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Physiological Studies on Candida albicans

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PHYSIOLOGICAL STUDIES ON *CANDIDA ALBICANS*

by

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Candida albicans is a common opportunistic, dimorphic human fungal pathogen. One of its virulence factors is the morphological switch between yeasts and hyphal or pseudohyphal forms, which can invade tissues and cause damage. Our studies focus on factors regulating pseudohyphae and epigenetic modifications of *C. albicans*. Regulating factors of pseudohyphae are aromatic alcohols and high phosphate. At low concentrations, exogenous aromatic alcohols induced pseudohyphae, as did high phosphate. For addressing the pathways involved in inducing pseudohyphae by aromatic alcohols or high phosphate, we used mutants defective in cAMP dependent PKA pathway (efg1/efg1), MAP kinase pathway (cph1/cph1), or both (cph1/cph1/efg1/efg1). These mutants failed to produce either hyphae or pseudohyphae in the presence of aromatic alcohols; but high phosphate still stimulated pseudohyphae. Gcn4, a transcription activator of more than 500 amino acid related genes, is turned-on in response to amino acid starvation. The accumulation of aromatic alcohols sends nitrogen starvation signals, which inhibit eIF2B, which in turn derepresses Gcn4p. High phosphate also induces pseudohyphae by derepressing Gcn4p, although the pathways involved are still unknown. In sum, aromatic alcohols and high phosphate induce pseudohyphae by derepressing Gcn4.

In this study we found a novel posttranslational histone modification in *C. albicans*, which is biotinylation. Western blot and Mass spectrometry techniques were used to find

that Histones H2B and H4 were biotinylated at every condition tested such as yeast vs. hyphae, aerobic growth vs. anaerobic growth, rich medium vs. defined medium. In *C. albicans* lysines K8, K11 in histone H4 and lysines K17, K18, K31 in histone H2B are biotin attachment sites as found using mass spectrometry. Biotin was also found to enhance the germ tube formation of *C. albicans*. Germ tube formation assays with biotin-starved cells as inoculum showed low percent of germ tubes (1-5%). Addition of biotin to the media showed 100% germ tubes. Biotinylation of histones were not detected from biotin-starved cells.

Appendix-A details work related to Farnesol quantification assays in several strains of *C.albicans* and *Ceratocystis ulmi,* and growth studies of class E VPS strains of *Saccharomyces Cerevisiae.*

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CHAPTER 1 - Aromatic alcohols and high phosphate stimulate pseudohyphal morphogenesis in *Candida albicans* by activating GCN4 and the General Amino Acid Control pathway

Swetha Tati, Suman Ghosh, Audrey L. Atkin, Kenneth W. Nickerson.

Abstract

Candida albicans is a polymorphic fungus that is capable of causing the life-threatening disease Candidiasis once it reaches the bloodstream of a susceptible host. One of the major virulence factors is its ability to switch from yeast to hyphal or pseudohyphal morphology. Yeast to hyphal transition is well studied and many environmental signals such as high temperature, alkaline pH, and presence of high CO_2 level, serum, N-Acetyl Glucosamine regulate hyphal morphogenesis by several well studied signal transduction pathways. Yeast to pseudohyphal development is relatively less understood. Earlier we have reported that aromatic amino acids are utilized by *C. albicans* in poor nitrogen conditions by fusel oil pathway, and as a byproduct, aromatic alcohols are secreted outside the cell. Humans cannot synthesize all the three aromatic amino acids viz. phenylalanine, tyrosine, and tryptophan; and have to obtain them through diet. Fungal pathogen *C. albicans* must synthesize them and therefore their regulation is critical for virulence. Here we report that aromatic alcohols secreted by the fungus *C. albicans*, send a signal back to the cell, which derepress the transcriptional factor Gcn4p. We show that in the presence of aromatic alcohols, *C. albicans* stimulate pseudohyphae that are dependent on both Efg1p and Cph1p. Earlier our group reported that high phosphate levels also stimulated pseudohyphae. In this report we show that high phosphate levels derepress Gcn4p similar to the aromatic alcohols. Further analysis revealed that high phosphate levels stimulated pseudohyphae in *cph1/cph1*, *efg1/efg1*, and *cph1/cph1 efg1/efg1* mutant cells as well. Our data suggest that high phosphate level stimulated pseudohyphal morphogenesis can be independent of MAPKinase pathway and cAMP/PKA pathway.

Introduction

Candida albicans is an opportunistic fungal pathogen that resides as commensal in normal individuals, but causes life-threatening disease, Candidiasis in immune-compromised patients. One of the major traits for *C. albicans* virulence is their ability to switch from yeast to hyphal or pseudohyphal morphology. Yeast cells are oval single cells and they divide by budding (axial or bipolar pattern), and give rise to two asymmetrical mother and daughter cells. The budding mechanism of *C. albicans* yeast cells is similar to *S. cerevisiae* (23). Like a true fungus *C. albicans* can also form germ tubes or hyphae. Germ tubes require induction and selection of the site for germ tube emergence followed by extension to an elongated hyphal filament separated by septae (5). Pseudohyphae are distinct state but the mechanism of pseudohyphae formation more closely resembles budding. Pseudohyphae are elongated cells connected in chains that resemble hyphae, but individually similar to yeast. They basically have delayed isotropic growth after bud emergence and they divide as unipolar budding pattern (5, 23).

