Transcription profiling of cyclic AMP signalling in *Candida albicans*

Doreen Harcus, André Nantel§, Anne Marcil, Tracey Rigby, and Malcolm Whiteway‡

Genetics Group, Biotechnology Research Institute, National Research Council of Canada, 6100 Royalmount Ave., Montreal, Quebec, Canada H4P 2R2
§ Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec, Canada H3A 2B2
‡ Department of Biology, McGill University, Montreal, Quebec, Canada H3A 1B1

Running title: cAMP signalling in *C. albicans*

[$]Corresponding author: Dr. Malcolm Whiteway
Genetics Group,
Biotechnology Research Institute,
National Research Council of Canada,
6100 Royalmount Ave.,
Montreal, Quebec,
Canada H4P 2R2
Tel: 514-496-6146
Fax: 514-496-6213
E-mail: malcolm.whiteway@cnrc-nrc.gc.ca

Keywords: adenylyl cyclase/CDC35/EFG1/microarray/RAS1

Abstract

We used transcription profiling in *Candida albicans* to investigate cellular regulation involving cAMP. We found that many genes require the adenylyl cyclase Cdc35p for proper expression. These include genes encoding ribosomal subunit proteins and RNA polymerase subunit proteins, suggesting that growth could be controlled in part by cAMP mediated modulation of gene expression. Other genes influenced by loss of adenylyl cyclase are involved in metabolism, the cell wall, and stress response, and include a group of genes of unknown function that are unique to *C. albicans*. The profiles generated by loss of the adenylyl cyclase regulator Ras1p and a downstream effector Efg1p were also examined. The loss of Ras1p function disturbs the expression of a subset of the genes regulated by adenylyl cyclase, suggesting both that the primary role of Ras1p in transcriptional regulation involves its influence on the function of Cdc35p and that there are Ras1p independent roles for Cdc35p. The transcription factor Efg1p is also needed for the expression of many genes; however, these genes are distinct from those modulated by Cdc35p with the exception of a class of hyphal specific genes. Therefore transcription profiling establishes that cAMP plays a key role in the overall regulation of gene expression in *C. albicans*, and enhances our detailed understanding of the circuitry controlling this regulation.

Introduction

cAMP is an important regulatory molecule in both prokaryotes and eukaryotes. In fungi this molecule has been implicated in a variety of cellular processes. For example, in *Magnaporthe grisea* cells with mutations in the gene encoding adenylyl cyclase have a reduced vegetative growth rate, are sterile, and are defective in forming appresoria and thus are unable to infect susceptible rice leaves (Choi and Dean, 1997). In *Ustilago maydis,* mutants of adenylyl cyclase cause constitutively filamentous growth, (Gold *et al.*, 1994) whereas strains with defects in the gene encoding the regulatory subunit of protein kinase A fail to induce tumors in plants (Gold *et al.*, 1997). In *Cryptococcus neoformans*, the cAMP signalling pathway regulates several important cellular processes including capsule production, melanin formation, mating, and virulence (Alspaugh *et al.*, 1997). In *Neurospora crassa,* cAMP regulates morphology, conidiation, mating, and stress responses (Lengeler *et al.*, 2000), while in *Schizosaccharomyces pombe* cAMP signalling plays a role in mating, sporulation, gluconeogenesis and entry into stationary phase (D'Souza and Heitman, 2001).

Perhaps the best studied fungal cAMP signalling network is that of the model eukaryote *Saccharomyces cerevisiae* (D'Souza and Heitman, 2001), where cAMP is essential for growth and regulates nutrient sensing, stress responses and pseudohyphal differentiation. Adenylyl cyclase, encoded by the *CDC35/CYR1* gene, appears primarily regulated by GTPases. These regulators include a pair of Ras homologs, encoded by *RAS1* and *RAS2* (Toda *et al.*, 1985), and a Gα subunit encoded by *GPA2* (Kubler *et al.*, 1997; Colombo *et*

al., 1998). Loss of adenylyl cyclase activity, or loss of Ras function is lethal to the cell, with cells arresting at the G1 phase of the cell cycle in a manner analogous to cells that have been nutrient starved (Toda *et al.*, 1985). A primary target of the yeast adenylyl cyclase is the cAMP-dependent protein kinase (PKA), made up of a regulatory subunit encoded by *BCY1*, (Matsumoto *et al.*, 1982), and a catalytic subunit encoded by three genes *TPK1*, *TPK2*, and *TPK3* (Toda *et al.*, 1987). Downstream of the kinase are a number of transcription regulators. These include the modulators of the stress response pathway Msn2p/Msn4p (Smith *et al.*, 1998) and Sko1p (Pascual-Ahuir *et al.*, 2001), as well as Gis1p involved in post-diauxic shift regulation (Pedruzzi *et al.*, 2000), and Sfl1p (Conlan and Tzamarias, 2001) and Sok2p (Ward *et al.*, 1995) involved in pseudohyphal development.

A regulatory system involving cAMP that appears related to the *S. cerevisiae* network has been identified in the fungal pathogen *Candida albicans*. Elements involved in the regulation of *C. albicans* cellular functions by cAMP include Cdc35p, the adenylyl cyclase that contributes to vegetative growth and is essential for yeast to hypha morphogenesis and virulence of C. *albicans* (Rocha *et al.*, 2001) and Ras1p, a GTPase required for the formation of hyphae but not pseudohyphae (Feng *et al.*, 1999; Leberer *et al.*, 2001). In addition, there are Tpk1p and Tpk2p, the catalytic subunits of the cAMP dependent protein kinase, which have redundant functions in growth and stress responses but exhibit functional differences in morphogenesis depending on environmental conditions (Bockmühl *et al.*, 2001; Cloutier *et al.*, 2003). Other components include Pde2p the high affinity phosphodiesterase (Bahn *et al.*, 2003), and Efg1p, a transcription

factor whose function is apparently controlled, at least in part, by cAMP-dependent protein kinase-directed phosphorylation (Bockmühl and Ernst, 2001).

Although many elements of the cAMP signalling network in *C. albicans* have been identified and disrupted, the overall structure and regulatory connections of the system are not well understood. Genetic studies are complicated by the diploid nature of the cells, and epistasis experiments involving protein overexpression have led to conflicting observations (Chen *et al.*, 2000; Bockmühl *et al.*, 2001; Leberer *et al.*, 2001). Although Ras1p is implicated in both the MAP kinase and adenylyl cyclase signalling pathways controlling hyphal development (Leberer *et al.*, 2001), loss of Ras function does not have as profound an effect on hyphal formation as does loss of adenylyl cyclase (Feng *et al.*, 1999; Rocha *et al.*, 2001). In *S. cerevisiae* there are many transcription factors acting downstream of Cdc35p, while in *C. albicans* only Efg1p has so far been convincingly identified as a downstream target of PKA activity (Liu, 2001). The observation that the highly similar adenylyl cyclase proteins of *S. cerevisiae* and *C. albicans* provide an essential function in one organism and not in the other suggests that it is the functions of the downstream targets that determine whether cAMP formation is required for viability (Rocha *et al.*, 2001).

To further the understanding of the role of cAMP in *C. albicans*, we have used transcription profiling to investigate the consequences of the absence of adenylyl cyclase and other key network components. We have studied deletion mutants of *CDC35, RAS1* and *EFG1* under conditions that direct either yeast or hyphal form growth. This study

provides the first comprehensive look at the transcriptional consequences of the deletion of adenylyl cyclase function in a eukaryotic cell, and develops the relationship among the components of this regulatory network and its downstream targets.

Materials and methods

Strains and growth conditions. The *C. albicans* strains used in this study were the wild-type clinical isolate SC5314 (Gillum *et al.*, 1984), the Caras1Δ CDH107 (*ura3/ura3 caras1::hisG-URA3-hisG/caras1::hisG;* (Leberer *et al.*, 2001)), the Cacdc35Δ CR216 (*ura3/ura3 cacdc35::hisG-URA3-hisG/cacdc35::hisG;* (Rocha *et al.*, 2001)**)** and the efg1Δ HLC52 *(ura3/ura3 efg1::hisG-URA3-hisG/ efg1::hisG*; (Lo *et al.*, 1997)). Overnight cultures were inoculated from a fresh colony and grown in 1% yeast extract, 2% peptone, 2% dextrose (YPD) medium (pH 6.2 - 6.5) at 30°C. Overnight cultures were diluted to an OD600 of 0.1 in YPD or YPD + 10% fetal bovine serum (FBS) and grown at 30°C or 37°C respectively, to an OD_{600} of approximately 0.8 (3 generations); this was approximately 4 to 5 hours for the wildtype, *efg1* and *ras1* strains and 8 to 9 hours for the *cdc35* strain. Cultures were harvested by centrifugation at 3000 x g for 4 min and the pellet rapidly frozen in a dry-ice/ethanol bath. FBS was from Invitrogen (Carlsbad, CA), and was incubated at 56°C for 30 min before use.

