$.

The filamentous and invasive growth assays showed that all *rho* mutants generated in the course of this study formed significantly reduced numbers of surface spread filaments and were less efficient in agar invasion compared with the *rho*$^{+}$ strain (Ref. I, Figure 1A, 1C). Filamentous and invasive growth of *rho* mutants, however, was not completely abolished. Compared with the *tec1Δ* strain that failed to show any characteristics of filamentous or invasive growth as reported before (Lorenz et al, 2000; Lorenz & Heitman, 1998; Mösch et al, 1999), the *rho* mutants retained some residual filament formation and invasion. Cells of the *rho* mutant colonies showed a length-to-width ratio that was comparable to *rho*$^{+}$ pseudohyphal cells (Ref I, Figure 1B). This indicates that although *rho*$^{0/-}$ cells are obviously defective in filamentous and invasive growth, they can still evoke some aspects of the morphological differentiation program that regulates cell elongation. No difference between filamentous response of *rho*^{0} and *rho*$^{-}$ strains was observed. This demonstrates that the presence of mtDNA *per se* is not sufficient to support wt levels of filamentation.

The filamentation defect of *rho* mutants can originate from a number of cellular rearrangements brought about by the loss of respiratory capacity. Two major differences between $rho^{0/-}$ and rho^{+} strains can be considered. First, an insufficient supply of metabolic energy or some metabolic intermediate could block the morphogenetic switch. Alternatively, dysfunctional mitochondria could modulate specific signaling pathways that regulate FG. To address the latter possibility, the transcriptional activation of a FG target was analyzed. The canonical marker of filamentous and invasive growth is the cell surface adhesin Flo11 required for both responses to occur at wt level (Lo & Dranginis, 1998; Lorenz et al, 2000). The P_{FLO11}::*lacZ* reporter and quantitative PCR analysis of *FLO11* mRNA level demonstrated that the expression of *FLO11* was severely reduced in *rho* mutants compared with the wt strain. Downregulation of *FLO11* was observed under all conditions tested, namely during exponential growth, under starvation conditions evoked on low ammonium SLAD medium, upon prolonged growth in SC $ura^{-}leu^{-}$ liquid medium and on YPD solid medium (Ref I, Figure 1D-G). These results demonstrate that in *rho* mutants the *FLO11* expression is not activated at transcriptional level.

Since the *rho* mutants fail to express *FLO11* at wt level, the effect of ectopically expressed *FLO11* on filamentous and invasive growth was examined. Expression of *FLO11* under control of the constitutive *TEF* promoter restored both responses in *rho* mutants (Ref I, Figure 1H, 1I). However, in the *tec1Δ* mutant strain the ectopic expression of *FLO11* was not sufficient to fully restore the FG (Ref I, Figure 1H). This suggests that other processes (besides regulation of *FLO11* expression) downstream of Tec1 are required for FG to occur at wt level. It also suggests that these processes are still operating in *rho* mutants. Since increasing the expression of *FLO11* is sufficient to restore filamentation and invasion phenotypes in respiratory deficient cells, it is evident that the metabolic status of *rho* cells is not limiting for these responses to occur. Instead, the results show that the morphological differentiation program is not induced at transcriptional level and suggest that mitochondrial dysfunction may interfere with signaling pathways that regulate filamentous and invasive growth.

3. The role of RTG signaling in FG (Ref I)

In respiratory deficient mutants extensive readjustments in cellular metabolism and gene expression take place to support the biosynthesis of glutamate and glutamine, the amino acids that serve as nitrogen donors in biosynthetic reactions (Liu & Butow, 2006). The mitochondria-to-nucleus RTG signaling pathway that mediates these changes is a sensor of both mitochondrial dysfunction and availability of certain nitrogen sources. The extent of RTG pathway activation in *rho* mutants depends on growth conditions (Liao et al, 1991) and yeast strain background (Kirchman et al, 1999). In addition, the pathway can be downregulated in rho^{0} cells by the addition of glutamate to the

growth medium (Liu & Butow, 1999), suggesting a negative feedback from restored cellular nitrogen pools. Since the RTG pathway regulates nitrogen homeostasis in *rho* mutants (Liao et al, 1991) and wt cells that are grown on certain nitrogen sources (Tate et al, 2002), a role for the RTG signaling in starvation response could be expected. Previous studies have indicated both positive and negative function for the RTG signaling in FG. It has been shown that the RTG pathway positive regulators *RTG1* and *RTG2* are required for invasive growth of rho^+ cells (Chavel et al, 2010). The expression of RTG pathway target gene *DLD3* (Chelstowska et al, 1999) is upregulated over 10-fold within the first 5h of isoamyl alcohol-induced FG and deletion of *DLD3* leads to reduced filament formation (Hauser et al, 2007). On the other hand, somewhat contradictory results have indicated that inactivation of the pathway in rho^0 cells can partially restore the butanol induced filament formation (Jin et al, 2008).

To monitor the effect of RTG signaling on filamentation and invasion response, *RTG2* was deleted from both wt cells and *rho* mutants. Rtg2 is the most proximal sensor of mitochondrial dysfunction described in the RTG pathway (Liao & Butow, 1993) (Figure 4). Deletion of *RTG2* did not rescue the filamentation and invasion defects of *rho* mutants and did not affect the invasion phenotype of rho^+ wt cells (Ref I, Figure 2B, C). However, reduced filament formation in rho^+ *rtg2Δ* colonies could be observed (Ref I, Figure 2B). These results were closely matched by the P_{FLO11}::*lacZ* reporter analysis. Deletion of *RTG2* failed to restore the *FLO11* reporter expression in *rho* mutants while in wt strain ~2–3 fold reduction in reporter expression was noted (Ref I, Figure 2D). These results demonstrate that inactivation of the RTG signaling in *rho* mutants is not sufficient to restore neither the filamentation and invasion phenotypes nor activate the signaling program necessary for *FLO11* expression. The results also show that the RTG pathway activity is required for a complete filamentous response of wt cells. Altogether, the findings support a positive role for the RTG pathway in filamentous growth response as suggested by Chavel et al (2010).

As mentioned above, the extent of the RTG pathway activation depends on the genetic background of the yeast strain and on specific growth conditions (Kirchman et al, 1999; Liao et al, 1991). Therefore, the pathway activity under starvation conditions in ∑1278b background was determined. In exponentially growing cells, ~2 fold increase in RTG reporter P_{CIT2}::*lacZ* activity was observed in *rho* mutants (Ref I, Figure 2E). This suggests a rather weak retrograde response under these conditions, as up to 10-fold induction has been reported in PSY142 rho^0 mutants compared with wt cells on raffinose medium (Liao et al, 1991). The measurement of *CIT2* mRNA level with quantitative PCR showed no difference between exponentially growing wt cells and *rho* mutants on YPD medium (Ref I, Figure 2G). This suggests that the RTG signaling operates at similar levels or only slightly higher in *rho* mutants compared with the wt strain during exponential growth. Under starvation

conditions where cells were grown in dense patches on selective solid media for 3 days, the RTG reporter indicated equal pathway activation in wt cells and *rho* mutants (Ref I, Figure 2F). In comparison to exponentially growing cells, the P_{CIT2}::*lacZ* reporter showed increased RTG signaling under starvation conditions (Ref I, compare Figure 2E and 2F). The measurements of *CIT2* mRNA levels supported the finding that the RTG pathway becomes activated in starvation conditions in both wt cells and *rho* mutants (Ref I, Figure 2G). This is in favor of a positive role of RTG signaling in starvation response in both wt cells and *rho* mutants.

The above results show a positive effect for the RTG signaling in FG as proposed by Chavel et al (2010) and as suggested by upregulation of RTG target gene *DLD3* in isoamyl alcohol-induced FG (Hauser et al, 2007). The RTG pathway is not only a sensor of mitochondrial dysfunction, but is also upregulated by certain nitrogen sources in wt cells (Tate et al, 2002). The fact that the RTG signaling becomes attenuated in rho^0 cells upon glutamate addition to the growth medium supports a view that a signal from nitrogen homeostasis is an important regulator of RTG pathway activity (Liu & Butow, 1999). Since nitrogen starvation is an inducer of filamentous growth, a role for the RTG signaling in the FG response could be expected. One explanation for results that show a negative role for *RTG2* in FG (Jin et al, 2008) could be offered. It has been reported that Rtg2 functions in the nucleus to suppress trinucleotide repeat instability (Bhattacharyya et al, 2002) and ERC formation (Borghouts et al, 2004). It is therefore possible that the phenotype observed by Jin et al (2008) could be due to compensatory secondary mutations arising in the *rtg2Δ* background due to increased instability of some nuclear loci. Indeed, upon *RTG2* deletion frequent revertant formation was observed on 1%-butanol containing SLAD plates (unpublished results). The formation of revertants was manifested by strongly exaggerated growth and filament formation and this occurred both in rho^+ and in $rho^{0/-}$ cells.