This yeast to hyphal morphogenesis is positively regulated by several signal transduction pathways such as the cAMP dependent PKA pathway, the MAPKinase pathway, and the Rim101p pathway, and negatively regulated by the Tup1p pathway. Tup1p acts in concert with Rfg1p or Nrg1p, (4, 6, 15, 21, 24, 33). However, not much is known about the yeast to pseudohyphal switch and its regulation. It is known that most cultures of *C. albicans* exhibit very low (<1%) pseudohyphal cells but that high levels of pseudohyphal development were observed in growth media supplemented with high phosphate (18), in rich growth media supplemented with 0.5% fusel alcohols (12, 13), and for fork head mutants of *C. albicans* (3). Dickinson's work on fusel alcohols inducing pseudohyphal

formation in yeasts (12, 13) primarily concerns *Saccharomyces. cerevisiae* but clearly states that fusel alcohols were observed to induce filament formation in rich nutrient conditions in every yeast species examined, including *C. albicans* (12, 13).

Another study suggests that tyrosol, one of the aromatic alcohols, can reduce the lag phase and accelerate germ tube formation in dilute cultures of *C. albicans* (10). In that study tyrosol failed to stimulate hypha formation at higher cell densities or in yeast inducing conditions (10). Another study by the same group suggested that phenethyl alcohol and tryptophol, but not tyrosol, stimulated morphogenesis in *S. cerevisiae* cells by inducing the expression of *FLO11* (important for flocculation) through a Tpk2p-dependent mechanism (9). Thus, these studies suggest that specific aromatic alcohols can induce filamentation in fungi and yeasts in a species-specific manner. We want to find out which aromatic alcohols can induce hyphal morphogenesis in *C. albicans* and to elucidate the mechanism of actions.

The other significant player in pseudohyphal development is Gcn4p (17, 32). Gcn4p is at the center of general amino acid control (GAAC). In *S. cerevisiae*, Gcn4p regulates more than 500 target genes involved with amino acid biosynthesis and the use of alternative nitrogen sources (17). The molecular events that induce *GCN4* transcription also reduce the rate of general protein synthesis by ca. one half (17). Gcn4p synthesis responds to many environmental signals including nitrogen starvation, amino acid starvation, and the presence of fusel alcohols (17). Tripathi et al (32) then showed that in *C. albicans* CaGcn4, the functional homolog of *S. cerevisiae* Gcn4, played a similar central role in coordinating morphogenetic and metabolic responses to amino acid starvation.

In the present study we showed that the aromatic alcohols viz. tyrosol, tryptophol, and phenethyl alcohol can induce pseudohyphae at a biologically relevant concentration. When farnesol and tyrosol were tested together in germ tube formation assays, the effect of/ farnesol was predominant over tyrosol. Most of the *C. albicans* cells remained in yeast form, although the percentage of pseudohyphae increased a little, it was statistically significant, suggesting a repression and activation of the same pathway. Farnesol predominantly acts on Ras1p-cAMP/PKA pathway (11). In this study we report for the first time that all the aromatic alcohols derepress Gcn4p in an eIF2α independent manner once their concentration builds up outside in the environment. The general amino acid control (GAAC) response pathway is known to induce pseudohyphae in pathogenic fungus *C. albicans* by activating both *CPH1* and *EFG1* (32). Here we report that in a similar mechanism aromatic alcohols induce pseudohyphae by derepressing the transcription factor Gcn4p. In doing so we have resolved the differences between the effects of farnesol, which prevents hyphae (28), and tyrosol, which enhances hyphae (9) and pseudohyphae.

Methods

Strains, media and growth condition

C.albicans wild type clinical isolate SC5314 and CAI4 (*ura3::imm434/ura3::imm434*) (14) were obtained from Dr. Alexander Johnson, University of California at San Francisco. JKC19 (*ura3::imm434*/*ura3::imm434*, *cph1::hisG*/*cph1::hisG*, *URA3::hisG*) (26), HLC52 (*ura3::imm434*/*ura3::imm434*, *efg1::hisG*/*efg1::hisG*, *URA3::hisG*) (26) and HLC54 (*ura3::imm434*/*ura3::imm434*, *cph1::hisG*/*cph1::hisG*,

efg1::hisG/*efg1::hisG*, *URA3::hisG*) (26) were obtained from Dr. Gerald R. Fink, Whitehead Institute at MIT, Massachusetts. GTC41 (*ura3::imm434/ura3::imm434, GCN4/gcn4::hisG-URA3-hisG*) (32) and GTC43 (*ura3::imm434/ura3::imm434, gcn4::hisG-URA3-hisG/gcn4::hisG*) (32) were obtained from Dr. Alistair J. P. Brown, Aberdeen UK. CR216 (*cyr1/cyr1*) was obtained from Malcolm Whiteway, McGill University, Montreal, Canada and CKY230 (*czf1/czf1*) and CKY283 (*czf1/czf1 efg1/efg1*) were obtained from Carol Kumamoto, Tufts University at Boston.

YPD medium (10 g of yeast extract, 5 g of peptone and 20 g of glucose per liter) at 30ºC was used for routine growth and maintenance of the *C. albicans* strains. Cells were also grown in two defined media: modified glucose-phoshate-proline (mGPP) (18, 19) and synthetic defined (SD) (10). Auxotrophic mutants were supplemented with 40 µg/ml of the required amino acid or pyrimidine.

