Dysfunctional mitochondria modulate cAMP-PKA signaling and filamentous and invasive growth of *Saccharomyces cerevisiae*

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ABSTRACT

Mitochondrial metabolism is targeted by conserved signaling pathways that mediate external information to the cell. However, less is known about whether mitochondrial dysfunction interferes with signaling and thereby modulates the cellular response to environmental changes. In this study, we analyzed defective filamentous and invasive growth of the yeast *Saccharomyces cerevisiae* strains that have a dysfunctional mitochondrial genome (*rho* mutants). We found that the morphogenetic defect of *rho* mutants was caused by specific downregulation of *FLO11*, the adhesin essential for invasive and filamentous growth, and did not result from general metabolic changes brought about by interorganellar retrograde signaling. Transcription of *FLO11* is known to be regulated by several signaling pathways including the filamentous-growth specific MAPK and cAMP-PKA pathways. Our analysis showed that the filamentous-growth specific MAPK pathway retained functionality in respiratory-deficient yeast cells. In contrast, the cAMP-PKA pathway was downregulated, explaining also various phenotypic traits observed in *rho* mutants. Thus, our results indicate that dysfunctional mitochondria modulate the output of the conserved cAMP-PKA signaling pathway.
INTRODUCTION

Mitochondrial metabolic activities are coordinated with the availability of nutrients through several conserved signaling pathways. In the budding yeast *Saccharomyces cerevisiae*, the cAMP activated protein kinase A (cAMP-PKA) pathway can modulate the enzyme content of mitochondria, reactive oxygen species generation, antioxidant defense system and mitochondrial protein import (Dejean et al. 2002; Hlavatá et al. 2003; Chevtzoff et al. 2005; Feliciello et al. 2005; Hlavatá et al. 2008; Schmidt et al. 2011). Downregulation of the target of rapamycin (TOR) pathway leads to an increase of mitochondrial respiratory complexes (Bonawitz et al. 2007; Pan and Shade 2009). The Snf1 pathway regulates the switch from glycolytic energy production to mitochondrial respiration in response to low glucose and ADP levels (Uley et al. 1994; Mayer et al. 2011).

Disturbed mitochondrial metabolism, in turn, can affect a broad range of cellular activities through aberrant fluxes of metabolites, formation of reactive oxygen species or through cellular signaling (Scheffler 2001; McBride et al. 2006). In yeast cells that lack the wild type mitochondrial genome (*rho* cells), the retrograde signaling pathway (RTG) is activated, leading to changes in nuclear gene expression and readjustments of carbohydrate and nitrogen metabolism (Liu and Butow 2006). Homologs of RTG genes have not been found in higher organisms, but the central stress regulator NF-κB has been proposed to fulfill similar functions (Srinivasan et al. 2010).

Two recent reports indicate that dysfunctional mitochondria can directly interfere with signaling pathways that mediate nutritional information to the yeast cell. In *rho* cells the main target of the TOR pathway – the Sch9 kinase – is dephosphorylated, suggesting downregulation of the pathway (Kawai et al. 2011). Mitochondrial dysfunction can also interfere with the regulation of autophagy by modulating the activity of the cAMP-PKA pathway (Graef and Nunnari 2011; Kawai et al. 2011).

Several of the signaling pathways that regulate mitochondrial metabolism are also required to activate an elaborate differentiation program leading to pseudohyphal or filamentous growth
(BRÜCKNER and MÖSCH 2012). Under specific nutrient-poor conditions, yeast cells switch to a unipolar budding pattern and form physically attached elongated cells that can invade the growth substrate (GIMENO et al. 1992; KRON et al. 1994; ROBERTS and FINK 1994). Pseudohyphal differentiation can be initiated by nitrogen starvation or in low glucose media (GIMENO et al. 1992; CULLEN and SPRAGUE 2000). It is also induced with fusel alcohols, the end products of amino acid catabolism in yeast, suggesting that intermediary metabolism can modulate the interpretation of nutritional signals received by the cells (DICKINSON 1996; LORENZ et al. 2000; JIN et al. 2008).

Nutritional clues are sensed and filament formation is regulated through the activation of complex and partially interconnected pathways, most notably the filamentous growth (FG) specific MAPK cascade and the cAMP-PKA pathway (LIU et al. 1993; ROBERTS and FINK 1994; ROBERTSON and FINK 1998; PAN and HEITMAN 1999). The pathways converge on FLO11 (MUC1), encoding a cell-surface glycoprotein that is essential for the morphogenetic switch (LIU et al. 1993; ROBERTS and FINK 1994; LO and DRANGINIS 1998; ROBERTSON and FINK 1998; PAN and HEITMAN 1999; RUPP et al. 1999).

Evidence that mitochondrial genes play a role in filamentation has been obtained from large-scale studies and genetic screens of filamentation-defective mutants (LORENZ et al. 2000; KANG and JIANG 2005; JIN et al. 2008). While respiratory-deficient yeast mutants appear to be defective in filament formation, the underlying mechanism remains unclear. Activation of the RTG pathway has been suggested to inhibit filamentous growth by changing nuclear gene expression in rho cells (LIU and BUTOW 2006; JIN et al. 2008). Somewhat contradictory results, however, have demonstrated that inactivation of the RTG signaling blocks invasive growth of respiratory-competent cells (CHAVEL et al. 2010).

Here we scrutinize the filamentous and invasive growth properties of rho mutants and show that they can undergo the morphogenetic change; however, the cells do not express the cell surface adhesin
Flo11. Analyses of the RTG, FG MAPK and cAMP-PKA signaling pathways indicate that the dysfunctional state of mitochondria modulates signaling through the cAMP-PKA pathway and this results in downregulation of *FLO11*. 
MATERIALS AND METHODS

Yeast strains and media: Yeast strains used in this study are listed in Table 1. Gene deletions were generated by one-step PCR-based gene disruption, replacing the complete ORFs with *kanMX6, natMX6* or *hphMX6* cassettes (JANKE et al. 2004; HENTGES et al. 2005). Respiration-deficient *rho−* and *rho0* strains were generated using two methods. First, deletions of *RPO41* or *MIP1* genes caused restructuring (*rho−*, strain SCΣ-146) or complete loss (*rho0*, strain SCΣ-139) of mitochondrial DNA (mtDNA), respectively. Second, ethidium bromide treatment was used to generate *rho−* and *rho0* mutants (strains SCΣ-160, SCΣ-150) (FOURY 2002). The respiratory deficiency of the strains was tested on a non-fermentable carbon source. Loss of mtDNA was verified by 4’,6-diamidino-2-phenylindole (DAPI) staining.