Microarrays - RNA extraction and labelling. The microarrays used in this study contained 6002 putative ORFs based on the preliminary sequence produced by the

Stanford Genome Technology Center. These are described in more detail (Nantel *et al.*, 2002) and at http://www.bri.nrc.gc.ca/business/microarraylab/index_e.html. A comprehensive annotation of the *Candida albicans* genome has recently been made available at http://candida.bri.nrc.ca. Isolation of mRNA, labelling and hybridization of the microarrays were performed as described (Nantel *et al.*, 2002). Transcription profiles for each condition represent the average of at least 4 to 9 independent hybridizations. These include dye-swap hybridizations (Cy3/Cy5 and Cy5/Cy3) from at least three independently produced RNA preparations.

Data Analysis. Quantitation and normalization of DNA microarrays was performed as described (Enjalbert *et al.*, 2003). To facilitate data interpretation and the number of transcripts that are presented in this manuscript, we used the "one class" algorithm from the "Significance Analysis of Microarrays" (SAM) package to isolate genes whose transcripts were significantly modulated by at least 1.4-fold and had a false discovery rate < 5%. This dataset was combined with transcription profiles from the yeast-to-hyphae transition induced by serum at 37°C (Nantel *et al.*, 2002; Lee *et al.*, 2004); although we used the average fold variation from both papers, we only used the list of hyphal-modulated genes that were identified in the earlier publication since the results obtained in Lee et al. (2004) were produced on a later version of the Candida array that contained additional genes. Most of the analysis was performed on a collection of 1168 genes whose transcript levels were significantly modulated under at least one of the conditions. Visualization and analysis of the transcript profiles and correlations with signalling pathways in *S. cerevisiae* was performed in GeneSpring (Silicon Genetics) as described

(Enjalbert *et al.*, 2003). Principal Components Analysis was performed in Matlab (The Mathworks, Natick, MA). All of the data can be obtained from http://cbr-rbc.nrc-cnrc.gc.ca/genetics/cAMP/.

Phenotypic Analysis. Strains were tested for Calcofluor White (CFW) sensitivity by diluting overnight cultures to an OD_{600} of 1.0 and plating 3 µl aliquots of 1:10 dilutions on YPD plates containing from 0 to 50µg/ml of CFW. The plates were incubated at 30°C and monitored for 4 days. Sensitivity of the strains to Zymolyase was tested by diluting exponentially growing cells to an OD_{600} of 0.5 in 10mM Tris-HCl (pH 7.5) containing 0 or 100 µg/ml of Zymolyase 100T. The cells were kept at 30°C with shaking and the OD_{600} was monitored over a period of 2.5 hr. For heat shock, early log phase cells grown at 30°C were put in a 50°C waterbath for 0, 10, 20, 30 or 60 minutes, 3 µl of a 1:10 dilution of the cells was plated on YPD, grown at 30°C and monitored for 3 days. For osmotic sensitivity, cells were grown to early log phase and 1:10 dilutions were spotted onto YPD plates containing 1.5M NaCl. The plates were incubated at 30°C and monitored for 3 days.

Results

In *Candida albicans,* previous studies have shown that loss of components in the cAMP regulatory circuit can have significant effects on growth and morphogenesis (Suppl. Figure 1), as well as on virulence (Whiteway, 2000). When cells that lack *CDC35* are

grown in conditions generating the yeast form, they tend to aggregate and exhibit a significantly reduced rate of proliferation relative to wild-type cells. In particular, the deletion of adenylyl cyclase blocked the serum induced yeast-to-hyphal transition (Rocha *et al.*, 2001), a process characterized by significant changes in gene expression (Nantel *et al.*, 2002). Therefore we asked both whether cAMP played a role in general control of gene expression, and more specifically in the transcriptional regulation of *C. albicans* morphological transitions. Deletion mutants of *RAS1* and *EFG1*, two other components of the cAMP regulon, show phenotypes distinct from that of the adenylyl cyclase null mutants. The *Ras1* mutant cells show a moderate reduction in the rate of cell proliferation, but do not appear to have any defects in cellular morphology while growing in the yeast form, while under hyphal-inducing conditions the cells fail to make true hyphae but can still grow as pseudohyphae (Feng *et al.*, 1999; Leberer *et al.*, 2001). Deletion mutants of *EFG1*, an effector of the pathway, grow at a normal rate but exhibit somewhat elongated rod-like cells in either yeast or hyphal growth conditions (Lo *et al.*, 1997; Stoldt *et al.*, 1997). Therefore we asked whether these differences were reflected in distinct transcription patterns in these mutant cells.

Transcription profiling and cluster analysis.

We initially measured the transcriptional effects of cells growing with and without adenylyl cyclase. Gene expression profiles of a disruption of *CDC35* were compared to profiles of wild-type cells during yeast form growth conditions (YPD at 30°C) and also under hyphal inducing conditions (YPD + serum at 37°C). In addition, the transcription profiles of cells undergoing the yeast to hyphal switch were measured by comparing

mRNA from the mutant or wild-type cells grown in YPD at 30°C to mRNA from the same cells grown in YPD at 37°C + serum. These profiles were compared with those generated from two other strains containing mutations in genes encoding other components of the cAMP pathway, a regulator Ras1p, and an effector Efg1p, under the conditions described above. The complete dataset can be obtained at http://cbr-rbc.nrc-cnrc.gc.ca/genetics/cAMP/. To facilitate presentation of our results, we selected 1168 genes that showed a statistically significant variation in transcript abundance (see methods for details) under one of the conditions measured in this study or during the yeast to hyphae transition induced by serum and high temperature in the wild type (Nantel *et al.*, 2002). A hierarchical cluster analysis of the transcript profiles for all experimental conditions in all strains is shown in Figure1. In such an analysis, genes (X axis) and comparisons (Y axis) that show a similar profile are located closer to each other. Dendrograms serve to represent the extent of profile similarities.

Among the mutant cells undergoing the yeast to hyphal transition, the *cdc35* and *ras1* strains clustered closely together and were separate from the profile of the *efg1* mutant. In addition, the *cdc35* and *ras1* profiles were linked together for both yeast and hyphal growth, while the *efg1* mutant clustered with itself under both growth conditions. Thus under all conditions examined, the *ras1* and cyclase profiles are in the same dendrogram sub-branch, while the profile of the *efg1* mutant is distinctly different. Similar results were observed following the separation of each experimental profile with a Principal Components Analysis (PCA), a different classification method that positions each of the comparisons on a three dimensional graph (Suppl. Figure 2). Morphologically, the three

mutants were distinct when growing in YPD at 30°C, and the *efg1* and *cdc35* mutants both remained non-hyphal when growing at 37°C in the presence of serum. Thus the transcription profiling provides a different picture of the relationships among the elements than did the cellular morphology.

Effects of cAMP on gene expression.

When cells of the *cdc35* mutant were grown in the yeast form condition and compared to wild-type cells differences in almost 600 transcripts were revealed. These differences included genes whose mRNA levels were either reduced or elevated in the absence of adenylyl cyclase function (Figure 2A). Among the genes that require *CDC35* for their normal transcript levels in yeast cells are those encoding a majority of ribosomal proteins for both the large and small subunits, as well as genes for subunits of the RNA polymerase holoenzymes (Suppl. Figure 3A). Another large group of genes whose message abundance is reduced by the loss of *CDC35* are associated with metabolic pathways such as the TCA cycle, pyrimidine metabolism and heme and sterol synthesis (Suppl. Figure 3A). This reduced expression of genes for the transcriptional and translational machinery, as well as for central metabolic pathways, correlates well with the reduced growth rate exhibited by these cells.

Many transcripts show a higher abundance in the *cdc35* mutant. A major group of these genes appear to be both unique to *C. albicans* and have as yet no known molecular function (Table I). We have designated three of the most highly influenced genes *ASR1*, *ASR2* and *ASR3* for adenylyl cyclase and stress responsive. In addition, a notable group

of the transcripts elevated in the absence of cAMP encode proteins involved in the formation and function of the cell wall (Table II). A variety of phenotypes suggest the wall of the mutant cells is different from that of wild-type cells; the *cdc35* mutant tends to aggregate, provides low yields of RNA and is difficult to transform. As shown in Figure 3A & B, *cdc35* cells are significantly more resistant than wild-type cells to Calcofluor White, which binds to chitin, as well as to Zymolyase, which is primarily a ß1-3 glucanase. Other, smaller groups of genes that are influenced positively and negatively by the loss of cAMP are involved in the cytoskeleton, cell cycle, iron uptake and multi-drug resistance (Suppl. Table I), and include as well a number of transcription factors (Suppl. Table II).

We observed a number of stress response genes influenced by the loss of cAMP. When we compared the list of adenylyl-cyclase-responsive genes during yeast form growth with the stress response data from our group (Enjalbert *et al.*, 2003), we observed a strong correlation between the *cdc35* profile and the profile observed in osmotically-shocked cells (Suppl. Figure 4A & Suppl. Table III). In addition, *cdc35* cells exhibited an increased sensitivity to osmotic stress (Figure 3C). It should be noted that the treatments used in Enjalbert et al. (2003) were not as stringent as the ones used here as the objective at the time was to produce a mild stress that would not result in excessive cellular mortality. Although we found little correlation between our array data and the stress response data for heat shock we observed that the *cdc35* mutant is slightly more resistant to increased temperature (Figure 3D), perhaps due to changes in the cell wall.