Pseudohyphal induction

Morphogenesis assays used cells grown in YPD at 37 °C to an OD 600 of 0.5 and then treated with 10mM 3-Amino Triazole (3AT) or 100µM phenethyl alcohol, tyrosol, or tryptophol for 2 hours. *C. albicans* cells were stained with 0.1% calcofluor white. Cell and colony morphology were analyzed by fluorescence microscopy (Steve Harris's).

Farnesol – Tyrosol competition assays.

C. albicans cells from stationary phase were transferred to mGPP (10mM GlcNAc in GPP (19) media with or without farnesol or tyrosol at 37 ºC for 4 hours. With no additions, these are germ tube inducing conditions. The final concentration of methanol was never greater than 1%. The assays were conducted in 25-ml Erlenmeyer flasks

using *C. albicans* inoculum which had been stored at 4°C in 50mM potassium phosphate buffer (pH 6.5). The cells were added in ~25µl aliquots to prewarmed (37°C) assay medium to give a final cell density of 10^7 cells/ml. The flasks were shaken on a New Brunswick Scientific G2 shaker at 37°C and 225rpm for 4hrs and examined for percent germ tube formation (GTF) by phase-contrast microscopy at different time points. At time zero, the inoculated cells were >98% undifferentiated with 0% germ tubes and 0% to 2% budding yeasts. The phenethyl alcohol was purchased from Aldrich Chemical Co., Milwaukee, WI; tyrosol from Avocado Research Chemicals, Ltd., Heysham, United Kingdom; and tryptophol from TCI-EP, Tokyo, Japan. These chemicals were prepared as 100mM stock solutions in methanol.

Student's t test was done for statistical analysis in each farnesol treated group comparing no tyrosol treatment to varying levels of tyrosol concentration (20µM – 80µM levels). *P-values* < 0.05 were considered statistically significant.

Western blot analysis.

Total cell lysates were prepared as described in Atkin et al. (2). The proteins in these lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10% gels, nitrocellulose membranes), followed by Western blotting. The Western blots were probed with Gcn4p (FL-281), rabbit polyclonal IgG and/or Anti-phospho-eIF-2 α ($pSer^{52}$), as described in Atkin et al. (2) and Bucci and Wente (8), respectively. Act1p was used as a loading control. The primary antibodies were detected with HRP conjugated secondary antibodies using the ECL Western Blotting Substrate (Pierce Biotechnology) on a Kodak Biomax XAR film.

Results

Aromatic alcohols induce pseudohyphae in C. albicans

We studied the morphology of *C. albicans* in the presence of exogenous aromatic alcohols. Resting phase cells were inoculated in YPD media at 37 ºC. After the yeast phase cells had reached 0.5 O.D. Phenethyl alcohol, tyrosol, or tryptophol were added to a final concentration of 100μM. After 2 hours the cells were stained with calcofluor white and cell morphology was evaluated by microscopy. These conditions were chosen to duplicate those of Tripathi et al (32) except that we treated the cells with aromatic alcohols instead of 3-amino triazole (3AT). Calcofluor white stains the chitin ring and is effective in differentiating hyphae and pseudohyphae (32). In the case of hyphae, the chitin ring appears in the filament whereas in case of pseudohyphae the chitin ring is at the constricted neck (32). We found that 100μM phenethyl alcohol, tyrosol, or tryptophol induced pseudohyphae (Fig. 1.1a). No pseudohyphae were observed with 10 or 20 μM of the aromatic alcohols. As a positive control for hyphae, we added 2.5 mM GlcNAc to the same actively growing cells (YPD at 37 ºC) and after 2 hours the added GlcNAc had induced hyphae (Fig. 1.1a); cells growing in YPD without additions remained in the yeast form at 37 ºC (Fig. 1.1a).

Aromatic alcohols induce pseudohyphae by derepressing Gcn4p

The accumulation of high levels of fusel alcohols sends a nitrogen starvation signal to *S. cerevisiae* cells, thus inhibiting eukaryotic translation initiation factor 2B (eIF2B), which in turn inhibits general protein synthesis and derepresses Gcn4p (17, 29). Previously we showed that fusel alcohol production in *C. albicans*, like that in *S. cerevisiae* (16) is

dependent on available nitrogen sources (15). In the presence of preferred nitrogen sources like ammonia, *C. albicans* cells inhibit the metabolism of aromatic amino acids so that fusel oil production is reduced whereas in the presence of poor nitrogen sources like proline, fusel oil secretion is much higher (15).