Cells were grown at 30°C unless specified otherwise in either YPD medium (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose) or synthetic complete medium without uracil and leucin (SC ura-leu−) to select for transformants (SHERMAN 2002). Agar (2%) was added to solid media. Filamentous growth was assayed on solid low-nitrogen (SLAD) medium (2% glucose, 50 μM ammonium sulfate, 0.17% yeast nitrogen base without amino acid and ammonium sulfate) supplemented with 1% isobutanol (LORENZ et al. 2000). Strains were complemented for auxotrophic mutations with pRS-series plasmids (SIKORSKI and HIETER 1989) and grown for 5-7 days to obtain equal colony size. Colony morphology was examined with an Olympus BX61 microscope using a 10x Plan Olympus objective and bright-field optics. The images were captured with an Olympus DP70 cooled CCD camera. For the invasive growth assay, the strains were grown on YPD plates for 3-7 days to obtain similar patch density. Plates were washed under a gentle stream of water and photographed before and after the wash (ROBERTS and FINK 1994). The *bcy1Δ* strains were analyzed at 25°C for invasive growth and glycogen accumulation.
**Plasmids:** Plasmids used in this study are listed in Table 2. Plasmids pLG669-Z *FLO11* 6/7 and 9/10 were constructed as described (Rupp et al. 1999). *FLO11* promoter fragments (-1400 to -1000 bp from *FLO11* start codon corresponds to fragment 6/7 and -2000 to -1600 bp to fragment 9/10, respectively) were amplified from genomic DNA and cloned into a XhoI-digested pLG669-Z (Guarente and Ptashne 1981). Genomic loci of *TPK2* (-579 to +566 bp) (Pan and Heitman 1999), *BCY1* (-420 to +400 bp), *SFL1* (-683 to +719 bp) and *FLO8* (-1032 to +497 bp) (Van Dyk et al. 2003) were amplified with PCR and cloned between XhoI and BamHI restriction sites into the pRS426 vector (Christianson et al. 1992). “–“ indicates nucleotides upstream from start codon and “+” indicates nucleotides downstream from stop codon of the respective gene.

**Cell elongation measurement:** Cell elongation was determined as described (Mösch and Fink 1997). Strains were grown on SLAD medium supplemented with 1% isobutanol for 2 (rho⁺) or 4 (rho mutant) days at 30°C to obtain equal colony size. The length to width (l/w) ratio of at least 200 cells from multiple colonies was quantified using light microscopy. Cells were divided into two classes: yeast form cells (YF) with a l/w ration of 1-2; pseudohyphal cells (PH) with a l/w ratio greater than 2.

**Reporter gene assay:** A reporter plasmid containing the *URA3* gene (Table 2) was transformed with pRS315 containing the *LEU2* gene (Sikorski and Hieter 1989) into yeast strains to compensate for auxotrophic mutations. Cells were pre-grown in SC ura′leu′ medium, inoculated into fresh medium to OD₆₀₀~0.3 and grown for 4-5h to OD₆₀₀~1.0-1.5 (exponential growth phase). For the 24h time point, cells were grown for 24h. For 5h growth on SLAD, cells were grown for 20h, washed twice with 2% glucose, transferred into SLAD medium and grown for 5h as described (Rupp et al. 1999). Starvation conditions were induced by plating ~10⁵ cells from exponentially growing cultures onto SC ura′leu′ plates and incubating for 3 days. Cells were scraped off the plate, washed twice with 2% glucose and assayed for reporter activity (Madhani and Fink 1997; Sabbagh et al. 2001). ß-galactosidase activity
was determined with the use of ONPG as a substrate and expressed as Miller Units (nmol of o-nitrophenol produced/ (mg of total protein*ml*min)) (ROSE and BOTSTEIN 1983).

**Quantitative PCR analysis:** Total RNA was isolated from cultures (15ml) grown exponentially in YPD medium or from patches grown for 3 or 7 days on YPD plates (MAI and BREEDEN 1997). 5μg of total RNA were treated with DNase I, followed by cDNA synthesis from 1μg of RNA with RevertAid M-MuLV Reverse Transcriptase (Fermentas) according to the manufacturer’s instructions. 1/20 of the synthesized cDNA was used as a template for quantitative real-time PCR. Quantitative PCR was performed in a 10μl reaction volume with the Maxima SYBR Green qPCR Master Mix (Fermentas) using an ABI Prism® 7900HT Fast Real-Time PCR System in standard conditions (denaturation at 95° for 15 min; 40 cycles: denaturation 95° 15s and annealing 60° 1 min). Melt curve profiles were generated to confirm specificity of amplified fragments. The length of PCR products was approx. 160bp, primer sequences used are available upon request. Gene expression was quantified by the comparative C_T method (SCHMITTGEN and LIVAK 2008). For normalization of mRNA levels, the geometric mean of two housekeeping genes (UBC6, ARP6) was used. Suitability of UBC6 and ARP6 as housekeeping genes was determined by the geNorm program (VANDESOMPELE et al. 2002; TESTE et al. 2009). The average and standard deviations were calculated from three independent experiments.

**Heat shock sensitivity:** Exponentially growing cells in YPD medium were exposed to 52° for 4-12 min and plated onto YPD plates. After growth at 30° for 3-5 days the colonies were counted. Viability was expressed as percentage of cells forming colonies after the heat shock relative to the number of colonies in the untreated samples (HLAVÁTÁ et al. 2003).

**Glycogen staining:** Cells were streaked onto YPD plates and grown for 3 to 6 days at 30° or 25°. Plates were exposed to iodine vapor until the appearance of a brown coloration indicating the presence of glycogen stores and then photographed.
Trehalase assay: Pre-cultures were diluted into fresh YPD medium and grown for 24h at 25° when the wild-type cells had passed the diauxic shift. Cell extracts were prepared and a trehalase assay was carried out as described (CARRILLO et al. 1994), except 10mM MES/KOH pH6.8 was used in reaction mixtures. Glucose concentration in the supernatant was determined with Glucose liquidcolor (Human GmbH). Protein concentration was determined with the Bradford method. Trehalase activity was expressed as nmol of glucose released/ (mg of total protein*min) (CARRILLO et al. 1994).

The average and standard deviations for reporter construct, temperature sensitivity and trehalase assays were calculated from three independent cultures, experiments were performed at least twice.
RESULTS

Functional mitochondrial genome is required for filamentous and invasive growth of budding yeast

Previous genome wide screens of genes required for yeast filamentous growth have indicated that respiratory-deficient yeast cells are defective in filament formation (KANG and JIANG 2005; JIN et al. 2008). However, it is unclear if respiratory deficiency generates defects in filamentous growth as a result of general remodeling of cellular metabolism or by interference with specific signaling pathways that regulate the filamentation response. To investigate the role of mitochondria in the filamentous and invasive growth in detail, we compared the ability of wild type rho+ and respiratory-deficient rho haploid cells of the Σ1278 background to form filaments and invade agar substrates. Mutant strains lacking the mitochondrial genome (rho0) or retaining nonfunctional short fragments of mitochondrial DNA (rho−) were constructed either by ethidium bromide mutagenesis or by disruption of RPO41 or MIP1 involved in mitochondrial genome maintenance (GENGA et al. 1986; FANGMAN et al. 1990).