We also compared the cyclase mutant cells to wild-type cells under hyphal growth conditions. As was found in cells growing in the yeast form, the absence of adenylyl cyclase had profound consequences on transcription profiles under these different growth conditions. Over 800 transcripts were affected by the loss of *CDC35* when compared to wild type at 37°C +FBS (Figure 2B), and there was a significant correlation between the profiles under both yeast and hyphal conditions in the *cdc35* mutant (Suppl. Figure 5A). Among the transcripts modulated in the *cdc35* mutant, general metabolic processes remain influenced as they were in the yeast form, although the magnitude of change in transcript levels was often lower in cells growing under hyphal inducing conditions. The genes involved in wall function that were activated in the yeast cells were also up-regulated in hyphal cells. Intriguingly however, there was a group of wall related genes not identified in yeast growth conditions that were repressed when grown under hyphal conditions (Table II). Hallmark genes of the cAMP pathway were also modulated in the *cdc35* mutant. In the absence of cAMP the transcript level of *BCY1* (the negative regulator of PKA) increased as it did in the yeast form, but surprisingly the levels of mRNA for a catalytic subunit, *TPK2*, and the transcription factor *EFG1* also increased.

Previous studies have shown that the ability to undergo the yeast-hyphal transition is blocked in cells that lack adenylyl cyclase (Rocha *et al.*, 2001). Our expression profiles show that during the yeast-to-hyphal transition almost all of the genes that were modulated in wild-type cells, including classic hyphal induced genes such as *ECE1*,

HWP1 and *SAP4,* are no longer responsive to the serum and heat signals in the adenylyl cyclase mutant (Table III). This suggests that most of the response to a shift from 30°C to 37°C + FBS in *C. albicans* is mediated by the cAMP pathway. However, a few transcripts, including *CHA2*, *GAP4, HMO1, RHD1, RHD3, SNZ1*, and *orf19.7531*, still respond as they did in the wild type (Table III), suggesting that a cAMP-independent pathway may contribute to morphogenesis under these conditions.

Effects of other cAMP network components on gene expression.

Ras function has been implicated as a regulator of adenylyl cyclase in fungal systems (Toda *et al.*, 1985), yet the loss of Ras1p function did not have as dramatic an effect on transcription as did loss of adenylyl cyclase. Only 72 transcripts were significantly more abundant in YPD-grown, *RAS1*- deleted cells compared to wild type, while four transcripts were less abundant (Figure 2A). However, the majority of the Ras1p-influenced transcripts are a subset of those that are modulated by cAMP. Many of the transcripts of the *Candida*-specific genes of unknown function that were more abundant in the absence of *CDC35* were also more abundant in the *ras1* mutant (Table I). A number of the cell wall genes whose transcript levels changed in the *cdc35* mutant showed the same behaviour in the *ras1* mutant, and in parallel, *ras1* cells were also more resistant than wild type to treatment with Calcofluor or Zymolyase (Fig. 3A & B). As with *cdc35* cells, *ras1* mutant cells were found to be slightly more resistant to heat shock (Figure 3D); this may also be a feature of changes in the cell wall as there was no strong correlation between data from the *ras1* mutant and data from heat shock response. In

contrast to *cdc35*, the *ras1* mutant is not sensitive to osmotic stress (Figure 3), and the profiles do not correlate with the osmotic stress response (Suppl. Figure 4B).

Loss of Ras1p function during hyphal inducing conditions modulated the expression of less than 100 transcripts, most of which were a subset of the transcripts modulated by the loss of *CDC35* (Figure 2B). In contrast to the situation during yeast growth conditions however, numerous transcripts with decreased levels were observed in *ras1* cells and many of these represent hyphal-induced genes.

The *ras1* mutant cells showed some differences from the *cdc35* mutant cells during analysis of the yeast-to-hyphal switch (Figure 2C). Some of the hyphal-specific genes, such as *ECE1*, *RBT1* and *HWP1,* whose transcript levels were clearly reduced when *ras1* was compared to the wild type in hyphal conditions, were still partially responsive in *ras1* but totally unresponsive in *cdc35* mutant cells (Table III). These genes could have a function in pseudohyphae development, an hypothesis that is supported by the fact that these transcripts were more abundant in the pseudohyphae resulting from the 37°C + FBS treatment of cells lacking the Sit4p phosphatase (Lee *et al.*, 2004).

In the current study we have extended the preliminary analysis (Nantel *et al.*, 2002) of the effects of an *EFG1* deletion on transcription profiles. Approximately 85 genes showed significant changes in transcript levels in the *efg1* mutant cells compared to wild-type cells growing under yeast growth conditions (Figure 2A). The majority of these Efg1p-modulated transcripts are distinct from those affected by the loss of *RAS1* or *CDC35*. A

notable group of decreased transcripts in the *efg1* mutant encoded enzymes involved in the glycolysis and gluconeogenesis pathways (Suppl. Figure 3B). As first described (Sohn *et al.*, 2003), the loss of Efg1p was found to modulate transcript levels of genes involved in cell wall maintenance. In our study only a few of the cell wall genes affected by the loss of Cdc35p are also affected by the loss of Efg1p (Table II). This transcript profile mirrored physiology in that *efg1* mutants were moderately sensitive to Calcofluor White and Zymolyase, rather than resistant as were the *ras1* and *cdc35* mutants (Figure 3A & B). We observed a small but significant correlation between the Efg1p-responsive transcripts and genes that were modulated during the white to opaque transition (Lan *et al.*, 2002) in *C. albicans* (Suppl. Figure 5B). This observation can be explained by the fact that levels of Efg1p are greatly reduced in opaque cells (Lan *et al.*, 2002). For the most part, many of the genes whose transcript levels were influenced by Efg1p did not fall into major classes of functionally similar elements, due at least in part to the fact that a large number of the Efg1p-responsive genes have currently unknown functions.

As in the case of cells grown in yeast form conditions, we found that within the group of genes influenced by the loss of *EFG1* in hyphal inducing conditions, the majority were distinct from those modulated by either *ras1* or *cdc35* mutations (Figure 2B). The small number of modulated transcripts that were commonly influenced in the *efg1*, *cdc35* and *ras1* mutants identified most of the highly modulated, hyphal-specific genes that were initially used to define this signalling pathway (Table III).

EFG1 showed its greatest influence when the mutant was undergoing the yeast-to-hyphal switch where close to 200 genes were affected (Figure 2C). We have previously demonstrated that cells lacking this transcription factor fail to transmit signals that are induced by the presence of serum (Nantel *et al.*, 2002). This may suggest Efg1p modulates a separate pathway that leads to changes in transcript profiles in response to an increase in temperature, and we also observed that the *efg1* mutant was sensitive to heat shock (Figure 3D). Approximately 75% of the *EFG1*-modulated transcript levels increase in abundance compared to wild type (Figure 2C), implying perhaps that Efg1p is primarily a transcriptional repressor during the yeast to hyphal transition (Giusani *et al.*, 2002).

Discussion

Both prokaryotic and eukaryotic cells use cAMP as an important regulatory molecule. In prokaryotes the best-studied systems show cAMP functioning as an allosteric regulator of a transcriptional control molecule (Botsford and Harman, 1992), while in eukaryotes cAMP provides intracellular regulation through its control of cAMP dependent protein kinase (Johnson *et al.*, 2001), and it can serve as an extracellular signal in processes such as slime mold chemotaxis (Saran *et al.*, 2002). *Candida albicans* cells become avirulent when they are defective in adenylyl cyclase (Rocha *et al.*, 2001), and so we have used global transcription profiling to examine the intracellular role of cAMP in this important human fungal pathogen.

Our findings establish that modification of cAMP metabolism had dramatic effects on the transcription patterns of *C. albicans* cells. Changes in the expression patterns of genes involved in transcription, translation and central metabolic pathways found in cells that lack cAMP can be correlated with modified growth rates. In addition, changes in the expression of genes involved in cell wall metabolism in cyclase defective cells are reflected by changes in cellular sensitivity to enzymes and compounds that disrupt the wall. As well, the *cdc35* mutant cells showed enhanced expression of osmotic stress response genes, though the cells were more sensitive to osmotic shock, perhaps because they could not handle this added challenge in their already activated state. Most dramatically, *C. albicans* cells that lack adenylyl cyclase function are profoundly defective in the ability to form hyphae, and our study revealed the transcriptional activation of virtually all the genes up-regulated during the yeast-to-hyphal switch is blocked in the *cdc35* mutant. Thus the changes in gene expression resulting from the inability to produce cAMP are correlated with significant changes in cellular physiology in this human pathogen. Because cyclase mutants are essentially avirulent in mouse models of systemic infection, it appears that the transcriptional effects resulting from loss of cAMP production impacts significantly on the ability of *C. albicans* to act as a pathogen.

Major changes in cell behaviour due to loss of cAMP regulation are not surprising. Morphogenetic regulation and virulence in *C. albicans* have also been intimately linked to metabolic control (Tripathi *et al.*, 2002), while in *S. cerevisiae*, cAMP plays a key role in cell cycle regulation (Matsumoto *et al.*, 1982) and loss of adenylyl cyclase activity

causes a cell cycle arrest that mimics that of cells responding to nutrient limitation (Kataoka *et al.*, 1985). Cell wall proteins play a key role in *C. albicans* morphogenesis (Calderone, 2002), and in *S. cerevisiae* the Ras/cAMP pathway has been shown to be required for maintenance of cell-wall integrity (Tomlin *et al.*, 2000). In addition, osmotolerance is strongly affected by cAMP-dependent protein kinase A in *S. cerevisiae* (Norbeck and Blomberg, 2000).