4. FG MAPK pathway is active in *rho* mutants (Ref I)

In order to gain insight into the mechanism of differential expression of *FLO11* in *rho* mutants and wt strain, the activities of two signaling pathways that are essential for *FLO11* expression were examined next. First, it was focused on the FG MAPK pathway activity. Induction of the pathway leads to cooperative binding of Ste12 and Tec1 transcription factors to specific filamentous response elements (FREs) in target gene promoters (Madhani & Fink, 1997) (Figure 6). It has been shown that *TEC1* is required for both isobutanol-induced filamentation and invasive growth (Lorenz et al, 2000; Mösch et al, 1999). Moreover, Tec1 is one of the core components of morphological differentiation program (Ryan et al, 2012).

To measure the signaling through the FG MAPK pathway, the activity of FRE-dependent P_{TEC1}::*lacZ* reporter was measured. Compared with wt cells, the reporter activity in *rho* mutants showed 1.4-fold decrease during exponential growth and 2.4-fold increase under starvation conditions (Ref I, Figure 3B, C). These results indicate that there is no major downregulation of FG MAPK signaling in *rho* cells and that under starvation conditions the pathway is activated even stronger in *rho* mutants compared with wt cells. In both wt cells and *rho* mutants the reporter activity was strictly dependent on Tec1. The activity of Tec1/Ste12-responsive fragment from *FLO11* promoter showed similar activation in wt cells and *rho* mutants (Ref I, Figure 4B). Altogether, the promoter fragment analysis indicates that the FG MAPK is activated in *rho* mutants in Tec1-dependent manner.

In *tec1Δ* strains the filamentous and invasive growth is completely absent (Ref I, Figure 1A and C) (Lorenz et al, 2000; Mösch & Fink, 1997; Mösch et al, 1999). In addition, the cells failed to acquire elongated morphology, a characteristic of pseudohyphal cells (Ref I, Figure 1B). In contrast, in respiratory deficient mutants the extent of cell elongation was rather similar to wt cells and some residual filamentation and invasion was observed. The fact that ectopic expression of *FLO11* fully restored filamentation response in *rho* mutants but not in *tec1Δ* strain (Ref I, Figure 1H) implicates that at least some Tec1 targets are functional in *rho* mutants. To address this possibility, $rho^{0/-}$ t*ec1Δ* double mutants were assayed for filamentous and invasive growth. Deletion of *TEC1* led to complete loss of residual filament formation and agar invasion of *rho* mutants (Ref I, Figure 3D, E). These results support the hypothesis that Tec1 activity is required for residual filamentous and invasive growth of respiratory deficient strains.

The finding that the FG MAPK still operates in *rho* mutants is consistent with a previous screen for FG MAPK modulators (Chavel et al, 2010). In this study, a gene deletion library was screened for mutants that influence secretion of the extracellular domain of Msb2. Msb2 functions at the head of the FG MAPK pathway and the release of the extracellular domain is required for pathway activation (Vadaie et al, 2008). Around 500 genes were identified that influenced Msb2 secretion. Among others, deletion of ~50 genes with various mitochondrial functions (TCA cycle, ETC components, mitochondrial translation, protein transport and processing) was found to increase Msb2 secretion (Chavel et al, 2010). These results are in accordance with direct measurements of pathway activities that indicate even slightly higher signaling through the FG MAPK cascade in *rho* mutants compared with wt strain under starvation conditions (Ref I, Figure 3C).

5. Mitochondrial dysfunction interferes with cAMP-PKA signaling in the context of filamentous and invasive growth (Ref I)

The cAMP-PKA pathway is another major regulator of filamentous and invasive growth (Brückner & Mösch, 2012; Cullen & Sprague, 2012) (Figure 6). Upon glucose depletion the PKA activity becomes downregulated, a process that is required for efficient adaptation to growth on non-fermentable carbon sources, upregulation of stress response, and entry into stationary phase (Santangelo, 2006; Thevelein & de Winde, 1999). Although FG is induced in starvation conditions, the cAMP-PKA pathway activity is still required for *FLO11* expression and filamentous and invasive phenotype (Pan & Heitman, 1999; Robertson & Fink, 1998). Moreover, it has been reported that in ∑1278b cells the Ras2/cAMP-PKA pathway is hyperactive, leading to suppression of stress-responsive genes that in turn promotes invasive growth (Stanhill et al, 1999). Previous reports on PKA activity in *rho* mutants can be separated into two categories. First, genome profiling of *rho* mutants indicates that a number of genes that are under repression of cAMP-PKA activity and that are induced upon diauxic shift are upregulated in rho^0 mutants (Traven et al, 2001). These results together with the finding that *rho* mutants exhibit a pleiotropic drug-resistance and increased resistance to heat shock suggest low activity of the cAMP-PKA pathway in rho^0 mutants (Traven et al, 2001). Moreover, it has been reported that loss of mtDNA is not tolerated in combination with deletion of *IRA2* and *PDE2*, inhibitors of the cAMP-PKA signaling (Dunn et al, 2006). On the other hand, it has been shown that mitochondrial respiratory deficiency suppresses autophagy during amino-acid starvation by stimulating the PKA activity (Graef & Nunnari, 2011). All the above mentioned studies that have pointed to altered PKA activity in *rho* mutants have been conducted in non-filamentous W303 genetic background.

Since cAMP-PKA signaling is one of the major regulators of filamentous growth and previous reports suggest modulation of the pathway activity in *rho* mutants, the activity of cAMP-PKA signaling in *rho* mutants of ∑1278b background was determined. First, the expression level of cAMP-PKA responsive *FLO11* promoter fragment fused to the *lacZ* reporter gene (Rupp et al, 1999) was monitored. The reporter showed significant ~8-fold decrease in *rho* mutants compared with the wt strain, suggesting strong downregulation of cAMP-PKA signaling (Ref I, Figure 4B).

Next the cAMP-PKA pathway activity was modulated genetically to examine the effects of pathway up- and downregulation on filamentous and invasion phenotypes of *rho* mutants. Since the reporter assay showed strong downregulation of the PKA pathway activity, activation of the pathway could be expected to lead to rescue of defective filamentous and invasive growth of *rho* mutants. To mimic PKA pathway activation, the downstream activator *FLO8* was overexpressed and the downstream inhibitor *SFL1* was deleted from

rho mutants and wt cells (Figure 6). As expected, the deletion of *SFL1* and overexpression of *FLO8* resulted in hyperfilamentation and hyperinvasion of both *rho* mutant and wt cells (Ref I, Figure 4E and F). Also, activation of the pathway by deletion of *BCY1* or overexpression of *TPK2* restored invasive growth of *rho* mutants. However, *TPK2* overexpression did not fully rescue the filamentation defect of *rho* mutants. It is possible that induction of filament formation may require higher threshold of PKA activity than invasive growth and *TPK2* overexpression does not lead to sufficient PKA activation to restore filamentation. Alternatively, input from other pathways could be required to restore efficient filament formation through modulation of Flo8 and Sfl1 activities. As described in Chapter 2 in Results and Discussion, filamentation was also not fully restored by *FLO11* overexpression in *tec1Δ* strain as opposed to invasive growth (Ref I, Figure 1H and 1I). Similarly, filament formation was moderately decreased by *RTG2* deletion in rho^{+} strain while there was no negative effect on invasive growth (Ref I, Figure 2B and 2C). These findings indicate that compared with invasion, the filamentation response is more susceptible to perturbation at activation level of at least some of the signaling pathways or may require more effectors. Indeed, genome wide deletion analysis has shown that more elaborate genetic program is required to induce complete filamentation compared with invasion as considerably more genes are connected to the former process (Ryan et al, 2012). However, the general conclusion from the PKA pathway activation assays (Ref I, Figure 4E and F) is that the upregulation of PKA signaling at the downstream transcriptional level leads to the restoration of filamentous and invasive growth of *rho* mutants.

If the cAMP-PKA pathway is indeed downregulated in *rho* mutants, it could be expected that downregulation of the pathway genetically has no major additive effect in *rho* mutants in comparison with wt cells. Simultaneous deletion of all three PKAs is lethal (Toda et al, 1987b). However, deletion of *TPK2* is sufficient to block the morphogenetic switch and haploid invasion (Jin et al, 2008; Robertson & Fink, 1998). Downregulation of PKA signaling was achieved by deletion of *TPK2* (Figure 6). Next the pathway was downregulated by overexpression of *BCY1* that leads to attenuation of cAMP-PKA pathway activity (Portela et al, 2001) and overexpression of transcription factor *SFL1* that results in downregulation of filamentation-specific signaling (Pan & Heitman, 2002). Decreased filamentation and invasion of rho^{+} cells was observed in all cases of pathway downregulation, by *TPK2* deletion or *BCY1* and *SFL1* overexpression, as expected (Ref I, Figure 4C, D). However, there was little additive effect on *rho* mutant phenotypes upon attenuation of the pathway activity with these genetic alterations. The filamentous growth of *rho* mutants was not affected with none of these genetic alterations (Ref I, Figure 4C, before wash) and overexpression of *BCY1* and *SFL1* in *rho* strains had also no notable effect on invasive growth (Ref I, Figure 4D, after wash). The only exception was the effect of *TPK2* deletion on residual invasion of *rho* mutants, which was completely lost (Ref I, Figure 4D, after wash; Figure 4C after wash).