We wanted to see if a similar response to fusel oils occurs in *C. albicans* cells, using western blot analysis with an antibody raised against *S. cerevisiae* Gcn4p. We treated the cells the same as we had for the morphological studies (Fig. 1.1b), and after 2 hours Gcn4p was derepressed when the cells were treated with 100μM phenethyl alcohol, Tyrosol, or Tryptophol (Fig. 1.1b). To confirm that the *S. cerevisiae* Gcn4p antibody was actually detecting *C. albicans* Gcn4p, we grew *C. albicans* strains CAF2 (*GCN4/GCN4*), GTC41 (*GCN4/gcn4*), and GTC43 (*gcn4/gcn4*) in YPD at 37 ºC and then, at an OD 600 of 0.5, added 40mM of the histidine analog 3-amino triazole (3AT). 3AT addition is a standard method for simulating amino acid starvation and thus derepressing Gcn4p (32). After 2 hours, Western blot analysis for GTC41 and CAF2 detected bands of the expected size (~ 35.3 kDa) for Gcn4p (Fig. 1.1c) but did not detect anything for the *gcn4/gcn4* mutant GTC43 (Fig. 1.1c). Together these data support the conclusion that Gcn4p is derepressed in the presence of 100μM aromatic alcohols (Fig. 1.1b).

To determine whether this elevated Gcn4p was eIF2a dependent or independent, we used an antibody that recognizes eIF2α phosphorylated at serine 52 but not the un-phosphorylated eIF2α (31). The eIF2α is just upstream of eIF2B and in *S. cerevisiae* the phosphorylated eIF2α inhibits eIF2B (17), which in turn represses Gcn4p. However, for *C. albicans* growing in YPD at 37°C with aromatic alcohols, phosphorylated eIF2α was

not detected (data not shown). This observation suggests that the aromatic alcohol activation Gcn4p is independent of eIF2α, exactly as reported by Ashe et al (1) for *S. cerevisiae* (17).

High phosphate induces both Gcn4p and pseudohyphae.

We previously showed that five strains of *C. albicans* (A72, MEN, 10261, SC5314, and CAI-4) formed pseudohyphae when grown in GPP supplemented with up to 600mM phosphate (18). Further, when tested in strain SC5314 (Fig. 1.3a), high phosphate (500mM or 600mM), in addition to causing pseudohyphal growth, caused Gcn4p expression at levels equivalent to those caused by 10mM 3-AT (Fig. 1.3b) or 100μM of tyrosol, tryptophol, or phenethyl alcohol (Fig. 1.1b). We did not determine whether this high phosphate expression of Gcn4p was eIF2 α dependent or not.

Regulatory pathways for pseudohyphae

We next addressed which regulatory pathways are necessary for the induction of pseudohyphae by aromatic alcohols. For this purpose we used mutants defective in the cAMP dependent PKA pathway (*efg1/efg1*), the MAP kinase pathway (*cph1/cph1*), or both pathways (*cph1/cph1 efg1/efg1*) as well as their CAI4 parent. These strains were tested in YPD at 37 ºC as described above and the morphology was monitored after 2 hours. As expected, the parental strain CAI4 formed hyphae in 2.5 mM GlcNAc, pseudohyphae in 100 μM Tyrosol, and stayed as budding yeasts in unsupplemented YPD, all at 37 ºC (Table 1 and Fig. 1.1). However, the three mutants which were defective in transcription factors for hyphal morphogenesis failed to produce either hyphae or pseudohyphae (Fig. 1.2), suggesting involvement of both the cAMP

dependent PKA kinase (Efg1p dependent) and MAP kinase pathways (Cph1p dependent) in aromatic alcohol induced morphogenesis. Significantly, these mutants can still undergo hyphal morphogenesis under other, non-standard conditions. The double mutant *efg1*/*efg1 cph1*/*cph1* can still filament in vivo (28) and under embedded agar conditions in vitro (7), and we found that 600mM phosphate stimulated pseudohyphal growth in both the single and double mutants for *efg1/efg1* and *cph1/cph1* (Table 1 and Fig 1.3a). In this regard, high phosphate is again acting similarly to the forkhead mutants (3). The *fκh2/ fκh2 cph1/cph1*, *fκh2/ fκh2 efg1/efg1*, and *fκh2/ fκh2 cph1/cph1 efg1/efg1* mutant strains formed pseudohyphal cells that resembled *fκh2/ fκh2* single mutants, indicating that fκh2p acts downstream of and/or parallel to the Cph1p and Efg1p transcription factors (3).

This contact-induced filamentous growth of *efg1/efg1 cph1/cphl* when in embedded agar is dependent on the Czf1 transcription factor (7a). Thus, two other strains missing Czf1 (*czf1/ czf 1* and *czf 1/ czf 1 efg1/efg1*) were tested for their ability to form pseudohyphae as triggered by aromatic alcohols or high phosphate (Table 1). Both strains still formed pseudohyphae with both stimuli (Table 1). However, the adenylcyclase mutant (*cyr1*/*cyr1*) was unable to form pseudohyphae; the cells remained as yeasts with both stimuli (Table 1).

Farnesol vs. Tyrosol

Both farnesol (19) and tyrosol (10) have been reported to be QSMs for *C. albicans* but the relationship between the two has been puzzling. The term quorum sensing was coined (14) to describe physiological differences observed between cells growing at high cell densities vs. low cell densities, with quorum sensing molecules (QSMs) being the

molecules which accumulate continuously during growth but only act once they have reached or exceeded a critical threshold concentration (14, 19). The ability of farnesol to block hyphal development at high cell densities fits these definitions (19) but the ability of tyrosol to promote hyphal development only occurs at cell densities $\leq 10^6$ per ml (10). Furthermore, Chen and Fink (9) later reported that filamentation in *C. albicans* was stimulated by tyrosol (10) but not by tryptophol or phenethylalcohol (9).