Filamentous growth of rho mutants and rho+ cells was assayed on low-nitrogen (SLAD) medium supplemented with 1% isobutanol (Figure 1A, middle panels). Invasive growth was monitored on the same medium or on YPD after washing non-adherent cells off the plates (Figure 1A, C, lower panels). All tested respiratory-deficient strains were defective in both filament formation and agar invasion, and no variation could be observed between different rho0 and rho− mutants. The rho cells formed fewer short and less branched filaments compared to rho+ strain (Figure 1B). However, we also noted, that despite of the obvious defect in filamentation, the rho mutant cells appeared to be elongated. 78% of rho cells had pseudohyphal geometry and this was only slightly less than the corresponding fraction of elongated cells of rho+ strain (84%) under similar growth conditions (Figure 1B). Compared to the rho+ tec1Δ strain the rho mutants retained some residual filament formation and invasion. TEC1
encodes a transcription factor activated by the FG MAPK cascade (Figure 3A) and is required for both isobutanol-induced filamentation and invasive growth (MÖSCH et al. 1996; LORENZ et al. 2000). Our results demonstrate that rho cells exhibit defective invasive growth. We also confirm that a functional mitochondrial genome is required for a wild type level of filamentation, as reported earlier (KANG and JIANG 2005; JIN et al. 2008).

Dysfunctional mitochondria interfere with signaling pathways regulating FLO11 transcription

The weak invasive phenotype and defective filament formation led us to ask if the adhesin FLO11, required for both filament formation and agar invasion (LAMBRECHTS et al. 1996; LO and DRANGINIS 1998; ROBERTSON and FINK 1998), is expressed in rho cells during starvation.

To analyze FLO11 promoter activity, we first exploited a P_FLO11::lacZ reporter carrying the whole 3kb promoter of FLO11 (RUPP et al. 1999). β-galactosidase activity was measured in cells grown either exponentially or under starvation conditions in SC ura-leu- or SLAD medium (Figure 1D-F). The activity of P_FLO11::lacZ reporter was severely reduced in rho mutants compared to the rho+ strain. Under all conditions tested the FLO11 reporter activity in rho mutants was similar to the rho+ tec1Δ strain.

To confirm these findings, the mRNA level of FLO11 was analyzed with quantitative PCR in rho+ and rho0 mip1Δ cells grown exponentially or patched onto YPD plates to induce invasive growth. We found that in the rho0 mip1Δ mutant, the FLO11 mRNA level was reduced approximately 4-fold in exponential cultures and 10-fold under invasive growth conditions (Figure 1G).

We next tested if respiratory-deficient mutants are able to shift to pseudohyphal growth by ectopically expressing FLO11. The expression of FLO11 under the control of the constitutive TEF promoter resulted in a filamentation and invasion phenotype of rho mutants that was indistinguishable from the rho+ strain (Figure 1H, I). In contrast, FLO11 expression in the rho+ tec1Δ strain only
partially restored filamentation, suggesting that other Tec1 targets are essential for filament formation at a wild type level.

Our results show that the expression of \textit{FLO11} is downregulated in respiratory-deficient yeast cells and ectopic expression of \textit{FLO11} can restore filamentous and invasive growth of the mutants. Since extensive rearrangements of nuclear gene expression patterns take place in \textit{rho} cells (EPSTEIN \textit{et al.} 2001; TRAVEN \textit{et al.} 2001), we next focused on the interorganellar (RTG) signaling pathway that can mediate these changes.

\textbf{The RTG pathway is activated in both \textit{rho}+ strains and \textit{rho} mutants during starvation}

Activation of the RTG signaling pathway in \textit{rho} mutants leads to compensatory readjustments in cellular metabolism and nuclear gene expression (LIU and BUTOW 2006). The sensor of mitochondrial dysfunction is the cytoplasmic protein Rtg2 that regulates the sub-cellular localization of the transcriptional activators Rtg1 and Rtg3 (Figure 2A) (SEKITO \textit{et al.} 2000). Two recent reports have reached conflicting conclusions as to whether the RTG pathway signaling is required for yeast invasive growth and filament formation or not. First, the RTG pathway regulators Rtg1 and Rtg2 have been shown to be required for invasive growth of \textit{rho}+ cells (CHAVEL \textit{et al.} 2010). In contrast, it was also suggested that in respiratory-deficient yeast strains, the activation of RTG signaling is at least partially responsible for defective filament formation (JIN \textit{et al.} 2008). Therefore, we next aimed to clarify the role of RTG signaling in filamentous growth in both the \textit{rho}+ strain and respiratory-deficient mutants.

We inactivated retrograde signaling by disrupting the pathway positive regulator \textit{RTG2} and then monitored filamentous and invasive growth (Figure 2B, C). Inactivation of the RTG pathway did not change the defective phenotype of respiratory-deficient mutants (Figure 2B, C). The invasion phenotype of the \textit{rho}+ \textit{rtg2A} strain was also not affected, but a reduction in filament formation was observed (Figure 2B, C). Different other mutants have been characterized where filament formation is
affected without reduction in invasive growth, including the bud-site selection and cell polarity mutants (MÖSCH and FINK 1997; LORENZ et al. 2000). Similarly, FLO11 overexpression in rho\(^+\) tec1Δ background restored invasive growth to wild type level in our hands but filament formation was rescued only partially (Figure 1H, I).

Next, we analyzed the effect of RTG pathway inactivation on FLO11 promoter activity by using the P\(_{FLO11}\)::lacZ reporter. The deletion of RTG2 did not increase the activity of the P\(_{FLO11}\)::lacZ reporter in respiratory-deficient strains (Figure 2D). Furthermore, the deletion led to a 3-fold decrease in the activity of the FLO11 reporter in the rho\(^+\) strain, supporting the observation that the RTG pathway is required for a complete filamentation response in wild type cells (Figure 2B) and for invasive growth as proposed earlier (CHAVEL et al. 2010).

RTG pathway activation in rho\(^0\) strains has mostly been studied under non-repressing conditions on raffinose medium using the non-invasive PSY142 strain (LIAO et al. 1991; LIAO and BUTOW 1993). Since most filamentation and invasion assays are performed in the Σ1278b background and on the repressive carbon source glucose, we next analyzed if the pathway is activated in Σ1278b strain under conditions that stimulate filamentation. The retrograde response was measured with the P\(_{CIT2}\)::lacZ reporter that reflects the expression level of the prototypic RTG pathway target CIT2 (LIAO et al. 1991). In exponentially growing cultures, only a modest 2-fold increase of β-galactosidase activity was observed in rho mutants compared to the rho\(^+\) strain, suggesting a weak retrograde response under these conditions (Figure 2E). In contrast, an approximately 10-fold increase has been reported for the PSY142 strain (LIAO et al. 1991). The activation of the pathway in the Σ1278b background was entirely dependent on RTG2, similarly to what has been reported for the PSY142 strain (Figure 2E, F) (LIAO and BUTOW 1993). Under starvation conditions on SC ura'leu' plates, the activity of P\(_{CIT2}\)::lacZ reporter was activated in rho mutants and the rho\(^+\) strain to comparable levels.
Compared to exponentially growing cultures the CIT2 reporter activity increased more than 3-fold in rho\(^+\) strain and approximately 1.3-fold in rho mutants (Figure 2F).