These results indicate that while in wt cells attenuation of the cAMP-PKA pathway results in reduced filament formation and agar invasion, in *rho* mutants there is no major additive effect on filamentous and invasive growth upon downregulation of the cAMP-PKA signaling. The only exception is the invasion response of *rho*$^{0/-}$ *tpk2Δ* strains. Since basal PKA signaling is required for viability, some Tpk-catalyzed, including Tpk2 mediated, phosphorylation is still expected in *rho* mutants. Apparently part of this activity is sufficient to induce residual invasion but not filament formation. This leaves room for speculation that overexpression of *BCY1* and *SFL1* does not result in complete abolishment of Tpk2 mediated effects. Notably, attenuation of cAMP-PKA signaling in wt cells closely resembles residual filamentation and invasion of *rho* mutant cells.

Altogether, the analysis of cAMP-PKA responsive *FLO11* promoter fragment and the experiments of cAMP-PKA pathway modulation in filamentous and invasive growth assays show that the PKA signaling in *rho* mutants is downregulated. An important question that remains unanswered is at which level is the cAMP-PKA pathway downregulated in respiratory deficient cells. It has been reported previously that *S. cerevisiae IRA2* and *PDE2* deletion strains become petite negative, indicating that the loss of mtDNA is not tolerated in strains with high Ras2 activity or increased cAMP levels (Dunn et al, 2006) (Figure 6). Interestingly, deletion of *BCY1* led to an inability of *rho* strains to grow on SLAD medium supplemented with 1% isobutanol (Ref I, Figure 4E). This demonstrates that activation of the cAMP-PKA pathway at Bcy1 regulatory level sensitizes *rho* mutants to isobutanol. Therefore, in *rho* mutants the downregulation of cAMP-PKA pathway and possibly the following induction of stress response may be important to maintain cellular integrity. Based on our findings and on the findings by Jensen and co-workers (Dunn et al, 2006) it can be proposed that the regulatory interaction between the cAMP-PKA signaling and mitochondrial dysfunction takes place upstream or at the level of the Bcy1 regulatory subunit.

It has been shown that in yeast *vps33Δ* mutant (*VPS33* is required for protein sorting and vesicle docking and fusion at the vacuole) the Ras2 protein localizes to the OMM (Wang & Deschenes, 2006). Since the *vps33Δ* cells also showed aberrant mitochondrial morphology and respiratory deficiency, it was supposed that the mitochondrial defect may contribute to Ras2 localization. Indeed, addition of azide that blocks ATP synthesis was sufficient to localize the GFP-Ras2 to mitochondria in wt cells. Altogether it was suggested that mitochondria participate in the normal trafficking of Ras2 (Wang & Deschenes, 2006). Recently it has been shown that active Ras2 colocalizes with mitochondria also in *whi2Δ* (*WHI2* is required for activation of the general stress response) and *cox4Δ* strains during nutrient depletion (Leadsham et al, 2009; Leadsham et al, 2013). Both of these mutants show defects in mitochondrial metabolism. The deletion of *WHI2* leads to aberrant mitochondrial morphology, decreased $\Delta\Psi_M$ and elevated ROS production (Leadsham et al, 2009). *COX4* is required for the formation of intact ETC, the maintenance of respiratory activity and $\Delta\Psi_M$ and

deletion of the gene leads to elevated ROS production (Leadsham et al, 2013). The relocalization of Ras2 from plasma membrane and nucleus to mitochondria could also be induced in wt cells by addition of proton-ionophore FCCP that dissipates the $\Delta\Psi_M$ (Leadsham et al, 2013). Altogether the data suggests that the loss of $\Delta\Psi_M$ leads to localization of RAS to the mitochondrial membranes where it signals to induce elevated ROS production and accelerate cell death (Leadsham et al, 2009; Leadsham et al, 2013). However, the RAS generated ROS production in *COX4* mutant was shown to be independent of PKA or RTG signaling, pointing to other downstream effectors (Leadsham et al, 2013). Altogether, it appears that in cells with dysfunctional mitochondria (mitochondria with decreased $\Delta\Psi_M$), active Ras2 becomes sequestered from plasma membrane and nucleus. Recently, nuclear localization of Ras2 was shown to be specifically required for invasive growth (Broggi et al, 2013b). Strains expressing Ras2 fused to the nuclear export signal failed to undergo invasive growth whereas other PKA-related phenotypes were unaffected (Broggi et al, 2013b). It is therefore possible that mitochondrial localization of active Ras2 in *rho* mutants sequester this GTP-ase from plasma membrane and nucleus, interfering with PKA signaling that affects invasive growth and stress response related phenotypes. While in *COX4* mutant the elevated ROS production was shown to be PKA independent, other PKA dependent aspects may be affected in parallel. Therefore, in *rho* mutants the PKA pathway activity may be regulated at the Ras2 level through re-localization of the Ras2 protein from other cellular compartments to mitochondria.

6. Analysis of cAMP-PKA activity in respiratory deficient mutants of W303 and ∑1278b genetic backgrounds (Ref I)

To assess the cAMP-PKA pathway activity in *rho* mutants in more detail, phenotypic traits mediated by the PKA signaling were analyzed next. cAMP-PKA signaling suppresses stress tolerance and it has been shown that mutants with reduced pathway activity are more resistant to heat shock (Hlavatá et al, 2003; Shin et al, 1987; Thevelein & de Winde, 1999). The analysis of cell viability after severe heat shock at 52°C demonstrated that the *rho* mutants of ∑1278b were considerably more tolerant compared with the wt strain (Ref I, Figure 5B) (Figure 10A). Activation of the pathway by *BCY1* deletion led to increased heat-shock sensitivity of both rho^+ and $rho^{0/-}$ strains: only 0.02% of cells formed colonies after 4min incubation at 52°C and further incubation lead to complete loss of viability (unpublished data).

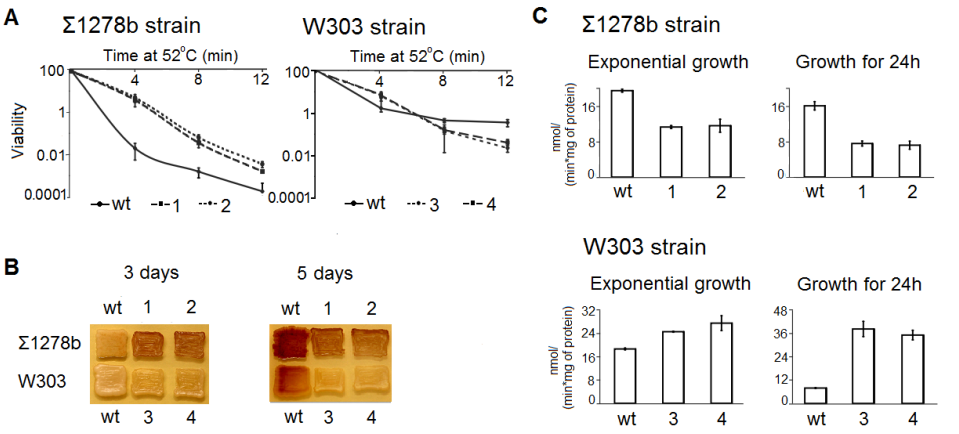


Figure 10. Respiratory deficiency influences the cAMP-PKA pathway activity depending on the genetic background of the strain. **A** – temperature sensitivity assay of strains Σ1278b and W303. Cells were grown exponentially in YPD medium and exposed to heat-shock at 52˚C for indicated times. Viability was expressed as percentage of cells forming colonies after the heat shock relatively to time point 0. **B** – glycogen staining. Strains of Σ1278b and W303 genetic background were patched onto YPD+Ade medium and incubated for 3 to 5 days at 30°C. Plates were exposed to iodine vapor and photographed. Dark coloration indicates presence of the glycogen stores. **C** – trehalase activity in total cell lysates. Strains were grown exponentially or for 24h in YPD medium, trehalase activity is expressed as nmol/ (min*mg of protein) glucose liberated. The assays were performed as described in Ref I under the Materials and Methods section. Strains denoted by numbers: 1 – *rho^0 mip1Δ* Σ1278b, 2 – *rho$^-$ rpo41Δ* Σ1278b, 3 – *rho^0–1* W303, 4 – *rho^0–2* W303.

The cAMP-PKA pathway inhibits accumulation of the reserve carbohydrate glycogen in actively dividing cells (François & Parrou, 2001). Glycogen can be detected upon incubation of wt cells for several days on solid medium by exposing the plates to iodine vapor. The visualization of glycogen storages indicated that this carbohydrate was accumulated rapidly and stored for a prolonged period of time in rho mutants (Ref I, Figure 5D) (Figure 10B). In wt cells accumulation was slower and became detectable by day 5. This difference points to earlier downregulation of the cAMP-PKA pathway in rho mutants. Activation of the pathway through BCY1 deletion completely abolished the glycogen staining of rho mutants, confirming the dependence on PKA activity (Ref I, Figure 5D).