In contrast, using YPD and a standard inoculum size of 10^7 cells per ml, we observed that each of the individual aromatic alcohols induced pseudohyphal development in *C. albicans* (Figs. 1.1-1.3). The germ-tube assays of Chen et al (10) were conducted in synthetic minimum medium (SD) at pH7 using an inoculum of 10^4 or 10^5 cells per ml, which had been grown in SD at pH 4.3(10). In an effort to resolve these differences, we treated *C. albicans* with the three aromatic alcohols when growing in YPD, GPP, or SD at cell densities of 10^4, 10^5, or 10^7 cells/ml. The results for growth in SD are presented in Tables 2 and 3.

Table 2 shows that tyrosol can promote hyphal development in SD medium (pH 7.0) when using inocula grown in SD medium (pH 4.3), thus repeating the observations of Chen et al (10). However, tyrosol did not promote hyphal development in GPP or mGPP (Ghosh and Nickerson, unpublished data) and in YPD all three aromatic alcohols promoted pseudohyphal development (Figs. 1.1). We next showed that the inoculum type was also important. The data in Tables 2 and 3 differ only in how the inocula were grown; cells for Table 2 were grown in SD (pH 4.3) whereas cells for Table 3 were grown in YPD. With the YPD grown inocula, tyrosol promoted pseudohyphae

exclusively (Table 3) and identical results were obtained with tyrosol, tryptophol, or phenethyl alcohol (data not shown).

Farnesol's mode of action is predominant over Tyrosol

Several previous studies from our lab show that farnesol can block germ tube formation at concentrations as low as 5 μM (19, 28). Thus, we wanted to test if the tyrosol-induced morphogenesis can override farnesol's action or *vice versa*. We used mGPP at 37 ºC for 4 hours and quantified the percentage of budding yeasts, hyphae and pseudohyphae. In these assay conditions mGPP alone gave 90% hyphal morphogenesis while the addition of 5 μM farnesol as expected was able to block the yeast to hyphal switch (Fig. 1.4). Increasing amounts of tyrosol (10, 20, 40, 60, and 80 μM) were used along with different levels of farnesol (5, 10, and 20 μM) for a farnesol tyrosol competition assay. We found that farnesol's effect was predominant; at all concentrations of farnesol tested, most (>65%) of the *C. albicans* cells grew as yeasts regardless of how much tyrosol was present (Fig. 1.4).

Last bar, 20μM farnesol still prevented germ tube formation when added 30 minutes after the cells were inoculated. Tyrosol was present from T_0. However, at higher levels of tyrosol, i.e. 40, 60, and 80 μM, the percentages of yeast cells were a little bit lower and the percentages of pseudohyphae were a little bit higher (Fig. 1.4). The percentages of pseudohyphae always increased in the presence of high levels of tyrosol, in a small but consistent manner (Fig. 1.4). Statistical analysis revealed that the small percentage increase of pseudohyphae in the presence of tyrosol over farnesol was significant, i.e. the germ tube inhibitory effect by farnesol was partially rescued by aromatic alcohols. Similarly, the percentage of yeast cells decreased in the presence of tyrosol over

farnesol. But, the percentage of hyphae did not change much suggesting tyrosol indeed have an effect on stimulation of pseudohyphae. The statistically significant (*P value* < 0.05) data are marked with asterisk.

This set of data confirms our previous observation (Fig. 1.1) that higher levels of the aromatic alcohols induce pseudohyphae in *C. albicans*. This also suggests that farnesol and aromatic alcohols probably acts in the same pathway. Farnesol blocks GTF by repressing one or more component in Ras1-cAMP pathway and the aromatic alcohols activate the same pathway (Fig 1.2).

Discussion

We examined the role of aromatic alcohols in pseudohyphae development in *C. albicans* cells. High concentrations of aromatic alcohols (100 μM) induced pseudohyphae by a mechanism that was dependent on both the cAMP/PKA pathway and the MAP kinase pathway. Further, aromatic alcohols induced Gcn4p in an eIF2α independent manner similar to *S. cerevisiae* cells. The three aromatic alcohols were roughly equivalent in their abilities to induce both pseudohyphae (Fig. 1.1a) and Gcn4p (Fig. 1.1b). In case of *C. albicans* cells, Gcn4p is also reported to induce hyphal morphogenesis by interacting with the Ras-cAMP pathway (32). Our observations that, in the presence of aromatic alcohols *C. albicans* cells induce pseudohyphae, as well as derepressing Gcn4p, fit beautifully with the two models (1, 32) and merge them together. Derepression of Gcn4p in the presence of aromatic alcohols also makes sense because when poor nitrogen sources are being used, the buildup of fusel alcohols externally sends a signal to the cells to activate the general amino acid control (GAAC) response by activating/elevating Gcn4p. Gcn4p then will activate amino acid biosynthetic genes. In case of *C. albicans*, Gcn4p also stimulates pseudohyphal development (32). In *S. cerevisiae*, Gcn4p synthesis is induced by amino acid deprivation, purine limitation, glucose limitation, growth on a nonfermentable carbon source, high salinity, and treatment with rapamycin or MMS (17), as well as the addition of fusel alcohols (17). The feedback regulation of aromatic amino acid metabolism and its regulation in filamentous morphogenesis are described in Fig. (1.5a).