To confirm these results, we compared the levels of CIT2 mRNA with quantitative PCR in the rho\(^0\) mip1\(\Delta\) mutant and rho\(^+\) strain. During exponential growth, no difference in CIT2 mRNA levels was detected between rho\(^0\) mip1\(\Delta\) mutant and rho\(^+\) strain (Figure 2G). Under starvation conditions, the level of CIT2 mRNA was increased in both the rho\(^+\) strain and rho\(^0\) mip1\(\Delta\) mutant compared to exponentially growing cells, indicating that the RTG pathway was activated upon glucose depletion in both strains (Figure 2G). Similar changes of CIT2 promoter activity were observed with the P\(_{CIT2}::\)lacZ reporter assay. However, we noticed that the CIT2 mRNA level in the rho\(^0\) mip1\(\Delta\) mutant was 3.3-fold elevated compared to the rho\(^+\) strain at the third day of growth on YPD plates (Figure 2G, 3 days on plate). No such difference was detected with the CIT2 reporter on SC ura\(^-\)leu\(^-\) media (Figure 2F). This could stem from intrinsic variations in the levels of RTG pathway activity at the beginning of starvation or may reflect the different rate of nutrient depletion during the growth of the rho\(^+\) and rho mutant strains. The difference disappeared after a prolonged incubation for 7 days under starvation conditions, when the level of CIT2 mRNA was approximately 10-fold higher compared to exponentially growing cells in both the rho\(^0\) mip1\(\Delta\) mutant and rho\(^+\) strain (Figure 2G, 7 days on plate).

In conclusion, we found that under starvation conditions the activity of RTG pathway in the rho\(^+\) strain was comparable to that of rho mutants, and that inactivation of the pathway did not restore filamentous and invasive growth of the respiratory deficient mutants. Furthermore, intact RTG signaling was required for FLO11 expression and extensive filament formation in the rho\(^+\) strain. Therefore the filamentous and invasive growth defects of rho mutants are not caused by active RTG signaling.

The FG MAPK pathway is active in rho mutants
Since inactivation of the RTG pathway does not restore *FLO11* expression in respiratory deficient mutants, we next examined the activities of two signaling pathways that are essential for *FLO11* expression: the FG MAPK pathway and the cAMP-PKA pathway.

The core FG MAPK cascade is composed of Ste11, Ste7 and the pathway-specific MAPK Kss1 (Liu *et al.* 1993; Roberts and Fink 1994). The pathway is regulated through transmembrane osmosensor Sho1 and mucin family member Msb2 (Figure 3A) (Cullen *et al.* 2000; Cullen *et al.* 2004). Signaling through the kinase cascade leads to activation of the transcription factors Tec1 and Ste12, followed by their cooperative binding to filamentation response (FRE) elements in target gene promoters (Madhani and Fink 1997). FRE elements are found in the promoters of *FLO11* and of *TEC1* itself (Madhani and Fink 1997; Lo and Dranginis 1998).

Differences in filamentous and invasive growth of rho mutants and the rho*+* tec1Δ strain (Figure 1A, F) implied that some Tec1 target(s) were expressed in rho mutants. Therefore, we hypothesized that the FG MAPK pathway is functional in rho mutants. This was verified by measuring FG MAPK signaling with the FRE-dependent reporter P*TEC1*:lacZ (Madhani and Fink 1997). Compared to the rho*+* strain the activity of the P*TEC1*:lacZ reporter in rho mutants was decreased 1.4-fold in exponentially growing cultures (Figure 3B), and increased 2.4-fold under starvation conditions (Figure 3C). In the tec1Δ strains, reporter activity always remained at background level.

We next checked if in rho mutants the FG MAPK signal reaches the *FLO11* promoter where specific regions respond to different input signals of filamentous and invasive growth (Rupp *et al.* 1999; Brückner and Mösch 2012). A promoter fragment known to be Tec1/Ste12-responsive (-1.6 to -2.0 kb upstream of initiator ATG (Rupp *et al.* 1999)) was fused to the lacZ gene and reporter activity was measured in exponentially growing cells. The FG MAPK-responsive fragment was activated in rho mutants and the rho*+* strain to comparable levels, with only slight 1.2-fold difference, confirming that the FG MAPK pathway was functional in rho mutants (Figure 4B).
Furthermore, inactivation of the FG MAPK pathway by disruption of TEC1 led to a complete loss of residual filamentation and invasion observed in rho mutants (Figure 3D, E, compare to Figure 1A). Therefore, we conclude that the FG MAPK pathway is active and is responsible for the residual filamentous and invasive growth of respiratory-deficient strains.

The cAMP-PKA pathway activity is modulated in respiratory-deficient strains

We next analyzed the functionality of the cAMP-PKA pathway in the regulation of filamentation response. Intra- and extracellular stimuli activate the adenylate cyclase Cyr1 through the GTP binding protein Ras2, Gpr1-Gpa2 receptor system or Mep2 ammonium permease (TODA et al. 1985; KÜBLER et al. 1997; LORENZ and HEITMAN 1997; LORENZ and HEITMAN 1998; KRAAKMAN et al. 1999; PAN and HEITMAN 1999). The resulting increased level of cAMP activates protein kinase A by dissociating a complex that consists of the inhibitory subunit Bcy1 and the catalytic subunits Tpk1/2/3 (Figure 4A) (TODA et al. 1987a; TODA et al. 1987b; THEVELEIN and DE WINDE 1999). Although redundant for viability (TODA et al. 1987b), the catalytic subunits differentially regulate filamentation response. While Tpk2 is required for pseudohyphal differentiation, the Tpk1 and Tpk3 inhibit it (ROBERTSON and FINK 1998; PAN and HEITMAN 1999).

First, the cAMP-PKA responsive FLO11 promoter fragment containing the Flo8 and Sfl1 binding sites (1.0 to -1.4 kb from the initiator ATG, (RUPP et al. 1999; PAN and HEITMAN 2002)) was analyzed as a fusion to the lacZ reporter gene. The reporter showed an approximately 8-fold decrease in activity in rho mutants compared to the wild type strain (Figure 4B), suggesting strong downregulation of the cAMP-PKA pathway in rho mutants. In comparison, the Tec1/Ste12 specific fragment showed only 1.2-fold decrease in exponential growth conditions in rho mutants (Figure 4B).

Subsequently, we deleted or overexpressed several cAMP-PKA pathway components in rho+ and rho strains and analyzed changes in filament formation and substrate invasion. We first inactivated
the signaling pathway by deleting TPK2, known to block the morphogenetic switch (ROBERTSON and FINK 1998; PAN and HEITMAN 1999; JIN et al. 2008). TPK2 disruption in rho mutants exerted modest effects on filamentous growth since residual filament formation was not lost as in case of TEC1 deletion (compare to Figure 3D), whereas residual invasion was completely abolished (Figure 4C). As PKA signaling at a basal level is required for viability, some Tpk2-catalyzed phosphorylation is expected in rho mutants. This activity is apparently required for residual invasion but not for filament formation.

Next, we overexpressed SFL1 or BCY1, leading to the downregulation of filamentation-specific signaling through the cAMP-PKA pathway in rho+ yeast cells. Sfl1 is a downstream repressor that reduces FLO11 expression (PAN and HEITMAN 1999) and Bcy1 is the inhibitory subunit of PKA (TODA et al. 1987a). As expected, the overexpression of SFL1 or BCY1 reduced invasion and filamentation of the rho+ strain. In rho mutants, however, residual filament formation and invasive growth was not affected (Figure 4C, D). Notably, the residual filamentous and invasive growth of rho mutants closely resembles rho+ strains with attenuated cAMP-PKA signaling, attained by overexpression of SFL1 or BCY1 (Figure 4C, D).