The activity of neutral trehalase Nth1 in cell lysates was determined next. Nth1 is directly phosphorylated by PKA and increased trehalase activity of lysates reflects the signaling through the cAMP-PKA pathway (Ptacek et al, 2005; Uno et al, 1983). Lysates were prepared from exponentially growing cells and cells grown for 24h in YPD. The 24h time point corresponds to late

logarithmic phase of growth where wt cells have passed diauxic shift and *rho* mutants undergo last divisions before final depletion of fermentable carbon source. In both time points lower trehalase activity in *rho* mutants compared with wt ∑1278b was detected (Ref I, Figure 5E) (Figure 10C). This indicates that the PKA activity is lower in respiratory deficient mutants compared with wt cells.

Altogether, the physiological readouts of the cAMP-PKA pathway indicate downregulation of the PKA signaling and support the results presented in Chapter 5 in Results and Discussion. The data are also in accordance with findings by Traven et al (2001) and Dunn et al (2006) where downregulation of the cAMP-PKA pathway activity in *rho* mutants and loss of tolerance to increased cAMP-PKA signaling, respectively were suggested. However, these findings contrast those reported by Graef and Nunnari (2011) where mitochondrial dysfunction was shown to upregulate PKA signaling. Graef and Nunnari (2011) demonstrated that respiratory dysfunction suppresses autophagy due to increased PKA activity in W303 genetic background. To address the difference between results presented in Ref I and in Graef and Nunnari (2011), the physiological readouts of PKA signaling in *rho* mutants of ∑1278b and W303 genetic backgrounds were directly compared (Figure 10). As previously described, the *rho* mutants of ∑1278b showed increased resistance to severe heat shock (Figure 10A, left panel), enhanced accumulation of glycogen (Figure 10B, upper rows) and decreased trehalase activity (Figure 10C, upper panels) in comparison to the respective wt strain. However, the *rho* mutants of W303 exhibited opposing phenotypes compared with isogenic wt cells. The W303 *rho* mutants and the wt parental strain showed similar resistance to heat shock or the mutant was even slightly more sensitive (Figure 10A, right panel). The W303 *rho* mutants did not accumulate glycogen by day 6 when wt cells had established detectable storages of the carbohydrate (Figure 10B, lower rows). The mutants also showed similar (in exponentially growing cells) or increased trehalase activity (in cells grown for 24h) compared with isogenic wt W303 strain (Figure 10C, lower panels). All these results show that the cAMP-PKA pathway is upregulated in *rho* cells of W303 background as suggested by Graef and Nunnari (2011).

The effect of respiratory deficiency on cAMP-PKA pathway activity appears to be dependent on the genetic background of the strain. It is likely that multigenic variations between W303 and ∑1278b play a role in this difference. First, the two strains differ in their auxotrophic markers. While ∑1278b is auxotrophic for uracil and leucine, W303 is auxotrophic also for adenine, histidine and tryptophane. It has been demonstrated that auxotrophic markers show complex interactions among each other in growth rate assays (Mülleder et al, 2012). Prototrophic S288c strain shows markedly increased CLS compared with an isogenic auxotrophic strain and several lethal phenotypes become compensated by restoring the prototrophic state (Mülleder et al, 2012). It is evident that the auxotrophic markers influence physiological parameters and essential phenotypes in complex ways. However, the strains ∑1278b and W303

bear variations in many other genomic loci as well. Sequencing data indicate that the genomes of Σ1278b and S288c differ with an average SNP density of 3.2 per kilobase (Dowell et al, 2010). These strain-specific variations can be expected to result in complex interactions and indeed, there is a 6% difference in conditional essential genes between the two strains (Dowell et al, 2010). Since W303 is more closely related to S288c than to Σ1278b, a considerable number of genetic alterations between Σ1278b and W303 are expected (Kvitek et al, 2008; Schacherer et al, 2007). These genetic variations can potentially influence many cellular processes and phenotypic traits. The cAMP levels and the cAMP-PKA pathway activity have been shown to be increased in Σ1278b compared with strains SP1 and SK1 (Stanhill et al, 1999; Strudwick et al, 2010). It has been proposed that differences in the cAMP-PKA pathway activity could explain the opposing behaviour of Σ1278b and SK1 in their ability to trigger pseudohyphal growth in the presence of a non-fermentable carbon source (Strudwick et al, 2010). Therefore the multigenic variations between Σ1278b and W303 combined with the differential regulation of cAMP-PKA pathway (that may stem from these variations) contribute most likely to the opposing influence of mitochondrial dysfunction on PKA activity in these two strains.

7. Transcriptional regulators of CLS (negative regulators) (Ref II)

When one (or more) essential nutrient(s) is (are) completely depleted from the growth medium, the yeast cells enter stationary growth-phase (Figure 5). The transcriptional mechanisms that control entry and maintenance of the stationary phase are complex, as at least one quarter of the yeast genome becomes transcriptionally reprogrammed during diauxic shift, postdiauxic slow growth, and stationary phase (DeRisi et al, 1997; Radonjic et al, 2005). Acquisition of stationary phase characteristics has been associated with signaling pathways, including the cAMP-PKA, TOR and Snf1 signaling that mediate the nutritional conditions to the cell (De Virgilio, 2012) (Figure 9). How the signals transmitted by these and possible other pathways are integrated to achieve a specific quiescence program that finally determines the CLS of yeast cells is still unclear.

With the computational method m:Explorer a prioritized list of candidate TRs that would govern specific gene expression patterns can be calculated (Ref II). m:Explorer integrates gene expression information from transcription factor (TF) knock-out strains (Hu et al, 2007), TF binding sites (Harbison et al, 2004; MacIsaac et al, 2006; Zhu et al, 2009), and nucleosome occupancy data (Kaplan et al, 2009) of gene promoters to assign specific list of target genes to 285 compiled TRs. To identify TRs that govern specific cellular programs, a gene list specific to that state is required. This list is compared with gene expression profiles assigned to each TR included in m:Explorer. To gain insight into

regulation of quiescence, the m:Explorer was used to predict candidate regulators of diauxic shift (process-specific gene list derived from (Radonjic et al, 2005)) and quiescent cell state (process specific gene list derived from (Aragon et al, 2008)). The diauxic shift data by Radonjic et al (2005) was included to predict quiescence entry regulators while the quiescence data by Aragon et al (2008) should predominantly reflect quiescence maintenance factors. However, it is expected that the factors controlling entry into quiescence and maintenance during quiescence would overlap to some extent. As a result, 97 potential regulators of diauxic shift and quiescent state were identified (Ref II, Figure 4C). This is ~1/3 of all TRs that are included into m:Explorer calculations. Since approximately one quarter of all yeast genes become differentially expressed in these conditions, a large number of potential quiescence regulators is not surprising.

To validate the prediction power of m:Explorer, the knock-out strains of 12 top ranking quiescence regulators (Table 2) were assayed in CLS experiment. The TR envolved potentially govern large part of gene expression changes observed in both entry and maintenance of the quiescence. CLS experiment was performed in liquid YPD medium, similarly to Radonjic et al (2005) and Aragon et al (2008). Viability was determined by plating the cells onto solid YPD media and calculating the percentage of cells that were able to form colonies. The total cell number was estimated from optical density of the cell culture.

Table 2. Gene list of respective knock-out strains included in CLS experiment (Ref II).

Gene	Respective gene function (according to www.yeastgenome.org, august 2014)	Regulator of diauxic shift/ quiescence (according to m:Explorer)	Phenotype of the deletion strain	Growth of the deletion strain on YPG
MGA2	ER membrane protein, regulates transcription of *OLE1*; activity repressed by unsaturated fatty acids	+/+	Increased CLS	+
CST6	Basic leucine zipper TF in ATF/CREB family, involved in utilization of non-optimal carbon sources and chromosome stability, regulates oleate responsive genes	+/+	Increased CLS	+
SWI3	Subunit of SWI/SNF chromatin remodeling complex	+/+	Decreased CLS	–
SDS3	Component of the Rpd3L histone deacetylase complex, required for catalytic activity	–/+	Increased CLS	+
SPT10	Putative histone acetylase with a role in transcriptional silencing, sequence-specific activator of histone genes	+/+	Increased CLS	+
SIN3	Component of both Rpd3S and Rpd3L histone deacetylase complexes, maintenance of chromosomal integrity	+/+	Decreased CLS	+
BAS1	Myb-realted transcription factor; regulation of genes of the purine and histidine biosynthesis pathways; meiotic recombination at specific genes	+/+	Increased CLS	+
SNF2	Catalytic subunit of the SWI/SNF chromatin remodeling complex	+/+	No viability deviation in late G0	–
SPT20	Subunit of the SAGA transcriptional regulatory complex	+/+	No viability deviation in late G0	–
HAA1	TF involved in adaptation to weak acid stress, relocalises to nucleus upon DNA replication stress	+/+	Decreased CLS	+
TUP1	General repressor of transcription, involved in the establishment of repressive chromatin structure	+/+	Decreased CLS	–
SNF11	Subunit of SWI/SNF chromatin remodeling complex	–/+	No viability deviation in late G0	+
ARD1	Subunit of protein N-terminal acetyltransferase NatA, affects telomeric silencing, cell cycle, heat-shock resistance, mating, sporulation	Positive control	Decreased CLS	+
MIP1	MtDNA polymerase gamma	Positive control	Decreased CLS	–
GAL3	TR, involved in activation of the GAL genes in response to galactose	Negative control	No deviation in CLS	+
PDR3	Transcriptional activator of the pleiotropic drug resistance network	Negative control	No deviation in CLS	+