Intriguingly, not only known cellular processes appear influenced by cAMP in *C. albicans*. A large number of genes whose transcript levels are increased in the absence of adenylyl cyclase are of unknown function and so far specific to *C. albicans*. Further study of this group of unique cAMP-repressed genes may reveal new and specialized functions for adenylyl cyclase in *Candida*. Several of these cAMP repressed genes also have significantly increased expression levels during osmotic stress; we have chosen three of the most highly influenced genes in this group (*orf19.2344, orf19.7284* and *orf19.842*) and have named them adenylyl cyclase and stress responsive genes *ASR1*, *ASR2* and *ASR3* respectively. Studies are underway to investigate more closely their regulation and cellular roles.

Studies in the yeast *S. cerevisiae* first suggested that Ras function can act as a regulator of adenylyl cyclase (Toda *et al.*, 1985). Our transcription profiling data in *C. albicans* support a similar connection between Ras1p and adenylyl cyclase in this somewhat distantly related organism, as the transcriptional consequences of loss of Ras1p and loss of Cdc35p function are similar. However, loss of *RAS1* influenced only a subset of the

cAMP-responsive genes, and among the genes whose modified regulation was common to both strains, the magnitude of change in expression was typically less for the *ras1* mutant. Overall, however, there were few genes whose regulation was uniquely modified in the *ras1* mutant, and this result suggests that the primary role of Ras1p in transcription regulation in *C. albicans* is to function as part of the cAMP regulatory circuit. Because epistatic relationships may change as a function of growth conditions, this observation does not rule out other regulatory patterns under environmental conditions different from those studied here.

The observation that loss of Ras1p had a less dramatic effect on the transcription profiles than did loss of Cdc35p, both in terms of the number of genes influenced and the magnitude of the induced changes, could be explained if loss of Ras1p only reduced, but did not eliminate, adenylyl cyclase activity. This would be consistent with Cdc35p having other regulators in addition to Ras1p (Figure 4). The *C. albicans* homolog of Gpa2p, a protein that acts as a regulator of adenylyl cyclase in *S. cerevisiae*, has been identified, although its role in cAMP signalling in the pathogen appears limited. However, a double mutant of Ras1p and Gpr1p, the *C. albicans* homolog of the *S. cerevisiae* glucose receptor that regulates adenylyl cyclase activity (Xue *et al.*, 1998), shows growth characteristics similar to the *C. albicans* adenylyl cyclase null mutant (our unpublished results).

The transcription factor Efg1p has been identified as a downstream target of the cAMP regulatory circuit based on its role in the process of hyphal development (Bockmühl and

Ernst, 2001). However, we found the set of genes whose transcript levels were influenced by the loss of *EFG1* to be generally distinct from those influenced by the loss of *CDC35* or *RAS1*. In fact, only the class of genes activated during the yeast-to-hyphal transition responds to the loss of all three elements. Intriguingly, one class of genes transcriptionally affected by loss of the cAMP pathway represents known or putative transcription factors. This fits with the wide impact of the loss of cyclase function on transcription profiles, and the recognition that much of this profile is independent of Efg1p (Figure 4).

Overall, transcription profiling provides a powerful tool to investigate organisms such as *C. albicans* that are not readily amenable to direct genetic analysis. In this study, we have probed the cAMP regulatory circuit of *C. albicans*, known to regulate morphogenesis and virulence, and have revealed its function in the regulation of gene expression involved in many cellular processes. This work suggests that there are still regulatory connections that need to be established; in particular the broad transcriptional effects of loss of adenylyl cyclase that are independent of Efg1p suggest that there are further transcriptional regulatory circuits that respond to cAMP.

Acknowledgements

We thank H. Lo for generously providing the Efg1 strain, members of the Whiteway lab for helpful discussion and Ursula Oberholzer and Marc Fortin for critical reading of the

manuscript. This project was funded by the Genomics and Health Initiative of the National Research Council of Canada, and CIHR grant MOP-42516. This is NRC publication number 46164.

References

Alspaugh, J.A., Perfect, J.R., and Heitman, J. (1997). Cryptococcus neoformans mating and virulence are regulated by the G-protein alpha subunit GPA1 and cAMP. Genes Dev *11*, 3206-3217.

Bahn, Y.S., Staab, J., and Sundstrom, P. (2003). Increased high-affinity phosphodiesterase PDE2 gene expression in germ tubes counteracts CAP1-dependent synthesis of cyclic AMP, limits hypha production and promotes virulence of Candida albicans. Mol Microbiol *50*, 391-409.

Bockmühl, D.P., and Ernst, J.F. (2001). A potential phosphorylation site for an A-type kinase in the Efg1 regulator protein contributes to hyphal morphogenesis of Candida albicans. Genetics *157*, 1523-1530.

Bockmühl, D.P., Krishnamurthy, S., Gerads, M., Sonneborn, A., and Ernst, J.F. (2001). Distinct and redundant roles of the two protein kinase A isoforms Tpk1p and Tpk2p in morphogenesis and growth of Candida albicans. Mol Microbiol *42*, 1243-1257.

Botsford, J.L., and Harman, J.G. (1992). Cyclic AMP in prokaryotes. Microbiol Rev *56*, 100-122.

Calderone, R. (2002). Candida and candidiasis. ASM Press.

Chen, J., Zhou, S., Wang, Q., Chen, X., Pan, T., and Liu, H. (2000). Crk1, a novel Cdc2-related protein kinase, is required for hyphal development and virulence in Candida albicans. Mol Cell Biol *20*, 8696-8708.

Choi, W., and Dean, R.A. (1997). The adenylate cyclase gene MAC1 of Magnaporthe grisea controls appressorium formation and other aspects of growth and development. Plant Cell *9*, 1973-1983.

Cloutier, M., Castilla, R., Bolduc, N., Zelada, A., Martineau, P., Bouillon, M., Magee, B.B., Passeron, S., Giasson, L., and Cantore, M.L. (2003). The two isoforms of the cAMP-dependent protein kinase catalytic subunit are involved in the control of dimorphism in the human fungal pathogen Candida albicans. Fungal Genet Biol *38*, 133-141.

Colombo, S., Ma, P., Cauwenberg, L., Winderickx, J., Crauwels, M., Teunissen, A., Nauwelaers, D., de Winde, J.H., Gorwa, M.F., Colavizza, D., and Thevelein, J.M. (1998). Involvement of distinct G-proteins, Gpa2 and Ras, in glucose- and intracellular acidification-induced cAMP signalling in the yeast Saccharomyces cerevisiae. Embo J *17*, 3326-3341.

Conlan, R.S., and Tzamarias, D. (2001). Sfl1 functions via the co-repressor Ssn6-Tup1 and the cAMP-dependent protein kinase Tpk2. J Mol Biol *309*, 1007-1015.

D'Souza, C.A., and Heitman, J. (2001). Conserved cAMP signaling cascades regulate fungal development and virulence. FEMS Microbiol Rev *25*, 349-364.
Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci U S A *95*, 14863-14868.
Enjalbert, B., Nantel, A., and Whiteway, M. (2003). Stress-induced gene expression in Candida albicans: absence of a general stress response. Mol Biol Cell *14*, 1460-1467.
Feng, Q., Summers, E., Guo, B., and Fink, G. (1999). Ras signaling is required for serum-induced hyphal differentiation in Candida albicans. J Bacteriol *181*, 6339-6346.
Gillum, A.M., Tsay, E.Y., and Kirsch, D.R. (1984). Isolation of the Candida albicans gene for orotidine-5'-phosphate decarboxylase by complementation of S. cerevisiae ura3 and E. coli pyrF mutations. Mol Gen Genet *198*, 179-182.
Giusani, A.D., Vinces, M., and Kumamoto, C.A. (2002). Invasive filamentous growth of Candida albicans is promoted by Czf1p-dependent relief of Efg1p-mediated repression. Genetics *160*, 1749-1753.
Gold, S., Duncan, G., Barrett, K., and Kronstad, J. (1994). cAMP regulates morphogenesis in the fungal pathogen Ustilago maydis. Genes Dev *8*, 2805-2816.
Gold, S.E., Brogdon, S.M., Mayorga, M.E., and Kronstad, J.W. (1997). The Ustilago maydis regulatory subunit of a cAMP-dependent protein kinase is required for gall formation in maize. Plant Cell *9*, 1585-1594.
Johnson, D.A., Akamine, P., Radzio-Andzelm, E., Madhusudan, M., and Taylor, S.S. (2001). Dynamics of cAMP-dependent protein kinase. Chem Rev *101*, 2243-2270.
Kataoka, T., Broek, D., and Wigler, M. (1985). DNA sequence and characterization of the S. cerevisiae gene encoding adenylate cyclase. Cell *43*, 493-505.
Kubler, E., Mosch, H.U., Rupp, S., and Lisanti, M.P. (1997). Gpa2p, a G-protein alpha-subunit, regulates growth and pseudohyphal development in Saccharomyces cerevisiae via a cAMP-dependent mechanism. J Biol Chem *272*, 20321-20323.
Lan, C.Y., Newport, G., Murillo, L.A., Jones, T., Scherer, S., Davis, R.W., and Agabian, N. (2002). Metabolic specialization associated with phenotypic switching in Candida albicans. Proc Natl Acad Sci U S A *99*, 14907-14912.
Leberer, E., Harcus, D., Dignard, D., Johnson, L., Ushinsky, S., Thomas, D.Y., and Schroppel, K. (2001). Ras links cellular morphogenesis to virulence by regulation of the MAP kinase and cAMP signalling pathways in the pathogenic fungus Candida albicans. Mol Microbiol *42*, 673-687.
Lee, C.M., Nantel, A., Jiang, L., Whiteway, M., and Shen, S.H. (2004). The serine/threonine protein phosphatase SIT4 modulates yeast-to-hypha morphogenesis and virulence in Candida albicans. Mol Microbiol *51*, 691-709.
Lengeler, K.B., Davidson, R.C., D'Souza, C., Harashima, T., Shen, W.C., Wang, P., Pan, X., Waugh, M., and Heitman, J. (2000). Signal transduction cascades regulating fungal development and virulence. Microbiol Mol Biol Rev *64*, 746-785.
Liu, H. (2001). Transcriptional control of dimorphism in Candida albicans. Curr Opin Microbiol *4*, 728-735.
Lo, H.J., Kohler, J.R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A., and Fink, G.R. (1997). Nonfilamentous C. albicans mutants are avirulent. Cell *90*, 939-949.
Matsumoto, K., Uno, I., Oshima, Y., and Ishikawa, T. (1982). Isolation and characterization of yeast mutants deficient in adenylate cyclase and cAMP-dependent protein kinase. Proc Natl Acad Sci U S A *79*, 2355-2359.