We next bypassed the requirement for cAMP-PKA activation by deleting the pathway downstream inhibitor SFL1 or by overexpressing the downstream activator FLO8, both resulting in hyperfilamentation in rho+ strains (LIU et al. 1996; ROBERTSON and FINK 1998). Both genes encode transcription factors that bind to the cAMP-PKA responsive region in the FLO11 promoter (RUPP et al. 1999; PAN and HEITMAN 2002). The rho mutants and rho+ cells both displayed strong filamentous and invasive growth phenotypes when SFL1 was deleted or FLO8 overexpressed (Figure 4E, F).

Last, activation of the pathway by the deletion of BCY1 or the overexpression of TPK2, known to lead to hyperfilamentation of the rho+ strain (PAN and HEITMAN 1999), restored invasive growth of rho mutants in our assays (Figure 4E, F). However, TPK2 overexpression did not enhance filament
formation of rho mutants, indicating that invasive growth and formation of filaments could be regulated differentially as observed earlier (Figure 1H, I; 2B, C) (LORENZ et al. 2000). Interestingly, the deletion of BCY1 led to inability of rho strains to grow on SLAD medium supplemented with 1% isobutanol (Figure 4E).

Analysis of the cAMP-PKA-responsive FLO11 promoter fragment and the effects of mutants that modulate the activity of cAMP-PKA signaling in filamentous and invasive growth assays suggest that signaling through the cAMP-PKA pathway is downregulated in rho mutants. It has been previously reported that *S. cerevisiae* does not tolerate loss of mtDNA combined with activation of the cAMP-PKA pathway by IRA2 or PDE2 deletion (DUNN et al. 2006). Our analysis demonstrates that activation of the pathway by BCY1 deletion is tolerated in rich growth media, but not under starvation conditions with additional stress. This further supports the notion that rho strains do not tolerate strong activation of the cAMP-PKA pathway.

Our data indicated that downregulation of cAMP-PKA signaling pathway could explain the defective filament formation of rho strains. Therefore we next analyzed physiological reporters of the cAMP-PKA pathway, like heat resistance, glycogen accumulation and trehalase activity, in rho+ strain and in rho mutants (Figure 5A).

The cAMP-PKA pathway suppresses stress tolerance; therefore, the mutants with a reduced pathway activity are more resistant to heat shock (SHIN et al. 1987; THEVELEIN and DE WINDE 1999; HLAVATÁ et al. 2003). To analyze the heat shock sensitivity of the respiratory-deficient strains, we exposed exponentially growing cultures to a severe heat shock at 52°. The heat shock tolerance of rho mutants was substantially increased compared to the respective rho+ strain (Figure 5B) that corresponds to the downregulated cAMP-PKA pathway. Activation of the pathway by BCY1 deletion led to increased heat shock sensitivity of both rho+ and respiratory-deficient strains. Only 0.02% of cells formed colonies on YPD plates after 4 minutes of incubation at 52°. We also tested temperature
sensitivity of rho mutants and the rho+ strain carrying bcylΔ. The double mutants mip1Δ bcylΔ and rpo41Δ bcylΔ both failed to grow above 34˚C (Figure 5C).

The cAMP-PKA pathway inhibits accumulation of reserve carbohydrate glycogen in actively dividing cells. Glycogen can be detected upon prolonged incubation of rho+ strains on YPD plates when the pathway becomes downregulated (François and Parrou 2001). Previously, it has been reported that the rho0 mutants of a non-invasive strain background mobilize glycogen, and by the third day of growth on YPD plates glycogen is not detectable (Enjalbert et al. 2000). Our analysis demonstrates that glycogen is readily accumulated and stored in respiratory-deficient Σ1278b mutants (Figure 5D, upper panel, days 3 and 6, respectively). In the rho+ Σ1278b strain, the accumulation of glycogen was slower and became detectable by day 6. This difference points to earlier downregulation of the cAMP-PKA pathway in rho mutants compared to the wild type strain. Activation of the pathway with BCY1 deletion completely abolished the glycogen staining of rho mutants, confirming that the glycogen content in rho mutants is dependent on cAMP-PKA pathway activity (Figure 5D, lower panel).

PKA activates the neutral trehalase NTH1 by direct phosphorylation (Uno et al. 1983; Shin et al. 1987; François and Parrou 2001). Enzymatic activity of neutral trehalase was measured in cultures grown for 24h when the rho+ strain has passed the diauxic shift and has presumably downregulated the cAMP-PKA pathway. The trehalase activity in rho mutants was decreased compared to the respective rho+ strain (Figure 5E), indicating that PKA activity is downregulated in rho mutants of the Σ1278b background. The disruption of BCY1 increases the trehalase activity in rho mutants to the levels observed in rho+ strains. As expected, the trehalase activity is also increased in rho+ bcylΔ strain.
DISCUSSION

Adaptation to nitrogen starvation or a poor carbon source requires the remodeling of cellular metabolism, and not surprisingly, the signaling pathways that regulate filamentation (e.g. cAMP-PKA and Snf1) also target mitochondria (ULERY et al. 1994; KUCHIN et al. 2003; FELICIELLO et al. 2005). Mitochondrial mass is increased in yeast filaments and genetic screens of mutants showing defects in filamentation have identified mitochondrial proteins, indicating that mitochondrial metabolism and filamentation response are linked (LORENZ et al. 2000; KERN et al. 2004; KANG and JIANG 2005; JIN et al. 2008). Our detailed analysis of Σ1278 rho mutant yeast strains revealed a defect in isobutanol-induced filament formation and agar invasion, demonstrating that mitochondrial function is critical for both processes (Figure 1).

Several explanations for the observed filamentation defect in rho strains can be considered. First, an insufficient supply of metabolic energy or the shortage of some metabolic intermediates could block the morphogenetic switch. Alternatively, dysfunctional mitochondria could trigger or interfere with specific signaling cascades that target the genes required for filamentous growth. The expression level of FLO11 in rho mutants was reduced and extensive filament formation and substrate invasion was restored by ectopic expression of FLO11 (Figure 1D-I). Therefore, we conclude that in rho cells a specific signal is either missing or inhibiting filament formation through the regulation of target genes, and there is no fundamental deficiency in metabolic building blocks required for pseudohyphal growth.

It is likely that an important role in the filamentation response is played by the interorganellar RTG cascade, known to reconfigure nuclear gene expression in yeast cells with dysfunctional mitochondria. The RTG pathway regulates the supply of glutamate used as a nitrogen source, and nitrogen starvation is one of the triggers of the filamentation response (GIMENO et al. 1992; LIU and BUTOW 1999; LIU and BUTOW 2006). Conflicting interpretations about the role of the RTG pathway in the filamentation response have been proposed. Downregulation of the RTG pathway by deletion of the
positive regulators RTG1 or RTG2 has been reported to inhibit substrate invasion of respiratory competent yeast cells (Chavel et al. 2010), indicating that RTG pathway is required for filamentous growth. Consistent with these observations, the expression of the pathway target gene DLD3 is upregulated during isoamyl alcohol-induced filamentation response (Chelstowska et al. 1999; Hauser et al. 2007). Our experiments demonstrate that the downregulation of RTG signaling does not restore the filamentous and invasive growth of respiratory-deficient strains. Moreover, we found that the RTG pathway is activated under starvation conditions not only in rho mutants but also in wild type cells, and the pathway stimulates the expression of FLO11 in WT cells (Figure 2). Therefore, our data supports the conclusion that the RTG pathway is needed for the filamentation response (Figure 6) (Chavel et al. 2010). It has been previously reported that rtg2Δ strains display genomic instability (Bhattacharyya et al. 2002). It is therefore possible that the reported mild filamentation rescue phenotype of rtg2Δ strains (Jin et al. 2008) could be due to compensatory secondary mutations frequently observed in the rtg2Δ background.