Under the assay conditions we employed we did not see any effect of these aromatic alcohols, in terms of pseudohyphal development, at lower concentrations (10 and 20

μM). These concentrations of aromatic alcohols (100μM) are biologically significant in a biofilm where the local micro-environment will be anaerobic and/or nutritionally poor. The requirement for high levels of aromatic alcohols for pseudohyphal development is consistent with the fact that *C. albicans* cells actually secrete very high levels of the aromatic alcohols. When grown under nitrogen poor conditions, *C. albicans* cells can secrete 1030, 2530, and 660 μg/g of dry weight of phenethyl alcohol, tyrosol, and tryptophol respectively at 37 ºC (15). We can expect similar or higher concentrations of the exogenous aromatic alcohols in local microenvironments such as inside a biofilm.

It is also interesting to contrast how the aromatic alcohols and high phosphate (>300 mM) cause *C. albicans* to grow predominantly as pseudohyphae (18). Both conditions derepress Gcn4p (Fig. 1.3b). The high phosphate phenotype of 100% pseudohyphae is the same as that observed by Bensen et al (3) for a mutant defective in the forkhead transcription factor. Perhaps high phosphate represses, inhibits, or inactivates the forkhead transcription factor (Fig. 5b.). This mode of action would in part explain one prominent difference between pseudohyphal induction by aromatic alcohols versus high phosphate, namely that the aromatic alcohols do not trigger pseudohyphae in the three CPH1 and EFG1 related mutants but high phosphate does (Table 1). In budding yeast, *S. cerevisiae*, Pho85p is known to have diverse roles in the regulation of cellular responses to nutrient levels (20) and negatively regulated in low phosphate conditions (25). Like budding yeast, high phosphate levels likely will activate cyclin-dependent kinase and thereby derepress Gcn4p. That would explain how high phosphate growth media triggers pseudohyphae by a very similar mechanism to aromatic alcohols stimulated pseudohyphal morphogenesis (18). *C. albicans* also secretes quorum sensing

molecule farnesol outside in the environment to negatively regulate hyphal morphogenesis (19).

The dominant theme of our work is that the three aromatic alcohols and high phosphate promote pseudohyphal growth concomitant with the activation of Gcn4p. In the process we have confirmed the observations of Chen et al (10) that within a narrow range of growth conditions (low cell densities in SD medium at pH 7.0) tyrosol can promote hyphal growth (Table 2). However, tyrosol is not a stronger inducer of hyphal growth and only effective at cell densities < 10^6/ml (H. Chen and G.R. Fink, personal communication) and thus probably should not be considered a quorum sensing molecule. Indeed, in direct competition experiments, the effects if tyrosol in promoting hyphal and pseudohyphal growth are totally overridden by only 5 μM farnesol (Fig. 4).

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Figure Legends

Figure 1.1. Aromatic alcohols induce pseudohyphae in wild type *C. albicans*.
Wild type *C. albicans* SC5314 cells were grown in YPD at 37 ºC until 0.5 O.D. and then treated with 2.5 mM N-acetyl glucosamine (GlcNAc) or 100 μM phenethyl alcohol (PEA), tyrosol (TOH), or tryptophol (TrpOH). After 2 hours, the cells were stained with calcofluor white. Photomicrographs (DIC and fluorescent) showing germ tube formation with GlcNAc treated cells whereas predominantly pseudohyphae formation with aromatic alcohol treated cells. Calcofluor white stains the chitin ring, which appears at the bud neck in pseudohyphae and inside of germ tube in case of true hyphae. B. Derepression of Gcn4p by aromatic alcohols in *C. albicans*. SC5314 cells were grown in YPD at 37 ºC till 0.5 O.D., and then treated with nothing, 100μM tyrosol (TOH), tryptophol (TrpOH), or phenethyl alcohol (PEA) respectively for 2 hours. C. CAI4 (*GCN4/GCN4*), GTC41 (*GCN4/gcn4*), and GTC43 (*gcn4/gcn4*) strains were grown in YPD at 37 ºC till 0.5 O.D., and then treated with 40 mM of 3-amino triazole (3AT) for 2 hours. The presence of Gcn4 protein is shown. Act1 levels were used as loading control.

Figure 1.2. Effect of tyrosol on morphogenesis of non-filamentous mutants. Photomicrographs showing germ tube assays for CAI4 (parent), JKC19 (*cph1/cph1*), HLC52 (*efg1/efg1*), and HLC54 (*cph1/cph1, efg1/efg1*) in the presence of 2.5 mM N-acetyl glucosamine (GlcNAc) (first column) and 100μM tyrosol (TOH) (second column) added to YPD at 37ºC after 4 hours. Representative photomicrographs are taken with a confocal microscope.