In addition to wt strain and mutant strains of 12 predicted regulators of quiescence, two positive and two negative control strains were included into list (Table 2). Positive control strains (*ard1Δ*, *mip1Δ*) were selected according to previously published results to include strains with predicted (negative) effects on CLS. Deletion of *ARD1* leads to failure to enter stationary phase (Whiteway & Szostak, 1985). Therefore, the reduced viability of *ard1Δ* strains in chronologically aging cultures is expected (Figure 11A). Among 12 potential quiescence regulators, four strains were respiratory deficient (*tup1Δ, swi3Δ, snf2Δ, spt20Δ*) as judged by the inability to grow on a non-fermentable carbon source (Ref II Table s3; Table 2). To provide a reference point to general respiratory deficiency in stationary phase viability, the respiratory deficient *mip1Δ* strain was included into the assay. *mip1Δ* cells are expected to display a defect in viability during prolonged incubation. This was indeed the case, as the viability curve of strain *mip1Δ* deviated from wt viability already at early time points (Ref II, Figure 5E). To exclude the possibility that deviations in CLS from wt strain result not only from specific gene deletions, but from integration of the selection marker (Kan::MX module) that is used in construction of the mutant collection (Giaever et al, 2002), *gal3Δ* and *pdr3Δ* strains were included as negative controls. Gal3 is involved in activation of the *GAL* genes in response to galactose (Torchia & Hopper, 1986), while Pdr3 is required for the induction of pleiotropic drug resistance network (Delaveau et al, 1994). Neither of these processes is expected to influence chronological aging in YPD medium. These two TR-s show non-significant scores in m:Explorer predictions of quiescence regulators. Accordingly, neither of the negative control strains showed significant deviation from wt strain viability (Ref II, Figure 5F for *pdr3Δ*; Figure 11B for *gal3Δ* strain) indicating that the Kan::MX module has no effect in the CLS assay.

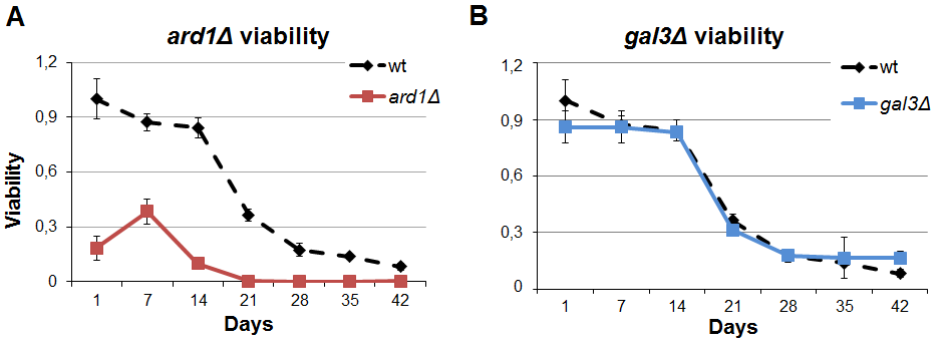


Figure 11. Viability curves of (**A**) *ard1Δ* and (**B**) *gal3Δ* strains. Deletion strains originate from EUROSCARF deletion collection in the BY4741 background (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). Liquid cultures were grown at least in triplicate at 30°C with aeration in YPD for 28 days and subsequently shifted to room temperature without aeration. Culture viability was determined by dividing CFU/ml (colony forming units per ml) with a total cell number per ml in corresponding culture (OD600 x 10^7). Error bars indicate standard deviation.

Viability of the strains was monitored over a six-week time-course. The stationary phase viability was considered to reflect chronological aging, i.e. the increased viability during late time points was taken as a measure of increased fitness and increased life span. Based on this criterion, the strains deleted for *in silico* predicted regulators of quiescence could be divided into three categories. The first category contained mutants with increased viability (*bas1Δ*, *cst6Δ*, *mga2Δ*, *sds3Δ*, *spt10Δ*). The second group of mutants had reduced viability (*haa1Δ, sin3Δ, swi3Δ, tup1Δ*). Viability of the third group (*snf2Δ, snf11Δ, spt20Δ*) did not deviate significantly from the wt strain at later time points (Ref II, Figure 5A; Table 2).

Studies of longevity have mostly focused on mutants with increased life span, since short-lived mutants can be defective in different aspects of growth (e.g. potential to divide) and not experience accelerated aging *per se* (Longo et al, 2012). Among the m:Explorer predicted regulators of quiescence, five strains (*bas1Δ*, *cst6Δ, mga2Δ*, *sds3Δ, spt10Δ*) displayed increased viability during stationary phase compared with wt strain. The strongest effect was observed with *bas1Δ* mutant (Ref II, Figure 5C). Whereas viability of the wt strain decreased to 50% by day 21 of stationary phase (3 weeks of growth), the viability of *bas1Δ* strain decreased to that level by day 35 (5 weeks of growth). The *cst6Δ* and *mga2Δ* strains maintained ~2 fold higher viability starting from the end of week 3 of the experiment (day 21) (Figure 12A). *sds3Δ* and *spt10Δ* strains showed higher viability compared with wt strain only at later time points of the experiment, starting from week 4 (day 28) (Figure 12B). The viability curve of *spt10Δ* strain differed from wt strain also at earlier time points, weeks 1–3, where it showed significantly reduced viability. It has been previously reported that *SPT10* deletion leads to slow growth (Natsoulis et al, 1994). However, the *spt10Δ* strain reached a similar optical density to wt two days after inoculation (data not shown), indicating that the final cell densities of *spt10Δ* and wt were similar in stationary phase. It seems that deletion of *SPT10* led to rapid loss of viability in a significant portion of the cells already at the exponential phase of growth. However, once the stationary phase was reached, the remaining viable cells lost the viability considerably slower compared with wt strain.

One of the strongest positive effects observed in CLS experiments upon single gene deletions are obtained with *sch9Δ* and *ras2Δ* mutants (Fabrizio et al, 2003; Fabrizio et al, 2001). In those mutants up to two fold increase in CLS (measured as the time the culture viability decreases to 0.1%) compared with wt strain has been observed. The results presented in Ref II are not directly comparable to these results since the growth conditions employed are different (growth in YPD in Ref II instead of SCM). Growth medium is known to have a major effect on the life span (Gray et al, 2004; Longo et al, 2012). Whereas in SCM medium the viability of yeast cells decreases to 10% with 10–15 days (Fabrizio et al, 2001; Ocampo et al, 2012), it takes up to four fold more time in YPD to decline to that level (Ref II). To compare the results presented in Ref II

with the *sch9Δ* and *ras2Δ* mutants longevity data, the timepoints when viability of the wt strain has decreased to 10% can be analyzed. Viability of *SCH9* and *RAS2* deletion mutants remains ~9 times higher compared with wt strain at that point in SCM medium (Fabrizio et al, 2003; Fabrizio et al, 2001). The strongest positive effect was observed with the *BAS1* deletion mutant in YPD medium that displayed ~4.5 fold increase in viability at the time when viability of the wt strain declined to 10% (at 42 days of growth) (Ref II, Figure 5C). Bas1 is a DNA-transacting TR, whereas Ras2 and Sch9 regulate several downstream effectors. Therefore, the effect that was observed with *bas1Δ* strain can be considered to be rather significant. Bas1 is required for expression of genes in histidine and purine biosynthetic pathways (Daignan-Fornier & Fink, 1992). It is possible that downregulation of these anabolic processes contributes to more efficient acquisition of stationary phase characteristics.

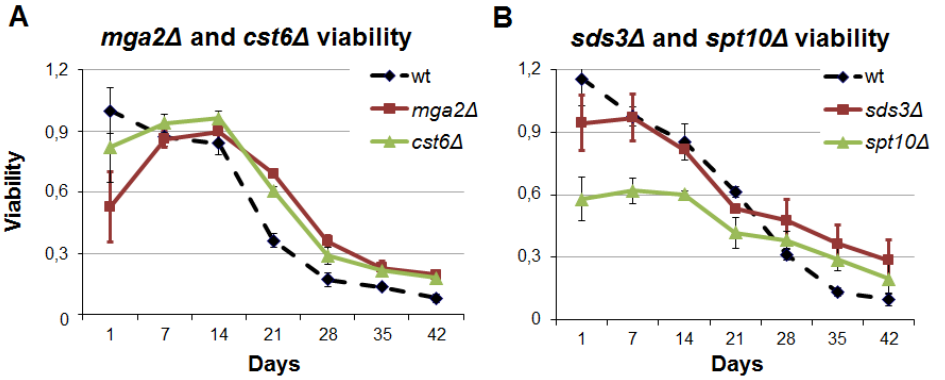


Figure 12. Viability curves of (**A**) *mga2Δ, cst6Δ* and (**B**) *sds3Δ, spt10Δ* strains. Experiments were performed as described in Figure 11.