The key regulators of FLO11 transcription are the FG MAPK and the cAMP-PKA pathways. Our analysis indicates that the FG MAPK pathway is functional in rho cells and the activity of the pathway is required for residual filamentation (Figure 3, 6). In contrast, the cAMP-PKA pathway is downregulated in Σ1278 rho strains. This conclusion is supported by the finding that the rho cells demonstrate several physiological characteristics typical for downregulated cAMP-PKA signaling, such as increased tolerance of stress, reduced trehalase activity and extensive accumulation of glycogen (Figure 5). Importantly, the activation of cAMP-PKA signaling by the overexpression of TPK2 or FLO8 and by the deletion of SFL1 or BCY1 rescued the morphogenetic differentiation program in rho cells, directly linking the defect of morphogenesis and the low level of cAMP-PKA signaling (Figure 4E, F; 6). Our data clearly indicate that the filamentation defect of rho mutants of the Σ1278b background can be explained by the downregulation of cAMP-PKA pathway. This is supported by the
analysis of the physiological readouts of the cAMP-PKA pathway in *rho* mutants. However, our findings contrast a recent report that the same pathway is upregulated in W303 *rho* cells during autophagy (GRAEF and NUNNARI 2011). It is possible that the different effects of dysfunctional mitochondria on cAMP-PKA signaling result from multigenic variations between the two strains.

Our data demonstrate that mitochondrial metabolism actively modulates the output of the cAMP-PKA signaling leading to the downregulation of the pathway in Σ1278 background (Figure 6). The RTG and FG MAPK pathways are functional in *rho* cells under nitrogen starvation conditions, whereas the decreased activity of cAMP-dependent signaling leads to the downregulation of *FLO11* transcription and thereby to the inhibition of filamentous growth (Figure 6).

Several pathways can modulate the expression of *FLO11* (BRÜCKNER and MÖSCH 2012) and therefore we do not rule out the possibility that other factors contribute to the reduced filamentation phenotype of *rho* mutants. However, we would like to speculate that two important regulators, Snf1 and Mss1 are probably functional in *rho* mutants. The Snf1 protein kinase pathway is required for glycogen accumulation that readily occurs in *rho* mutants (Figure 5D) (THOMPSON-JAEGGER et al. 1991; CULLEN and SPRAGUE 2000; FRANÇOIS and PARROU 2001). Mss11 is required for the invasion response of *rho*+ cells when *FLO8* is overexpressed or *SFL1* deleted (GAGIANO et al. 1999; VAN DYK et al. 2005). Since invasive growth is restored in *sfl1Δ* cells or by the overexpression of *FLO8* in the *rho* background (Figure 4E, F), it is likely that *Mss11* is functional in *rho* strains.

Interesting question remaining unanswered is at which level the cAMP-PKA pathway is downregulated in *rho* cells. It has been reported that *ira2Δ* and *pde2Δ* mutants do not tolerate the loss of mtDNA (DUNN et al. 2006). This suggests that the hyperactivation of Ras2 or the elevated levels of cAMP are not tolerated in respiratory-deficient strains. Our analysis indicates that the deletion of *BCY1* in *rho* mutants causes the temperature sensitive growth defect at 34° (Figure 5C) and it is lethal under nitrogen starvation conditions in the presence of isobutanol (Figure 4E). Therefore, we propose that the
regulatory interaction between the cAMP-PKA pathway and mitochondrial function takes place upstream or at the level of the Bcy1 regulatory subunit.

Our results demonstrate that mitochondrial function can actively modulate cAMP-PKA signaling and this model has potential implications for higher eukaryotes. In mammalian cells, AKAPs (A-Kinase-Anchoring Proteins) localize PKA near the sites of cAMP generation or to PKA targets (Carlucci et al. 2008). Different AKAP1 RNA splice variants have been shown to localize to the outer mitochondrial membrane (OMM) (Lin et al. 1995; Chen et al. 1997). AKAPs potentially contribute to enhanced localization and translation of mRNAs targeted to mitochondria. In the context of our data it is tempting to speculate that the tethering of PKA signaling to OMM could also be important for sensing the functional state of mitochondria and for the modulation of PKA activity.
ACKNOWLEDGEMENTS

We thank Gerald R. Fink (MIT, USA), Chris A. Kaiser (MIT, USA), Steffen Rupp (HKI, Germany), and NBRP of the MEXT (Japan) for plasmids and strains; Maie Loorits for technical assistance; Arnold Kristjuhan for critical reading of the manuscript; Laura Sedman for language corrections and members of the Sedman lab for fruitful discussions. This work was supported by grants 7013, 8845 (J.S.) and 9210 (T.T.) from Estonian Science Foundation.
REFERENCES


Kraakman, L., K. Lemaire, P. Ma, A. W. Teunissen, M. C. Donaton et al., 1999 A Saccharomyces cerevisiae G-protein coupled receptor, Gpr1, is specifically required for glucose activation of the cAMP pathway during the transition to growth on glucose. Mol Microbiol 32: 1002-1012.


Lorenz, M. C., and J. Heitman, 1997 Yeast pseudohyphal growth is regulated by GPA2, a G protein alpha homolog. EMBO J 16: 7008-7018.

Lorenz, M. C., and J. Heitman, 1998 The MEP2 ammonium permease regulates pseudohyphal differentiation in Saccharomyces cerevisiae. EMBO J 17: 1236-1247.


Toda, T., S. Cameron, P. Sass, M. Zoller, J. D. Scott et al., 1987a Cloning and characterization of BCY1, a locus encoding a regulatory subunit of the cyclic AMP-dependent protein kinase in Saccharomyces cerevisiae. Mol Cell Biol 7: 1371-1377.
FIGURE LEGENDS

FIGURE 1

Dysfunctional mitochondria interfere with signaling pathways regulating FLO11 transcription. (A) Filamentous growth of rho+ (SCΣ-48), rho+ tec1Δ (SCΣ-114) and respiratory-deficient rho mutants (SCΣ-139, -146, -150, -160). Yeast cells were grown for 3 (rho+ strains) or 5 (rho mutants) days on a low-nitrogen (SLAD) medium (upper panel) or SLAD supplemented with 1% isobutanol (middle panel). Invasion into agar was visualized after washing the plates under a stream of water (lower panel). (B) Magnification of colonies marked in (A). Percentage of long pseudohyphal (PH) cells with length to width ratio >2 is indicated below panels (n=200). (C) Invasive growth assay. Strains were grown on YPD plates for 3 (rho+ strains) or 7 (rho mutants) days. Plates were washed under a stream of water and photographed before (upper panel) and after (lower panel) the wash. (D-F) P_{FLO11::lacZ} reporter activity in rho+ cells, rho mutants and respective tec1Δ strains (SCΣ-114, -248, -250, -246, -271) carrying pYEp355-FLO11::lacZ. β-galactosidase activity was measured from cells growing exponentially (D) or for 24h (E) in SC ura’leu’ medium or for 5h on SLAD medium (F). (G) Analysis of FLO11 transcript levels with quantitative PCR in rho+ (SCΣ-48) and rho0 mip1Δ (SCΣ-139). Strains were grown exponentially in YPD or patched onto YPD plates for 3 and 7 days before total RNA isolation. The results were normalized to the geometric average of two housekeeping genes (UBC6 and ARP6) and expressed relative to the exponentially growing rho+ strain. (D-G) Error bars represent standard deviations of three independent measurements. (H, I) Filamentous and invasive growth of strains ectopically expressing FLO11 (+P_{TEF::FLO11}). Strains were transformed with pB4126 and assayed for haploid filamentation and invasion as described for (A) and (C). Bars, 100 μm.