Nantel, A., Dignard, D., Bachewich, C., Harcus, D., Marcil, A., Bouin, A.P., Sensen, C.W., Hogues, H., van het Hoog, M., Gordon, P., Rigby, T., Benoit, F., Tessier, D.C., Thomas, D.Y., and Whiteway, M. (2002). Transcription profiling of Candida albicans cells undergoing the yeast-to-hyphal transition. Mol Biol Cell *13*, 3452-3465.
Norbeck, J., and Blomberg, A. (2000). The level of cAMP-dependent protein kinase A activity strongly affects osmotolerance and osmo-instigated gene expression changes in Saccharomyces cerevisiae. Yeast *16*, 121-137.
Pascual-Ahuir, A., Posas, F., Serrano, R., and Proft, M. (2001). Multiple levels of control regulate the yeast cAMP-response element-binding protein repressor Sko1p in response to stress. J Biol Chem *276*, 37373-37378.
Pedruzzi, I., Burckert, N., Egger, P., and De Virgilio, C. (2000). Saccharomyces cerevisiae Ras/cAMP pathway controls post-diauxic shift element-dependent transcription through the zinc finger protein Gis1. Embo J *19*, 2569-2579.
Rocha, C.R., Schroppel, K., Harcus, D., Marcil, A., Dignard, D., Taylor, B.N., Thomas, D.Y., Whiteway, M., and Leberer, E. (2001). Signaling through adenylyl cyclase is essential for hyphal growth and virulence in the pathogenic fungus Candida albicans. Mol Biol Cell *12*, 3631-3643.
Saran, S., Meima, M.E., Alvarez-Curto, E., Weening, K.E., Rozen, D.E., and Schaap, P. (2002). cAMP signaling in Dictyostelium. Complexity of cAMP synthesis, degradation and detection. J Muscle Res Cell Motil *23*, 793-802.
Smith, A., Ward, M.P., and Garrett, S. (1998). Yeast PKA represses Msn2p/Msn4p-dependent gene expression to regulate growth, stress response and glycogen accumulation. Embo J *17*, 3556-3564.
Sohn, K., Urban, C., Brunner, H., and Rupp, S. (2003). EFG1 is a major regulator of cell wall dynamics in Candida albicans as revealed by DNA microarrays. Mol Microbiol *47*, 89-102.
Stoldt, V.R., Sonneborn, A., Leuker, C.E., and Ernst, J.F. (1997). Efg1p, an essential regulator of morphogenesis of the human pathogen Candida albicans, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. Embo J *16*, 1982-1991.
Toda, T., Cameron, S., Sass, P., Zoller, M., and Wigler, M. (1987). Three different genes in S. cerevisiae encode the catalytic subunits of the cAMP-dependent protein kinase. Cell *50*, 277-287.
Toda, T., Uno, I., Ishikawa, T., Powers, S., Kataoka, T., Broek, D., Cameron, S., Broach, J., Matsumoto, K., and Wigler, M. (1985). In yeast, RAS proteins are controlling elements of adenylate cyclase. Cell *40*, 27-36.
Tomlin, G.C., Hamilton, G.E., Gardner, D.C., Walmsley, R.M., Stateva, L.I., and Oliver, S.G. (2000). Suppression of sorbitol dependence in a strain bearing a mutation in the SRB1/PSA1/VIG9 gene encoding GDP-mannose pyrophosphorylase by PDE2 overexpression suggests a role for the Ras/cAMP signal-transduction pathway in the control of yeast cell-wall biogenesis. Microbiology *146 (Pt 9)*, 2133-2146.
Tripathi, G., Wiltshire, C., Macaskill, S., Tournu, H., Budge, S., and Brown, A.J. (2002). Gcn4 co-ordinates morphogenetic and metabolic responses to amino acid starvation in Candida albicans. Embo J *21*, 5448-5456.

Ward, M.P., Gimeno, C.J., Fink, G.R., and Garrett, S. (1995). SOK2 may regulate cyclic AMP-dependent protein kinase-stimulated growth and pseudohyphal development by repressing transcription. Mol Cell Biol *15*, 6854-6863.
Whiteway, M. (2000). Transcriptional control of cell type and morphogenesis in Candida albicans. Curr Opin Microbiol *3*, 582-588.
Xue, Y., Batlle, M., and Hirsch, J.P. (1998). GPR1 encodes a putative G protein-coupled receptor that associates with the Gpa2p Galpha subunit and functions in a Ras-independent pathway. Embo J *17*, 1996-2007.

Figure legends

Figure 1. Cluster analysis of transcription profiles. We selected 1168 transcripts that exhibited a statistically significant variation in abundance of at least 1.4-fold and had a false discovery rate < 5% (see methods for details). These were separated by two-dimensional hierarchical clustering (Eisen *et al.*, 1998) that groups genes and experiments with similar profiles. Normalized fluorescence ratios between the experimental and reference samples are represented as a green to red color scale. Similarity between each gene's transcription profile is represented by the horizontal dendrogram while the vertical dendrogram represents the similarity between experiments.

Figure 2. Comparison of differentially expressed transcripts in the mutants with the wild type. The Venn diagrams show the overlap between groups of genes that show increased (top number) and decreased (bottom number) transcript levels in various experiments. Effects of the *ras1*, *cdc35* and *efg1* mutations when compared to the wild type under (A) yeast form conditions, (B) hyphal inducing conditions or (C) the change in transcript abundance during the yeast to hyphal switch in *ras1*, *cdc35* and *efg1* mutants (these numbers refer to increases or decreases in the hyphal condition). Statistically significant (p-value$<10^{-10}$) overlaps between gene lists are indicated with an asterisk (*).

Figure 3. Comparison of sensitivities to Calcofluor white, Zymolyase and environmental stress in the wild type (SC5314) and *ras1*, *cdc35* and *efg1* mutants. (A) Comparison of sensitivity to Calcofluor white. Strains were spotted on YPD plates containing 0 or 50 µg/ml of Calcofluor white, and monitored after three days of growth. (B) Sensitivity to Zymolyase. Strains were incubated in 100 µg/ml of Zymolase 100T and the OD_{600}nm was monitored for 2.5 hr. Data represents mean values ± SD of at least three independent experiments. (C) Sensitivity to osmotic shock. Strains were spotted on YPD plates containing 0 or 1.5M NaCl. (D) Sensitivity to heat shock. Strains grown at 30°C were immersed in a 50°C waterbath for intervals of 0 to 60 minutes and then spotted on a YPD plate and incubated at 30°C. For both stresses, results were monitored after two days of growth. Cell concentrations of the *cdc35* mutant were adjusted to compensate for different growth rates.

Figure 4. Model of the *C. albicans* cAMP signalling pathway. The schematic representation shows the role of three components of the cAMP pathway: Ras1p, Cdc35p and Efg1p. The model proposes that Ras1p is a primary regulator of adenylyl cyclase but that there are other regulators of this pathway since the loss of Ras1p modulates only a subset of genes affected by the loss of Cdc35p, and Ras1p defective strains can still produce pseudohyphae. It appears that all three components are involved in the morphogenetic switch, but the cAMP pathway mediates regulation of other cellular processes independent of Efg1p, and these other targets of the cAMP pathway may or may not be regulated through PKA. In addition, we suggest that Efg1p also exerts effects on the transcription of genes outside the cAMP regulatory circuit. The asterisk (*) shows that the cell wall processes affected by cAMP and Efg1 are different.