The two top ranking regulators in m:Explorer prediction, Mga2 and Cst6, have been implicated in regulation of *OLE1* (a key fatty acid desaturase) and oleate responsive genes, respectively (Chellappa et al, 2001; Saleem et al, 2010). These results are intriguing in the light of recent reports that indicate a role for sphingolipid metabolite balance in lifespan determination (Huang et al, 2014). It has been shown that genetic and pharmacological downregulation of sphingolipid synthesis increases yeast CLS (Huang et al, 2012). This effect was shown to be partly mediated through the Sch9 protein kinase. It has also been shown that Sch9 is a regulator of sphingolipid metabolism and upon *SCH9* deletion the changes in specific sphingolipid levels resemble those observed in stationary phase cultures (Swinnen et al, 2014). Therefore, the lifespan promoting effects of *MGA2* and *CST6* mutants may at least in part derive from effects on lipid metabolism and/or Sch9 regulation.

A more general role for Sds3 and Spt10 in curtailing lifespan in later time points of stationary phase is difficult to interpret. While Sds3 is a component of Rpd3L histone deacetylase complex (Vannier et al, 1996), Spt10 has been assigned as putative histone acetylase with a broad role in transcription and chromatin structure (Chang & Winston, 2011). Therefore they participate in somewhat opposing biological processes. In addition, while deletion of *SDS3* leads to increased viability, deletion of another component of Rpd3L complex, *SIN3*, results in slightly decreased viability (Ref II, Figure 5A). It can therefore be speculated that transcriptional regulation of specific targets (rather than general histone acetylation/ deacetylation) mediated by these factors plays a role in modulation of CLS.

8. Respiratory deficient mutants display varying viability in stationary phase (Ref II)

Among the top 12 regulators of quiescence predicted by m:Explorer, four (*TUP1, SWI3, SNF2,* and *SPT20*) are required to maintain respiratory capacity of the cell (Table 2). The fact that cellular respiration plays a positive role in life span extension is well known (De Virgilio, 2012) (Chapter 4.2 in Review of Literature). Genome wide transcriptional profiling indicates that genes with various mitochondrial functions are upregulated upon entry and during the stationary phase (Martinez et al, 2004). Furthermore, functional mitochondria are important for retention of viability for extended periods of time (Bonawitz et al, 2007) and there seems to be a certain minimal threshold of respiration that is required to achieve the lifespan of wt cells (Ocampo et al, 2012).

The viability of respiratory defective mutants of *in silico* predicted regulators of quiescence in comparison with wt (Ref II, Figure 5A) and *mip1Δ* strains (Figure 13) was analyzed next. The *mip1Δ* strain was included as a reference of respiratory deficiency. Contrary to what was expected, not all respiratory deficient mutants showed reduced viability at later time points of stationary phase in comparison to wt strain (Figure 13B). The viability curves of mutants also differed from the viability curve of the *mip1Δ* strain. In fact, viability curves of all the respiratory deficient mutants showed different dynamics. The quickest decline in viability was observed for the *mip1Δ* control strain that showed viability less than 0.3% at 28 days of growth (Figure 13A). Similar results were obtained for a *TUP1* deletion mutant at that time point. However, the rapid loss of viability in the *tup1Δ* strain occurred approximately one week later compared with *mip1Δ* strain, as on day 14 the *tup1Δ* strain showed 60% viability, whereas only 6% of *mip1Δ* cells were viable on day 14. The *swi3Δ* mutants maintained up to 50% viability for even 21 days. However, the mutant demonstrated less than 0.3% viability by 42 days of growth. At that time, ~8% of wt cells were still viable (Figure 13A). It has been noticed before that the loss of viability in CLS experiment occurs at different time points for various

respiratory deficient mutants when introduced to mild heat stress (Martinez et al, 2004). The results in Ref II, Figure 5A and Figure 13 confirm that inability of a specific mutant to respire does not lead to uniform viability dynamics in CLS experiment. It rather appears that specific mutations that lead to respiratory deficiency can affect the viability in a certain range, depending on the type or magnitude of exact rearrangements that occur in the cell.

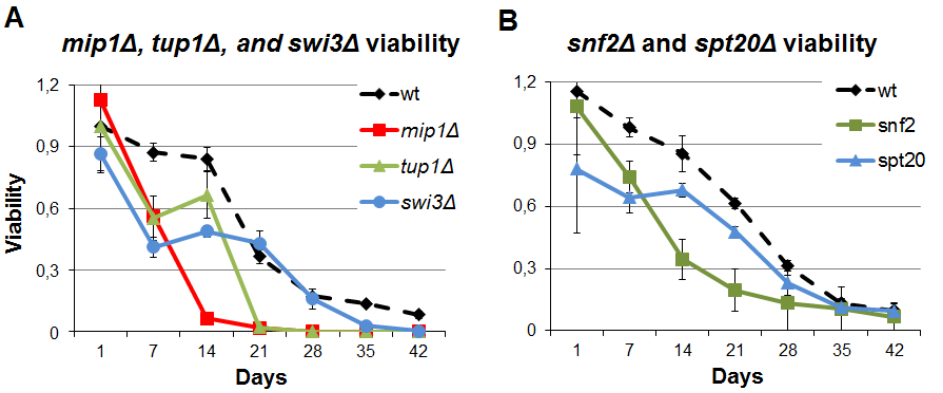


Figure 13. Viability curves of respiratory deficient mutants. Experiments were performed as described in Figure 11.

The *snf2Δ* and *spt20Δ* strains, although with decreased viability at earlier time points, showed no significant difference in viability from the wt strain starting from day 28 of growth (Figure 13B). This result is somewhat unexpected, since the respiratory capacity is considered as a prerequisite for efficient entry and maintenance of stationary phase. Since *snf2Δ* mutants are able to form micro-colonies on YPG medium, it is likely that low level of respiration is still maintained in the mutant. This could explain higher viability at late time points compared with *mip1Δ*, *tup1Δ*, and *swi3Δ* strains. However, no such explanation can be offered for the *SPT20* deletion strain.

There are at least two possibilities how to interpret the results obtained with respiratory deficient mutants. First, the deleterious effect of mitochondrial dysfunction could be similar in all five respiratory deficient strains but becomes alleviated by rearrangement of gene expression at transcriptional or chromatin remodeling level in TR deletion mutants. In this case the functional state of mitochondria can be viewed as a signal that modulates viability and can be interfered without restoration of that specific mitochondrial function. If this scenario holds true, the transcriptional program evoked by *TUP1, SWI3*, *SNF2*, and *SPT20* deletion triggers rearrangements that lead to increased viability of respiratory impaired cells. This can be achieved, for example, through induction of stress responsive genes or upregulation of storage carbohydrate metabolism. Recent epigenetic findings by Schroeder et al (2013) provide another potential

point of interference to the quiescence program. They demonstrated that mtROS elicits DNA damage response independently of DNA damage, leading to repression of subtelomeric transcription and increased CLS. The chromatin remodeling complex components Swi3 and Snf2 can therefore potentially affect the quiescence program at chromatin remodeling level.

The second possibility is that the mitochondrial defects of four predicted regulators could affect different aspects of mitochondrial function (e.g. specific ETC complexes, mitochondrial translation, mitochondrial protein turnover, mitochondrial fusion or fission etc.) that all lead to respiratory deficiency, but maintain other aspects of mitochondrial function that contribute to lifespan retention in a positive manner (e.g. specific aspects of mitochondrial translation, certain threshold of $\Delta\Psi_M$, generation of hormetic ROS). In this case, the *mip1Δ* strain has the strongest phenotype as it has no mtDNA, no mitochondrial translation, aberrant respiratory chain, and appreciably decreased $\Delta\Psi_M$. If in other mutants, however, only some of the rearrangements take place that occur in *mip1Δ*, it is possible that the induction of stress resistance, accumulation of reserve energy storage or some other important aspects of quiescence characteristics are still available to some extent. This in turn would lead to increased viability compared with *mip1Δ*.

Altogether, the experimental data of Ref II supported the validity of m:Explorer to have high *in silico* prediction power and to provide potentially novel regulators of quiescence. The results give first insight into the potential role of Bas1, Mga2, and Cst6 in regulation of specific cellular processes that affect viability of stationary phase cells. The results imply a more general role for the SWI/SNF chromatin remodeling complex and general transcriptional silencing in stationary phase regulation. It can further be concluded that respiratory deficiency of different mutant strains in general does not lead to uniformly decreased viability dynamics in CLS experiments. Rather, the exact type of mitochondrial dysfunction or accompanying cellular rearrangements occurring in specific mutants play a role in longevity determination.