FIGURE 2

RTG pathway is activated in both rho+ cells and rho mutants during starvation. (A) Signal transduction through the RTG pathway. Ovals indicate positive, rectangles negative regulators of the pathway, gray marks mutant used in this study. (B, C) The effect of RTG2 deletion on filamentous (B) and invasive (C) growth. Filamentation and invasion of rho+ (SCΣ-48), rho mutants (SCΣ-139, -146) and respective rtg2Δ strains (SCΣ-272, -276, -289) were analyzed as described for Fig. 1 (A) and (C). Bar, 100 μm. (D) P_{FLO11::lacZ} reporter activity
in cells carrying pYEp355-\textit{FLO11::lacZ}. (E, F) \( P_{\text{CIT2}}::\text{lacZ} \) reporter activity in cells carrying pEC261. \( \beta \)-galactosidase activity was measured from cells growing exponentially in SC ura\textsuperscript{+} leu\textsuperscript{-} medium (D-E) and on selective plates for 3 days (F). (G) Analysis of \( \text{CIT2} \) transcript levels with quantitative PCR in \( \text{rho}^\ast \) (SC\Sigma-48) and \( \text{rho}^0 \text{mip1}\Delta \) (SC\Sigma-139). Strains were grown exponentially in YPD or patched onto YPD plates for 3 and 7 days. Data was normalized and expressed as described for Fig. 1 (G). (D-G) Error bars indicate standard deviations of three independent measurements.

**FIGURE 3**

FG MAPK pathway is active and responsible for residual filamentation in \( \text{rho} \) mutants. (A) Signal transduction through the FG MAPK pathway. Ovals indicate positive regulators of the pathway, gray marks mutant used in this study. (B, C) \( P_{\text{TEC1}}::\text{lacZ} \) reporter activity in \( \text{rho}^\ast \) (SC\Sigma-48), \( \text{rho} \) mutants (SC\Sigma-139, -146) and respective \( \text{tec1}\Delta \) strains (SC\Sigma-114, -248, -250) carrying pBHM275. \( \beta \)-galactosidase activity was measured from cells growing exponentially in SC ura\textsuperscript{+} leu\textsuperscript{-} medium (B) and on selective plates for 3 days (C). Error bars indicate standard deviations of three independent measurements. (D, E) Filamentous (D) and invasive (E) growth of \( \text{rho}^\ast \) cells, \( \text{rho} \) mutants and respective \( \text{tec1}\Delta \) strains were analyzed as described for Fig. 1 (A) and (C). Bar, 100 \( \mu \text{m} \).

**FIGURE 4**

Activation of the cAMP-PKA pathway restores filamentous and invasive growth of respiratory-deficient strains. (A) Signal transduction through the cAMP-PKA pathway. Ovals indicate positive, rectangles negative regulators of the pathway, gray marks mutants used in this study. (B) Activity of cAMP-PKA and FG MAPK-responsive \( P_{\text{FLO11 fragment}}::\text{lacZ} \) reporters in \( \text{rho}^\ast \) (SC\Sigma-48), \( \text{rho}^0 \text{mip1}\Delta \) (SC\Sigma-139) and \( \text{rho}^\ast \text{rpo41}\Delta \) (SC\Sigma-146). Scheme of the \( \text{FLO11} \) locus indicates positions of cAMP-PKA pathway responsive (-1.0 to -1.4 kb from \( \text{FLO11} \) start codon, named 6/7) and FG MAPK responsive (-1.6 to -2.0 kb from \( \text{FLO11} \) start codon, named 9/10) promoter fragments. Numbers mark nucleotides in kb. \( \beta \)-galactosidase activity was measured from cells growing exponentially in SC ura\textsuperscript{+} leu\textsuperscript{-} medium and carrying either pLG669-Z \( \text{FLO11} \) 6/7, pLG669-Z \( \text{FLO11} \) 9/10 or pLG669-Z (no insert). Error bars indicate standard deviations of three independent measurements. (C, D) The effect of cAMP-PKA pathway downregulation on filamentous and invasive growth. Colony morphology and invasive growth were analysed in \( \text{rho}^\ast \) cells and \( \text{rho} \) mutants overexpressing \( \text{SFL1} \) (pRS426-\textit{SFL1}) or \( \text{BCY1} \)
(pRS426-BCY1), or containing the tpk2Δ mutation (SCΣ-312, -326, -328). (E, F) The effect of cAMP-PKA pathway activation on filamentous and invasive growth. Colony morphology and invasive growth were analysed in rho⁺ cells and rho mutants overexpressing FLO8 (pRS426-FLO8) or TPK2 (pRS426-TPK2), or containing sfl1Δ (SCΣ-320, -335, -332) or bcy1Δ (SCΣ-316, -346, -344) mutations. Filamentation (C, E) and invasion (D, F) were analyzed as described for Fig. 1 (A) and (C). Bars, 100 μm.

**FIGURE 5**

**cAMP-PKA pathway activity is downregulated in rho mutants.** (A) Cellular processes controlled by PKA-mediated phosphorylation. (B) Heat shock sensitivity of rho⁺ cells (SCΣ-48) and rho mutants (SCΣ-139, -146). Yeast cells were grown exponentially in YPD and exposed to heat shock at 52°C for indicated times. Viability was expressed as the percentage of cells forming colonies after heat shock relative to untreated cells. (C) Serial dilution spot test of rho⁺ cells, rho mutants, and respective bcy1Δ strains (SCΣ-316, -346, -344) grown on YPD medium at the indicated temperatures. (D) Glycogen staining of rho⁺ cells, rho mutants, and respective bcy1Δ strains. Yeast cells were patched onto YPD and incubated for 3 and 6 days at 30°C or 25°C. Plates were exposed to iodine vapour. The dark colour indicates the presence of glycogen stores. (D) Trehalase activity of rho⁺ cells and rho mutants and respective bcy1Δ strains. Yeast cells were grown in YPD at 25°C. Error bars indicate standard deviations of three independent measurements.