Table I Genes unique to *Candida albicans* whose transcript levels increase in the absence of adenylyl cyclase

		Expression ratio (mutant/WT)		
Orf	Description	Δcdc35Y	Δras1Y	Δefg1Y
orf19.2344	No good BLAST hits	19.6	9.7	1.0
orf19.7284	"	12.1	6.3	0.7
orf19.6420	"	6.0	1.0	1.7
orf19.842	"	5.9	3.0	0.9
orf19.7350	"	5.3	3.5	0.5
orf19.675	"	5.1	2.1	0.9
orf19.2030	"	4.8	2.6	1.3
orf19.3740	"	4.4	1.7	1.1
orf19.3793	"	4.2	1.9	0.9
orf19.2769	"	3.9	2.2	1.0
orf19.2765	"	3.8	2.3	0.9
orf19.682	"	3.3	1.5	0.7
orf19.4706	"	2.9	1.8	1.1
orf19.5642	"	2.4	1.7	0.8
orf19.7502	"	2.4	1.4	1.1
orf19.3439	"	2.2	1.6	1.6
orf19.6329	"	2.2	1.3	1.1
orf19.199	"	2.1	1.2	1.1
orf19.1246	"	2.1	1.5	0.9
orf19.5158	"	2.1	1.3	1.1
orf19.5070	"	2.0	1.2	1.1
orf19.2685	"	2.0	1.0	1.6
orf19.5843	"	1.9	1.3	1.0
orf19.1597	"	1.9	1.2	1.0
orf19.5785	"	1.9	2.5	1.4
orf19.2701	"	1.9	1.4	1.1
orf19.1120	"	1.8	1.3	1.1
orf19.6737	"	1.8	1.5	2.0
orf19.3469	"	1.7	1.3	1.2
orf19.3644	"	1.6	1.4	0.9
orf19.4651	"	1.6	1.5	0.8
orf19.5876	"	1.6	1.1	1.1
orf19.6604	"	1.6	1.3	1.0
orf19.5587	"	1.6	1.3	1.0
orf19.822	"	1.6	1.9	0.8
orf19.305	"	1.5	1.1	1.0
orf19.446	"	1.5	1.2	1.0
orf19.4282	"	1.5	1.0	1.3
orf19.3742	"	1.5	1.1	1.0
orf19.4988	"	1.4	1.0	0.9

Red = transcript level increased
Green = transcript level decreased
White = not modulated in a cutoff of 1.4 fold
Grey = did not pass SAM

Table II Cell wall genes whose transcripts are modulated in the absence of adenylyl cyclase

Common Name	Orf	Description	Expression ratio (mutant/WT) Δcdc35 yeast	Δcdc35 hyphae	Δras1 yeast	Δras1 hyphae	Δefg1 yeast	Δefg1 hyphae
CHS6	orf19.5155	chitin synthesis	0.8	0.6	1.1	0.9	1.0	1.0
CHT2	orf19.3895	chitinase 2 precursor	0.4	0.6	0.9	1.0	1.5	1.8
ECM1	orf19.5299	cell wall structure or biosynthesis	0.6	0.6	0.8	1.2	1.1	1.2
EXG1	orf19.2952	exo-1,3-beta-glucanase	0.9	0.4	0.8	0.8	1.2	0.7
HWP1	orf19.1321	hyphal wall protein	1.1	0.06	1.2	0.2	0.7	0.2
HYR1	orf19.87	glutathione peroxidase	0.9	0.5	1.2	0.9	0.9	0.8
PHR1	orf19.3829	pH regulated GPI-anchored membrane protein	1.2	0.2	1.4	0.5	1.6	0.7
RBT1	orf19.1327	repressed by TUP1 protein 1	1.3	0.25	*1.4	0.4	1	0.3
SMI1	orf19.5058	regulator of 1,3-beta glucan synthesis	0.8	0.7	0.8	0.8	0.9	0.8
BGL2	orf19.4565	beta-glucosidase 2 precursor	2.6	1.9	1.5	1.3	1.0	1.0
CCW14	orf19.1969	cell wall mannoprotein involved in cell stress response	3.1	1.8	1.9	1.1	1.1	1.0
CHS7	orf19.2444	involved in chitin synthesis	1.7	1.6	*1.4	1.3	1.1	1.0
CHT3	orf19.7586	chitinase 3 precursor	1.3	1.5	*1.4	1.2	1.2	1.5
DCW1	orf19.1989	Defective Cell Wall	1.6	1.7	1.0	1.0	1.0	1.0
ECM17	orf19.5477	sulfite reductase involved in cell wall biogenesis and architectu	1.1	1.9	1.2	1.2	1.0	*1.4
ECM33	orf19.4255	protoplast-secreted	3.3	1.2	2.3	0.7	1.4	0.9
ECM4	orf19.2613	involved in cell wall biogenesis and architecture	3.3	2.4	2.1	1.5	0.8	0.8
KRE6	orf19.7363	glucan synthase subunit involved in cell wall assembly	2	1.6	1.3	1	0.9	1
KRE9	orf19.5861	cell wall synthesis protein | cell wall beta-glucan assembly	1.8	1.6	1.6	1	1.1	1.2
KTR1	orf19.1010	mannosyltransferase involved in O- and N-linked glycosylation	1.9	2.0	1.1	1.3	0.8	1
PHR2	orf19.6081	pH regulated cell wall protein	1.6	1.8	1.3	0.9	0.8	0.6
SCW10	orf19.1779	soluble cell wall protein similar to mannoprotein MP65	2.3	2.5	1.8	1.2	1.1	0.9
SIM1	orf19.5032	protein involved in control of DNA replication	1.7	2.5	1.7	1.6	1.3	1.5
WSC1	orf19.5867	cell wall protein	*1.4	1.5	1.1	0.9	1	0.9

Red = transcript level increased
Green = transcript level decreased
White = not modulated in a cutoff of 1.4 fold
Grey = did not pass SAM
* these made the 1.4 fold cutoff when rounded to one decimal point