9. MtDNA topology of petite-negative yeast *Candida albicans* (Ref III)

As described in Chapters 2–6 in Results and Discussion, the mitochondrial functional state is tightly linked to cellular cAMP-PKA signaling and filamentous and invasive growth of *S. cerevisiae*. Data from our laboratory further suggests that some specific factors involved in maintenance of *S. cerevisiae* mtDNA may be connected to the cellular signaling pathways involved in regulation of morphogenetic switch (unpublished results). In *C. albicans*, related processes such as dimorphic switch from yeast to hyphal growth and regulation of adhesive and invasive properties of the cell are considered to be important virulence factors (Mayer et al, 2013). Mitochondrial

(respiratory) function plays an important role in determination of the *C. albicans* virulence and drug resistance (Shingu-Vazquez & Traven, 2011). The understanding of mitochondrial involvement in virulence related phenotypes would therefore offer mechanistic insights into the course of pathogenesis (Shingu-Vazquez & Traven, 2011). In a first step, the description of replication mechanism and factors involved in the maintenance of the *C. albicans* mtDNA is an essential precondition to investigate downstream mechanisms and possible interference points.

Although mtDNA maintenance factors and respiratory function have been rather well studied in the classical model yeast *S. cerevisiae*, mitochondrial function of several fungi has escaped the detailed scrutiny because they belong to the group of petite-negative yeasts. These yeasts do not produce cytoplasmic spontaneous petite mutants and do not (or very rarely) form respiratory deficient mutants upon treatment of cells with mutagenizing agents (Bulder, 1964). The ability to survive loss of functional mitochondrial genome is probably the most extensively used property of *S. cerevisiae* model in studies of mitochondrial function. That tool cannot be applied to petite-negative strains. However, screens with several mutagenizing agents have been performed to produce and describe some respiratory deficient cells of *C. albicans* (Roth-Ben Arie et al, 1998). Altogether, little is known about the replication and maintenance mechanisms of mitochondrial genome of *C. albicans*. In our laboratory, a strain deleted for the mtDNA helicase *CaHMI1* has been constructed and described (Jõers et al, 2007). Although this strain is respiratory competent as indicated by the ability to grow on glycerol media, the helicase CaHmi1 is required to maintain wt mtDNA organization. Deletion of both alleles of *CaHMI1* from the obligatory diploid genome of *C. albicans* leads to fragmentation of the mitochondrial genome and accumulation of certain mtDNA fragments (Jõers et al, 2007).

To introduce a system for wt mtDNA maintenance studies in a petite-negative yeast and to gain insight into the replication mechanism of wt yeast mtDNA, the detailed description of *C. albicans* mtDNA was undertaken (Ref III).

The unit size of *C. albicans* mtDNA is 40.4 kb (Jones et al, 2004). Among other components of respiratory complexes, it encodes NADH dehydrogenase subunits that are not found in *S. cerevisiae* (Jones et al, 2004). The mtDNA of *C. albicans* appears to consist of a network of complex branched molecules (Jõers et al, 2007). Possible replication origins and mechanisms of mtDNA replication are not known. However, since the deletion of *CaHMI1* leads to accumulation of mtDNA fragments containing *COX2* gene region, enhanced replication initiation within or close to that fragment has been suggested (Jõers et al, 2007). To characterize the organization, topology, replication and recombination intermediates of *C. albicans* mtDNA, various gel electrophoretic methods were employed (Ref III).

To separate topologically different mtDNA molecules of *C. albicans*, PFGE analysis was performed (Ref III, Figure 1C). The PFGE conditions were chosen to optimize the separation of linear molecules of unit sizes up to 200 kb and to facilitate the migration of large circular DNA molecules in the gel. Agarose embedded cells, embedded purified mitochondria and purified mtDNA were simultaneously analyzed to assess the quality of DNA purification. The amount of well-bound mtDNA was less for purified mtDNA compared with embedded cells and purified mitochondria (Figure 1C, lanes 5, 6, 7 compared with lanes 1, 2 and 3, 4). Reduction in the well-bound fraction is probably due to breakage of large complex DNA structures during preparation, resulting in more mtDNA migrating as smaller molecules. However, there was no major difference in DNA running pattern whether the purified mtDNA, purified mitochondria or whole cells were used. Most of the mtDNA signal was detected in the well-bound fraction and as a smear of molecules with sizes corresponding to linear ~12 to 49 kb DNA fragments (Figure 1C, lanes 1,3,5). Cleaving the DNA with single cutting *Nco*I restriction enzyme resulted in a shift of the hybridization signal to faster migrating forms between sizes ~8 and 40 kb. Since the unit size of *C. albicans* mitochondrial genome is 40.4 kb, an enhanced signal in that region after *Nco*I treatment indicates that head-to-tail concatemers exist that stay in the wel- bound fraction of non-cut preparations.

To address whether supercoiled circular molecules migrate in the gel, the DNA preparations were treated with topoisomerase I (TopoI) which catalyzes the relaxation of negatively supercoiled DNA. Relaxation of supercoiled circular molecules that migrate in the gel by TopoI would lead to immobilization of these molecules. Therefore, the signal in the respective gel lane would shift to the well-bound fraction. After treatment with TopoI a reduction of the signal in the well and increased DNA signal in faster migrating DNA species in size range between ~15 kb and 48.5 kb was observed (Figure 1C, lane 7). The TopoI treatment therefore strongly suggests absence of supercoiled circular molecules.

Since the running pattern of untreated mtDNA samples indicates the presence of large, complex, possibly branched structures, the purified mtDNA was next treated with phage T7 endonuclease I (T7EndoI) (Ref III, Figure 1C, lane 8). T7EndoI cleaves cruciform, branched, non-perfectly matched DNA, and more slowly, nicked double-stranded DNA structures. The treatment with T7EndoI had the most pronounced effect on DNA running patterns. It degraded molecules of the well-bound fraction and of sizes larger than 20 kb. Remaining molecules of sizes between ~5 to 20 kb are assumed to be linear double-stranded DNA. Since after the treatment no residual signal could be detected in the well, molecules of 20 kb and smaller are most likely generated by cleavage of branched molecules. It can be concluded that the majority of *C. albicans* mtDNA forms a complex and branched network containing head-to-tail concatemers. Neither supercoiled circulars DNA molecules (that would increase the well-bound fraction after TopoI and migrate as slow mobility smear after

T7EndoI treatment) nor relaxed circular DNA molecules (would be well-bound after T7EndoI) could be detected.

Systematic analysis of mtDNA with 2D gel electrophoresis revealed the presence of passing replication forks, four-stranded DNA structures and complex branched molecules on entire mitochondrial genome (Ref III). In addition, on all radiographs significant portion of linear dsDNA molecules with unit sizes less than corresponding 1N fragment and ssDNA arcs were detected. Presence of these structures strongly indicates that recombination driven replication (RDR) plays a key role in mtDNA maintenance in *C. albicans*. It was proposed that mtDNA replication is initiated by recombinative end invasions of homologous DNA fragments all over the mitochondrial genome (Ref III).

Relative copy numbers of various mtDNA regions of the *CaHMI1* double deletion mutant strain (PJ387) were analyzed in more detail next (Ref III, Figure 6). A significant copy number elevation was observed at the *nad1* region in small coding region of the mtDNA with progressively decreasing DNA levels in the nearby inverted repeat in the mutant strain. The regions further away showed copy numbers significantly below wt level. The DNA copy number elevation at *nad1* in PJ387 mutant strain suggests frequent replication initiation within or nearby of that fragment. Specific Y arc patterns were also observed close to this region with 2D gel electrophoresis (Ref III, Figure 3) and frequent strand invasion was therefore proposed to occur in the inverted repeat, suggesting a special role in replication initiation. The significant copy number elevation in the *CaHMI1* mutant strain near the proposed replication initiation sites suggests that while the homologous recombination driven replication is initiated readily in the mutant, the elongation to regions further away seems to be impaired.

Altogether, the *C. albicans* was introduced as suitable model organism to study wt mtDNA maintenance in yeast. A first systematic description of mtDNA topology and DNA metabolic intermediates of that petite-negative yeast species was provided. Data from various 2D gel electrophoresis methods demonstrated that recombination plays a key role in mtDNA maintenance in *C. albicans*. Since findings in mitochondria of plants (Manchekar et al, 2006; Oldenburg & Bendich, 2001) and human heart (Pohjoismäki et al, 2009) also point to a homologous recombination initiated DNA replication, the results support a more universal mechanism for recombination driven replication in mtDNA maintenance. Further, deletion of *CaHMI1* was shown to results in substantial fragmentation and accumulation of specific fragments of the mtDNA. It has been proposed that factors involved in the metabolism of *C. albicans* mtDNA provide one possible mechanism for interference with pathogenicity determinants (Shingu-Vazquez & Traven, 2011). The increasing incidence of multidrug resistance during treatment of opportunistic fungal infections has raised concerns about limited number of clinically useful antifungal drugs available and a role for mitochondria regulated processes (ROS

generation, iron and lipid homeostasis) are proposed to be among possible new targets for drug discovery (Hameed & Fatima, 2013). Yeast genes that lack human homologues are considered especially promising candidates for broad-spectrum anti-fungal targets. The CaHmi1 has no homologues in higher organisms, making it potential yeast-specific target. Although deletion of *CaHMI1* does not lead to lethality or loss of respiratory function (Jõers et al, 2007), it results in substantial rearrangements in the mtDNA. It is yet to be determined weather this affects the fitness and virulence determinants of *C. albicans*.