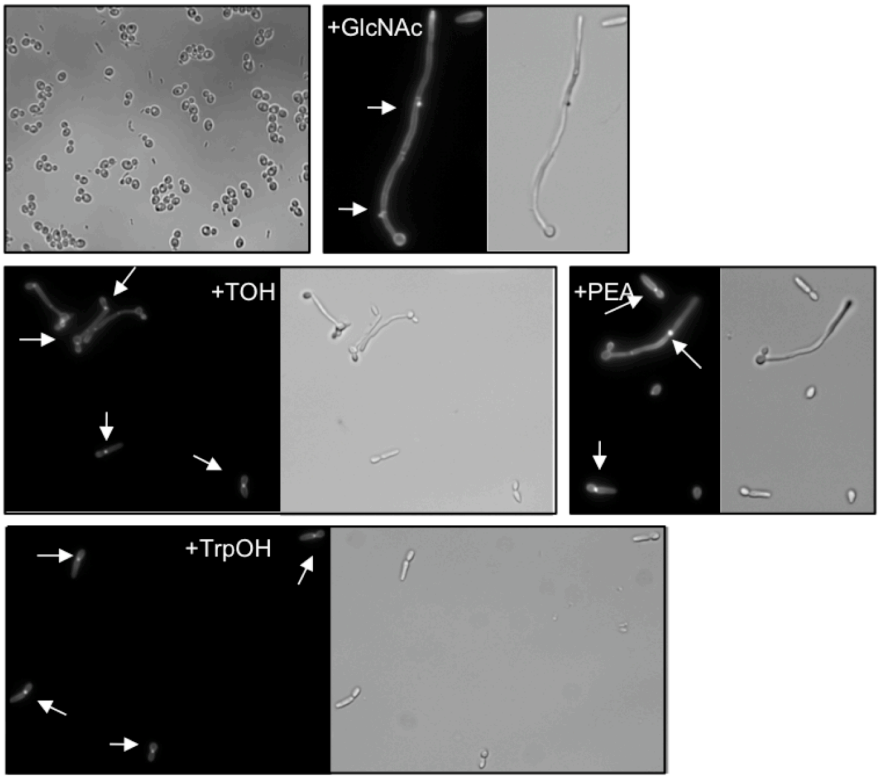
Figure 1.3. High phosphate (600mM) induces both *GCN4* and pseudohyphae. A. Four strains of *C. albicans*, CAI4 (parent), JKC19 (*cph1/cph1*), HLC52 (*efg1/efg*), and HLC54 (double mutant) were grown in both GPP and GPP with 600 mM phosphate and stained with calcofluor white. B. Western blots for wild type *C. albicans* SC5314 grown at 37°C in GPP with and without 3-amino-triazole (3AT) or high phosphate (500 or 600 nM)

Figure 1.4. Farnesol tyrosol competition in morphogenesis in *C. albicans*. The percentage (%) germ tube bioassay was conducted in GPP+GlcNAc (pH 6.5) at 37˚C with 0, 5, 10, and 20 μM farnesol (F) and 0, 10, 20, 40, 60 and 80 μM tyrosol (T). The percentages of yeasts (Y), pseudohyphae (P), and mycelia (M) were calculated after 4 hours. Student's t tests were performed for each set with the same amount of farnesol treated cells and were compared for no tyrosol added with varying level of tyrosol added. Statistically significant data (*P value* < 0.05) were marked with asterisks.

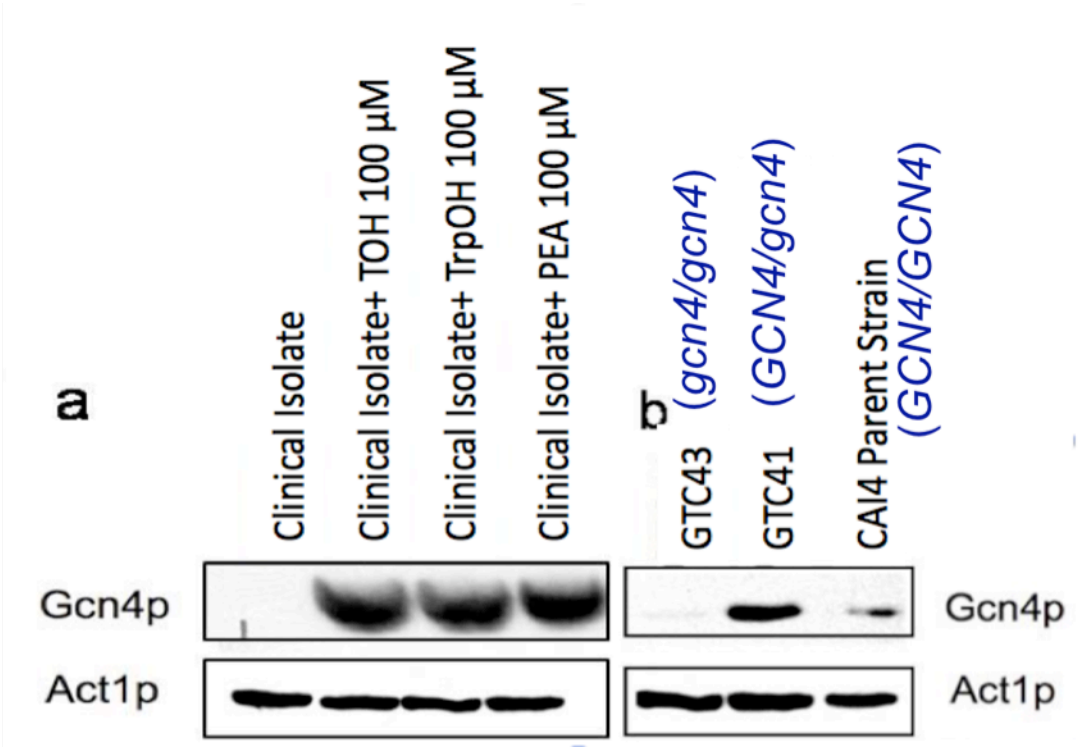
Figure 1.5. Model for feedback control of amino acid biosynthesis and morphogenesis by fusel alcohols in C. albicans. a). *C. albicans* can use the aromatic amino acids tryptophan, phenylalanine and tyrosine as cellular nitrogen sources. This results in the production of tryptophol, phenylethanol and tyrosol, collectively known as fusel oils (15). Accumulated aromatic alcohols can then derepress Gcn4p to activate amino acid biosynthetic genes. Gcn4p also has a role in hyphal morphogenesis in *CPH1* and *EFG1* dependent manner. b) Genes are in boxes; enzymes/proteins are in ellipses. The scheme is based on our findings, as well as on pathways reported for both *S. cerevisiae* and *C. albicans* by other groups.