Table III Role of cAMP regulating components in the yeast to hyphal transition

Common Name	Orf	Description	Expression ratio (hyphae/yeast) WT	Δcdc35	Δras1	Δefg1
ECE1	orf19.3374	secreted cell elongation protein	33.3	1.1	8.1	2.5
SAP4	orf19.5716	candidapepsin 4 precursor	9.3	1.1	1.0	1.3
SAP5	orf19.5585	secreted aspartyl proteinase 5	9.1	0.7	1.0	1.6
HWP1	orf19.1321	hyphal wall protein	6.1	0.8	3.1	1.6
SOD5	orf19.2060	copper-zinc superoxide dismutase	5.7	1.1	2.0	1.1
SAP6	orf19.5542	candidapepsin 6 precursor	4.2	1.3	0.8	1.1
IHD1	orf19.5760	Induced in Hyphal Development;membrane protein	4.0	*1.4	4.4	1.3
RBT1	orf19.1327	repressed by TUP1 protein 1	3.8	0.9	1.9	1.4
CHA2	orf19.1996	catabolic serine/threonine dehydratase	3.2	2.7	4.4	4.5
DDR48	orf19.4082	flocculent specific protein;contains NNDDNSYG motif	2.5	0.4	0.4	1.0
PHR1	orf19.3829	pH regulated GPI-anchored membrane protein that is required for morphogenesis	2.5	1.2	1.7	1.5
GAP4	orf19.4456	amino acid permease	2.4	1.5	1.4	1.0
PTP3	orf19.7610	protein tyrosine phosphatase	2.3	1.2	1.6	1.2
	orf19.6705	conserved hypothetical protein	2.3	0.9	1.7	1.2
PRY4	orf19.6202	pathogenesis related protein, repressed by TUP1 protein 4	2.3	0.7	0.9	0.9
KRE1	orf19.4377	cell wall beta-1,6-glucan assembly	2.2	0.9	0.7	0.9
PST1	orf19.2241	1,4-benzoquinone reductase;brefeldin A resistance protein;Protoplast-Secreted protein	2.2	0.8	1.1	1.4
	orf19.4666	hypothetical protein	2.1	1.2	1.3	1.4
IHD2	orf19.6021	Induced in Hyphal Development	2.1	1.2	1.2	1.1
	orf19.2685	hypothetical protein	2.1	1.1	1.2	0.7
	orf19.4749	hypothetical membrane protein	2.0	1.0	1.8	1.2
	orf19.7531	conserved hypothetical protein	2.0	2.1	1.6	*1.4
	orf19.4316	conserved hypothetical protein	2.0	0.9	1.0	1.2
SNZ1	orf19.2947	stationary phase protein	1.9	2.3	2.2	2.1
	orf19.815	DOCK180 protein	1.8	1.5	1.5	1.0
RBT8	orf19.5674	glycosyl-phosphatidylinositol protein;similar to RBT5	1.8	1.3	1.4	1.1
ARO10	orf19.1847	pyruvate decarboxylase	1.8	1.5	1.5	2.5
ALR1	orf19.1607	putative divalent cation transporter	1.7	1.1	1.4	1.4
IRO1	orf19.1715	transcription factor	1.7	0.8	1.1	1.0
TUB2	orf19.6034	beta- tubulin	1.7	0.9	1.0	0.7
GRP4	orf19.3150	similar to plant dihydroflavonol 4-reductase	1.7	2.2	0.9	1.8
	orf19.3475	Gag protein	1.7	0.8	0.9	1.0
	orf19.1105.2	hypothetical protein	1.6	1.0	1.1	1.3
YKE2	orf19.6601.1	non-native actin binding complex polypeptide	1.6	1.5	1.7	1.4
NIP7	orf19.3478	nucleolar protein required for 60S ribosome subunit genesis	1.6	1.2	1.1	0.7
	orf19.4607	conserved hypothetical protein	1.5	0.9	1.2	1.4
RNH1	orf19.5563	ribonuclease H	1.5	1.2	1.0	0.8
	orf19.2903	conserved hypothetical protein	1.5	1.1	1.2	1.5
EXG1	orf19.2952	exo-1,3-beta-glucanase	1.5	1.0	1.1	1.3
	orf19.4521	weak similarity to reductases	1.5	1.2	1.2	1.0
	orf19.2769	hypothetical protein	1.5	1.2	1.0	1.6
	orf19.5588	conserved hypothetical protein	1.5	1.1	1.2	1.1
	orf19.199	hypothetical protein	1.5	1.1	0.4	1.0
NOP15	orf19.7050	nucleolar protein 15;ribosome biogenesis	1.5	1.1	1.0	1.0
GLG2	orf19.7434	glycogenin;self-glucosylating initiator of glycogen synthesis	1.5	0.9	1.3	1.1
IFD1	orf19.1048	conserved aryl-alcohol dehydrogenase	1.5	1.1	1.4	1.9
CTA2	orf19.7544	transcriptional activation	1.5	1.6	1.4	2.2
PFY1	orf19.5076	profilin	1.5	0.9	1.0	1.0
RAX1	orf19.5903	similar to B-chains of the insulin- related superfamily;may be involved in bud site selection	1.5	1.2	1.0	0.8
HIS3	orf19.183	imidazoleglycerol-phosphate dehydratase	1.4	1.4	1.1	1.2
YAL60	orf19.5288	Zn-containing alcohol dehydrogenase	0.1	0.8	0.7	2.1
DHA12	orf19.4737	membrane transporter of the MFS-MDR family	0.3	0.8	0.7	1.6
STF2	orf19.2107.1	ATP synthase regulatory factor	0.3	1.3	1.2	1.9
YHB1	orf19.3707	flavohemoglobin;dihydropteridine reductase	0.3	1.1	2.5	2.1
	orf19.2659	conserved hypothetical protein	0.3	1.4	2.6	2.7
RHD1	orf19.54	Repressed in Hyphal Development;family of conserved protein of unknown function	0.3	0.5	0.3	0.9
YTH1	orf19.6881	cleavage and polyadenylation specificity factor	0.4	1.2	1.2	1.3
	orf19.2668	gag-pol polyprotein	0.4	0.9	0.4	0.5
GPM2	orf19.1067	phosphoglycerate mutase	0.4	1.0	1.0	1.5
OPT9	orf19.2584	oligopeptide transporter specific for tetra- and pentapeptides;possible pseudogene	0.4	0.8	0.2	0.5
YWP1	orf19.3618	putative cell wall protein	0.4	1.0	1.2	2.4
	orf19.1353	hypothetical protein	0.4	1.7	2.7	1.5
CSP37	orf19.2531	cell surface protein	0.5	0.8	0.7	1.3
GPP1	orf19.5437	DL-glycerol-3-phosphatase	0.5	0.8	1.2	2.5
TYE7	orf19.4941	basic helix-loop-helix transcription factor	0.5	2.0	1.6	1.8
PTC4	orf19.6638	ser/thr protein phosphatase PP2C	0.5	1.2	1.1	1.0
RHD3	orf19.5305	conserved protein reressed in hyphal development	0.5	0.6	0.5	0.5
	orf19.951	hypothetical protein	0.5	1.0	1.1	1.2
	orf19.449	predicted phosphatidyl synthase	0.5	1.3	1.2	1.1
FCY1	orf19.4195.1	cytosine deaminase	0.5	1.2	1.0	0.9
DAK2	orf19.4777	dihydroxyacetone kinase	0.5	1.0	1.0	1.0
CBF1	orf19.2876	centromere-binding kinetochore protein	0.5	1.2	1.2	1.5
RPS24	orf19.5466	ribosomal protein S24	0.5	1.3	0.9	0.7
ALD5	orf19.5806	aldehyde dehydrogenase	0.5	1.3	0.9	0.6
PCL7	orf19.6225	cyclin like protein interacting with PHO85	0.5	1.0	1.0	1.1
MEP1	orf19.1614	low affinity high capacity ammonium permease	0.5	0.9	0.9	0.9
GDH3	orf19.4716	NADP-glutamate dehydrogenase	0.5	1.1	0.7	1.0
AGP2	orf19.4679	amino-acid permease	0.5	0.7	0.6	0.6
PHO15	orf19.4444	4-nitrophenyl phosphatase	0.5	0.9	1.0	1.4
MUQ1	orf19.2107	choline phosphate cytidylyltransferase;phosphoethanolamine cytidylyltransferase	0.5	1.0	0.9	1.0
PCK1	orf19.7514	phosphoenolpyruvate carboxykinase	0.5	1.5	1.8	1.5
PFK2	orf19.6540	Phosphofructokinase	0.5	1.5	1.4	2.1
LEU1	orf19.7498	3-isopropylmalate dehydratase	0.6	1.0	1.0	1.3
HSP122	orf19.4216	heat shock protein	0.6	1.2	0.7	1.0
HYP2	orf19.3426	translation initiation factor eIF5A.1	0.6	0.8	0.9	0.8
	orf19.400	hypothetical protein	0.6	1.0	1.0	0.9
	orf19.1353	hypothetical protein	0.6	1.3	1.9	1.6
	orf19.3302	hypothetical protein	0.6	1.0	0.7	1.0
	orf19.633	putative methyltransferase	0.6	1.0	1.0	1.0
MIG1	orf19.4318	transcriptional regulator	0.6	1.0	1.1	1.0
ADK12	orf19.3391	adenylate kinase	0.6	1.0	0.9	0.9
MRP20	orf19.3350	mitochondrial ribosomal protein	0.6	1.2	1.3	0.8
	orf19.5282	hypothetical protein	0.6	1.1	1.1	1.1
VTC4	orf19.3363	polyphosphate synthetase	0.6	1.2	1.2	1.3
GSY1	orf19.3278	glycogen(starch) synthase	0.6	0.8	0.8	1.5
HRT2	orf19.4624	Ty3 transposition effector	0.6	1.0	1.0	1.3
MAF1	orf19.2173	nuclear-cytoplasmic transport;negative effector of Pol III synthesis	0.6	1.5	1.1	1.1