**FIGURE 6**

**Model showing the role of mitochondrial function in the regulation of cAMP-PKA signaling.** Mitochondrial dysfunction triggers downregulation of the cAMP-PKA pathway in S1278b cells. This leads to a defective filamentation response despite the wild type level activity of the RTG and FG MAPK pathways. Downregulation of the cAMP-PKA pathway results in modulation of several cellular processes in addition to filamentation in respiratory-deficient mutants. Activation is shown by arrows and inhibition by T-bars.
### TABLE 1

Yeast strains used in this study

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\[ \text{SC-316 } \rho^+ \text{ bcy1Δ} \quad \text{MATa [ρ^+] } \text{ura3-52 leu2Δ0 bcy1Δ::hphMX6} \quad \text{This study} \]

\[ \text{SC-346 } \rho^0 \text{ mip1Δ bcy1Δ} \quad \text{MATa [ρ^0] } \text{ura3-52 leu2Δ0 mip1Δ::natMX6 bcy1Δ::hphMX6} \quad \text{This study} \]

\[ \text{SC-344 } \rho^- \text{ rpo41Δ bcy1Δ} \quad \text{MATa [ρ^-] } \text{ura3-52 leu2Δ0 rpo41Δ::kanMX6} \quad \text{This study} \]

\[ \text{bcy1Δ::hphMX6} \]

\[ \text{SC-348 } \rho^0 \text{ bcy1Δ} \quad \text{MATa [ρ^0] } \text{ura3-52 leu2Δ0 bcy1Δ::hphMX6} \quad \text{This study} \]

\[ \text{SC-350 } \rho^- \text{ bcy1Δ} \quad \text{MATa [ρ^-] } \text{ura3-52 leu2Δ0 bcy1Δ::hphMX6} \quad \text{This study} \]

\[ \text{SC-320 } \rho^+ \text{ sfl1Δ} \quad \text{MATa [ρ^+] } \text{ura3-52 leu2Δ0 sfl1Δ::hphMX6} \quad \text{This study} \]

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\[ \text{SC-332 } \rho^- \text{ rpo41Δ sfl1Δ} \quad \text{MATa [ρ^-] } \text{ura3-52 leu2Δ0 rpo41Δ::kanMX6 sfl1Δ::hphMX6} \quad \text{This study} \]

\[ \text{SC-334 } \rho^0 \text{ sfl1Δ} \quad \text{MATa [ρ^0] } \text{ura3-52 leu2Δ0 sfl1Δ::hphMX6} \quad \text{This study} \]

\[ \text{SC-337 } \rho^- \text{ sfl1Δ} \quad \text{MATa [ρ^-] } \text{ura3-52 leu2Δ0 sfl1Δ::hphMX6} \quad \text{This study} \]

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FIGURE 2
Aun et al.

A

- Restoration
- Rtg2
- Lst8
- Mks1
- Rtg3
- Rtg1

Nitrogen metabolism
Glutamate/glutamine synthesis

B

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<td>rtp2\textsuperscript{Δ}</td>
<td>rtp2\textsuperscript{Δ}</td>
<td>rtp2\textsuperscript{Δ}</td>
</tr>
<tr>
<td>Before wash</td>
<td>Before wash</td>
<td>Before wash</td>
<td>Before wash</td>
<td>Before wash</td>
</tr>
<tr>
<td>After wash</td>
<td>After wash</td>
<td>After wash</td>
<td>After wash</td>
<td>After wash</td>
</tr>
</tbody>
</table>

C

- Before wash
- RTG2
- rtp2\textsuperscript{Δ}
- rtp2\textsuperscript{Δ} rtp41\textsuperscript{Δ} mip1\textsuperscript{Δ}

D

\[ P_{\text{rho}:\text{lacZ}} \]
Exponential growth

- β-gal (Miller Units)
- ρ\textsuperscript{o}, ρ\textsuperscript{o} mip1\textsuperscript{Δ}, ρ\textsuperscript{o} rtp41\textsuperscript{Δ}

- RTG2
- rtp2\textsuperscript{Δ}

E

\[ P_{\text{rho}:\text{lacZ}} \]
Exponential growth

- β-gal (Miller Units)
- ρ\textsuperscript{o}, ρ\textsuperscript{o} mip1\textsuperscript{Δ}, ρ\textsuperscript{o} rtp41\textsuperscript{Δ}

- RTG2
- rtp2\textsuperscript{Δ}

F

\[ P_{\text{cro}:\text{lacZ}} \]
Starvation conditions

- β-gal (Miller Units)
- ρ\textsuperscript{o}, ρ\textsuperscript{o} mip1\textsuperscript{Δ}, ρ\textsuperscript{o} rtp41\textsuperscript{Δ}

- RTG2
- rtp2\textsuperscript{Δ}

G

Relative CIT2 mRNA

- CIT2 (Arbitrary Units)
- Exponential growth, 3 days on plate, 7 days on plate

- ρ\textsuperscript{o}, ρ\textsuperscript{o} mip1\textsuperscript{Δ}
FIGURE 4
Aun et al.

A

Gpr1

Ras2 → Cyr1 ← Gpa2

Ira1/2

cAMP → Pde1/2 → Bcy1

Tpkl Tpkl Tpkl

Flol Stl1

Filamentous growth

B

P_FLO11::lacZ

Exponential growth

β-gal (Miller Units)

-1.6 to -2.0

-1.0 to -1.4

No insert

FG MAPK pathway

PKA pathway

FLO11

C

Before wash

After wash

ρo^− ρo^− mip1Δ ρo^− rpo41Δ ρo^−

+SFL1

tpk2Δ

+BCY1

D

Before wash

After wash

ρo^− ρo^− ρo^− mip1Δ rpo41Δ ρo^− rpo41Δ

+SFL1

tpk2Δ

+BCY1

E

Before wash

After wash

ρo^− ρo^− ρo^− mip1Δ rpo41Δ ρo^− rpo41Δ

+FLO8

sfl1Δ

+TPK2

bcy1Δ

F

Before wash

After wash

ρo^− ρo^− ρo^− mip1Δ rpo41Δ ρo^− rpo41Δ

+FLO8

sfl1Δ

+TPK2

bcy1Δ
FIGURE 5
Aun et al.

A

\[ \text{cAMP-PKA pathway} \]

\[ \downarrow \]

Stress (heat) resistance
Trehalase activation
Glycogen and trehalose content
Filamentous growth

B

\[ \text{Time at 52° (min)} \]

\[ \text{Viability} \]

\[ \text{\( \rho^\circ \) rpo41\Delta} \]
\[ \text{\( \rho^\circ \) mip1\Delta} \]
\[ \text{\( \rho^\circ \) mip1\Delta rpo41\Delta} \]

C

30°

25°

3 days

3 days

6 days

BCY1

bcy1\Delta

D

Exponential growth

Growth for 24h

\[ \text{nmol glucose released} \]

\[ (\text{min} \times \text{mg of protein}) \]

\[ \text{\( \rho^\circ \)} \]
\[ \text{\( \rho^\circ \) mip1\Delta} \]
\[ \text{\( \rho^\circ \) rpo41\Delta} \]
\[ \text{\( \rho^\circ \) mip1\Delta rpo41\Delta} \]
FIGURE 6
Aun et al.

Glycogen accumulation
Stress resistance
Trehalase activation

Respiration → cAMP-PKA pathway → Filamentation → RTG pathway → FG MAPK pathway