CONCLUSIONS

In recent years several reports have demonstrated that a signal from the functional state of mitochondria feeds into conserved signaling pathways to modulate the outcome of diverse cellular responses (Graef & Nunnari, 2011; Kawai et al, 2011; Leadsham et al, 2013; Schroeder et al, 2013). By scrutinizing the effect of mitochondrial dysfunction on various phenotypic traits in starvation stress conditions additional evidence for such a mechanism to occur in the model yeast *S. cerevisiae* is provided in this thesis. It is also shown that specific cellular context and strain background can influence the interaction between mitochondrial function or mitochondria generated signal and final phenotypic traits in profound way. Following conclusions can be drawn from the present study:

1. In the context of filamentous and invasive growth, the functional state of mitochondria serves as a signal that interferes with morphological differentiation program at transcriptional level. More precisely, it was shown that in the respiratory deficient mutants the transcription of well described filamentous growth target *FLO11* is downregulated and induction of *FLO11* expression restores both responses. These results indicate that insufficient supply of metabolic energy or some other metabolic intermediate is not limiting for initiation of the morphological differentiation in *rho* mutants.
2. The RTG signaling pathway induced in rho^0 cells plays a positive role in filamentous growth response and the RTG signaling is induced in wt cells in starvation conditions. In addition, the FG specific MAPK cascade is functional in rho^0 mutants. It can be concluded that inactivity of neither FG MAPK or RTG pathway is responsible for the strong filamentation and invasion defect of *rho* mutants.
3. Mitochondrial dysfunction specifically downregulates the cAMP-PKA signaling pathway in ∑1278b strain. Analysis of phenotypic traits indicate that the effect of respiratory dysfunction on cAMP-PKA signaling activity depends on genetic background of the strain and is probably interpreted according to specific cellular metabolic context.
4. Validation of computational method m:Explorer revealed several new candidate regulators of diauxic shift and quiescent state of the cell. The most pronounced effects were observed with *BAS1*, *MGA2*, *CST6* deletion strains where significant CLS extension was demonstrated. It was further shown that respiratory deficiency of different mutants in general does not lead to uniformly decreased viability dynamics and respiratory dysfunction can modulate CLS by varying degree depending on specific TF perturbation. The exact type of mitochondrial dysfunction, or accompanying cellular rearrangements of specific mutants appear to play a role in longevity determination. The latter possibility stresses that the signal from

mitochondrial (dys)function affects cell faith according to specific cellular context.

5. Topological analysis of *C. albicans* mtDNA indicates that the majority of the mitochondrial genome is present as complex and branched network that contains head-to-tail concatemers. The topological studies provide framework for identification and characterization of mtDNA maintenance factors.

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SUMMARY IN ESTONIAN

Mitokondrid kui rakulise signaaliülekande moduleerijad

Mitokondrid on eukarüootse raku organellid, kus toimub raku elutegevueks vajaliku energia tootmine oksüdatiivse fosforüleerimise teel. Samuti mängivad mitokondrid olulist rolli raku metabolismi koordineerimises, kuna mitmete metaboolsete vaheühendite süntees ja lagundamine toimub mitokondrite sisemuses. Lisaks nendele eluks hädavajalikele protsessidele osalevad mitokondrid rakulises signaaliülekandes. Ühelt poolt on mitmed signaaliülekande rajad vajalikud mitokondrites toimuvate protsesside reguleerimiseks. Näiteks pärsivad pagaripärmis *Saccharomyces cerevisiae* mitokondriaalset hingamist väliskeskkonna toitainete tingimusi seiravad rapamütsiini märklaua (TOR) ja tsüklilise AMP sõltuva valgu kinaasi A (cAMP-PKA) signaalirajad. Snf1 signaalirada osaleb aga mitokondriaalse hingamise aktiveerimises. Teisalt on üha enam tõendeid selle kohta, et mitokondrite funktsionaalne seisund võib ise mõjutada teatud konserveerunud signaaliradade aktiivsust. *S. cerevisiae* puhul on näidatud, et autofaagiat esile kutsuvates näljatingimustes põhjustab mitokondrite väärtalitlus cAMP-PKA raja aktiveerimise (Graef & Nunnari, 2011). Samuti on näidatud, et mitokondri väärtalitlusega pärmi mutantides vähendatakse TOR raja aktiivsust (Kawai et al, 2011).

Vastusena teatud näljatingimustele osalevad eelpool mainitud TOR, cAMP-PKA ja Snf1 signaalirajad pagaripärmi elutegevuses ja morfoloogia ümberkujundamises, põhjustades pärmi filamentset ja invasiivset kasvu. Kuna samad signaalirajad reguleerivad ka mitokondrite talitlust, on tõenäoline, et mitokondrites toimuvate protsesside reguleerimine on üks osa morfoloogilise ümberlülitamise keerukast protsessist. Kuigi mitokondrite normaalse talitluse hädavajalikkus filamentse kasvu esilekutsumises on korduvalt kinnitust leidnud (Jin et al, 2008; Kang & Jiang, 2005; Lorenz et al, 2000), ei ole teada, mil moel mitokondrite funktsionaalne seisund morfoloogilist ümberlülitamist mõjutab. Käesoleva uurimustöö peamiseks eesmärgiks oli iseloomustada häiritud mitokondriaalse funktsiooniga rakkude käitumist näljatingimustes. Samuti oli käesoleva töö osaks arvutusliku meetodi m:Explorer poolt ennustatud statsionaarse faasi elulemuse säilitamiseks oluliste transkriptsiooni regulaatorite olulisuse kinnitamine katselisel teel. Uurimustöö hõlmab ka pärmide mitokondriaalse DNA topoloogia iseloomustamist.

Käesoleva töö peamised tulemused:

1. Mitokondrite funktsionaalne seisund mõjutab rakulist signaaliülekannet ja sekkub seeläbi filamenteerumise ja invasiivse kasvu ühe märklaudgeeni *FLO11* ekspressiooni regulatsiooni. Töös näidati, et mitokondri väärtalitlusega pärmi mutantides, millel puudub funktsionaalne mitokondriaalne DNA (*rho* rakud) ja mis pole seetõttu võimelised läbi viima mitokondriaalset hingamist ning teatud tsitraaditsükli metaboolseid reaktsioone, on *FLO11*

üleekspresseerimine piisav, et esile kutsuda nii filamentset kui invasiivset kasvu. Sellest võib järeldada, et mitokondrites toodetud energia ja/või metaboolsed vaheühendid ei ole morfoloogilise ümberlülitamise esilekutsumiseks hädavajalikud. Samas mõjutab mitkondrite väärtalitlus olulisel määral *FLO11* geeni ekspressiooni, mis on reguleeritud väga paljude erinevate signaaliradade poolt.

2. Retrograadne (RTG) signaaliülekande rada, mis aktiveeritakse *rho* rakkudes, osaleb filamentse kasvu esilekutsumises positiivse regulaatorina. Samuti aktiveeritakse RTG rada näljatingimustes metsikut tüüpi rakkudes. Lisaks on *rho* rakkudes säilinud filamenteerumise spetsiifilise mitogeeni aktiveeritud valgu kinaasi (FG MAPK) raja aktiivsus.
3. Filamenteerumisvõimelises ∑1278b pärmitüves põhjustab mitokondrite väärtalitlus cAMP-PKA raja aktiivsuse vähenemise. cAMP-PKA raja poolt mõjutatud fenotüübiliste tunnuste võrdlemisel ∑1278b ja W303 pärmitüves selgus, et mitkondrite väärtalitluse mõju cAMP-PKA raja aktiivsusele sõltub konkreetsest tüvest.
4. Arvutusliku meetodi m:Explorer valideerimise tulemusena tuvastati mitmed uued statsionaarse faasi elulemust mõjutavad regulaatorid. Suurim positiivne mõju kronoloogilise vananemise katsetes oli pärmitüvedel, mille genoomist olid kustutatud *BAS1*, *MGA2* ja *CST6* geenid. Lisaks analüüsiti selliseid transkriptsiooni regulaatoreid, mille genoomist kustutamine viis muuhulgas *rho* fenotüübini. Nende puhul sõltus mõju kronoloogilisele vananemisele konkreetsest mutandist. Seega on *rho* rakkude puhul oluline kas rakuline kontekst, mille ümberkujundamisega on võimalik mõjutada mitokondrite väärtalitluse mõju vananemisprotsessile, või konkreetne mitokondri defekt.
5. *C. albicansi* mitokondriaalse DNA topoloogia analüüs näitas, et enamik selle pärmi mitokondri genoomist esineb keeruka ja hargnenud võrguna, kus esinevad üksteisega seotud ühe genoomse korduse pikkused molekulid. Mitokondriaalse DNA topoloogiline kirjeldus *C. albicansis* pakub raamistiku järgnevaks mitokondriaalse DNA metabolismiga seotud valguliste komponentide tuvastamiseks.

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