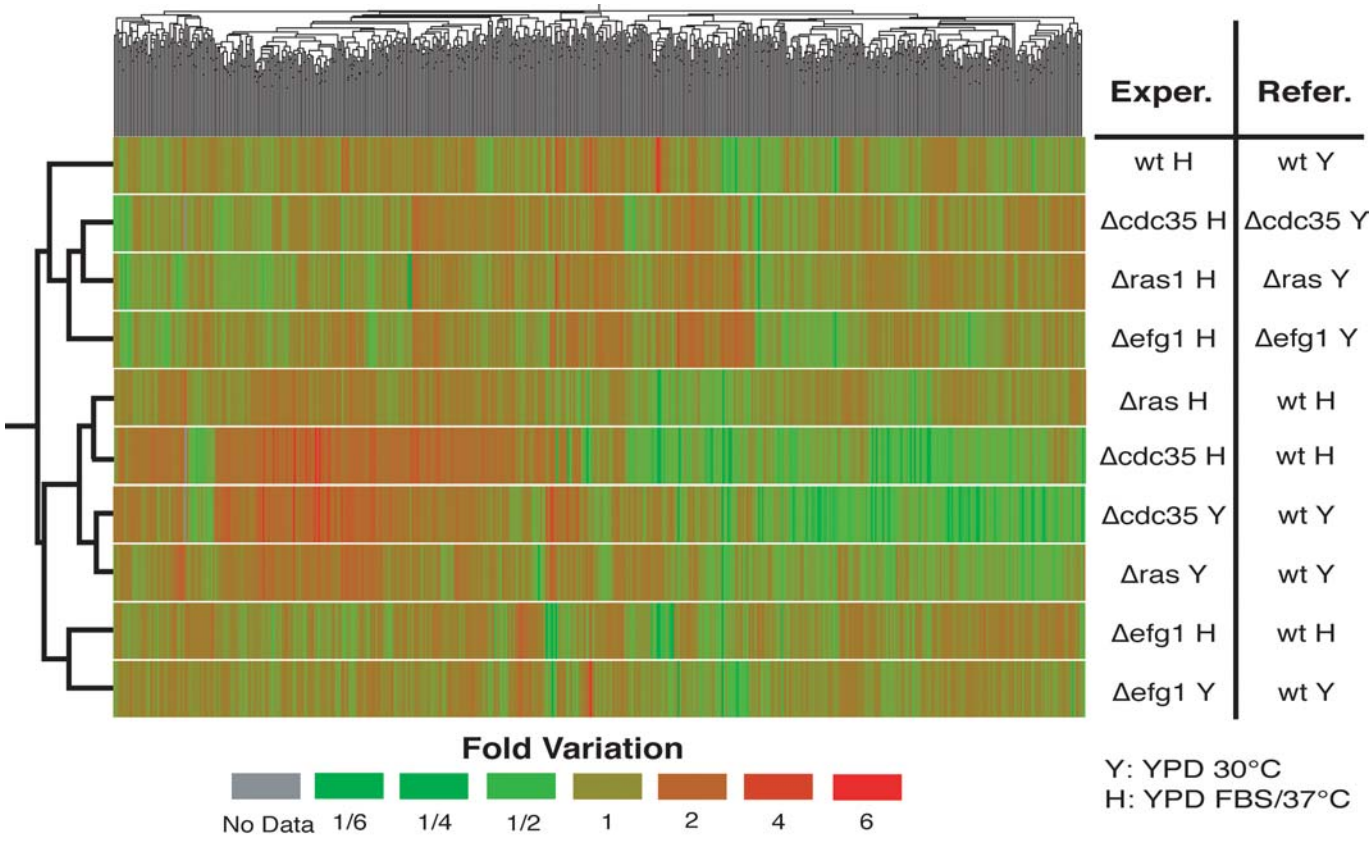

Figure 1
Harcus et al., 2004

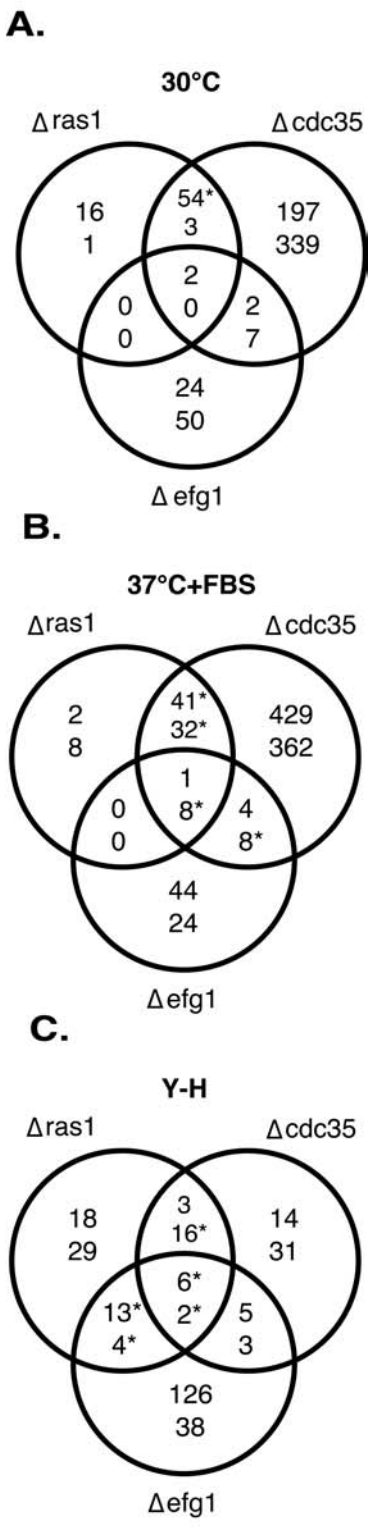

Figure 2
Harcus et al., 2004

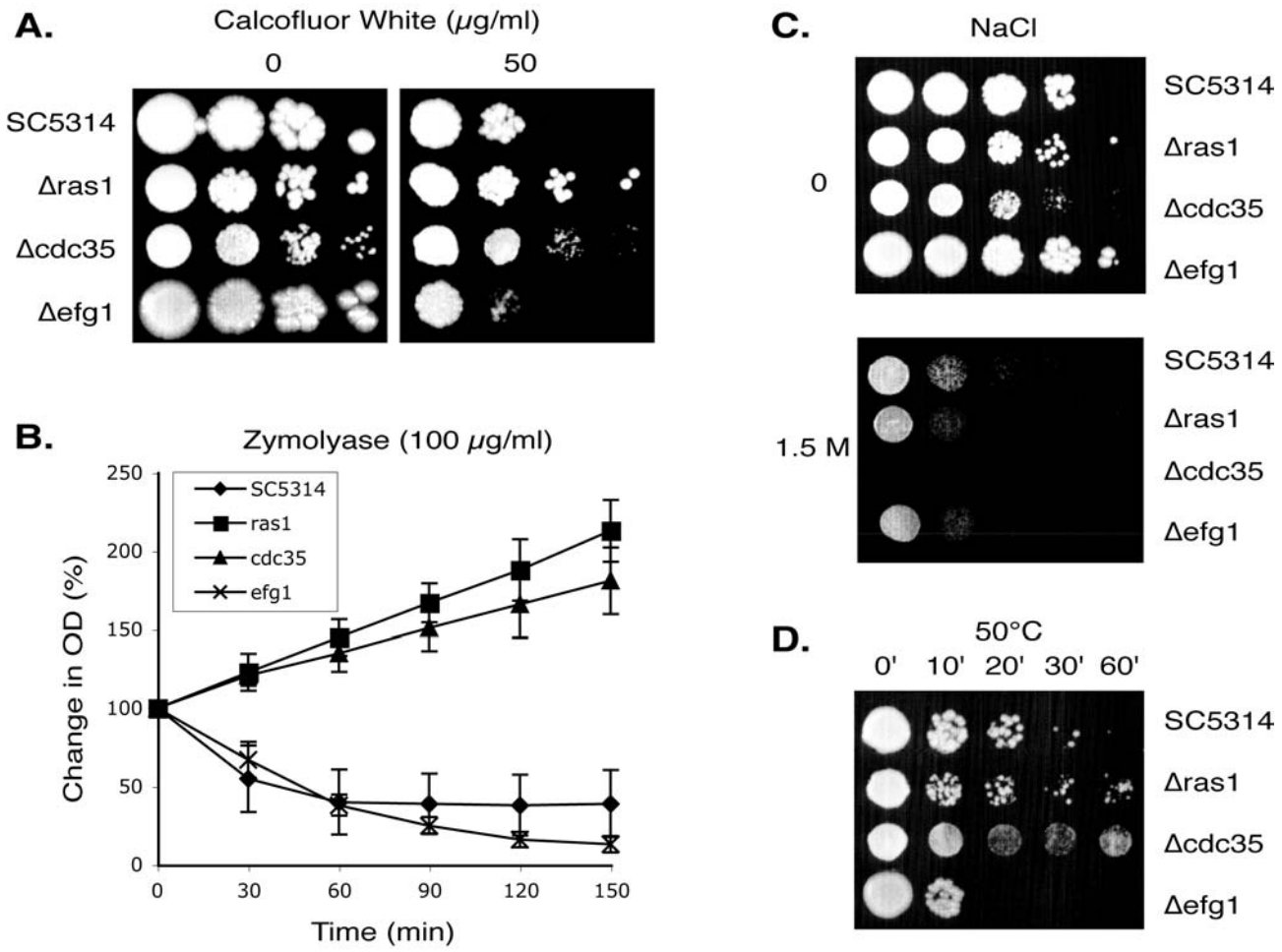

Figure 3
Harcus et al., 2004

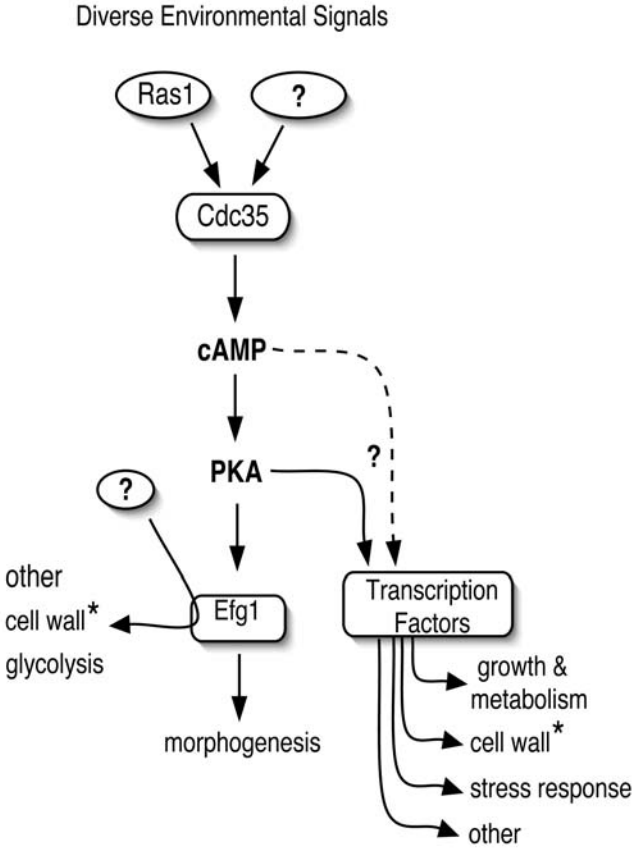

Figure 4
Harcus et al., 2004