Figures

Figure 1.1 Aromatic alcohols induce pseudohyphae in wild type *C. albicans*



Source: Suman Ghosh, 2009. Physiology, Regulation and Pathogenesis of nitrogen metabolism in the opportunistic fungal pathogen *Candida albicans*.



Source: Suman Ghosh, 2009. Physiology, Regulation and Pathogenesis of nitrogen metabolism in the opportunistic fungal pathogen *Candida albicans*.

Figure 1.2 Effect of tyrosol on morphogenesis of non-filamentous mutants

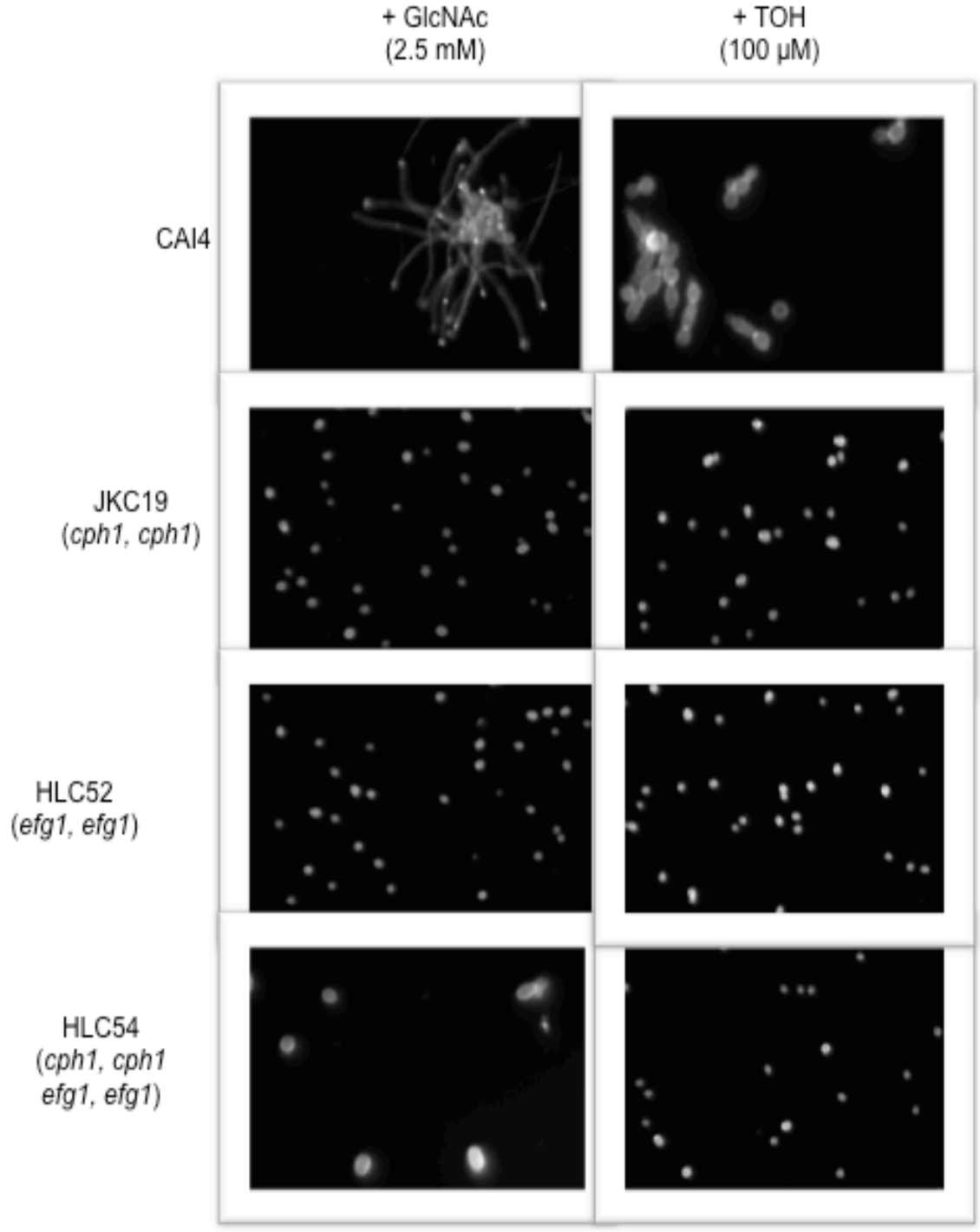
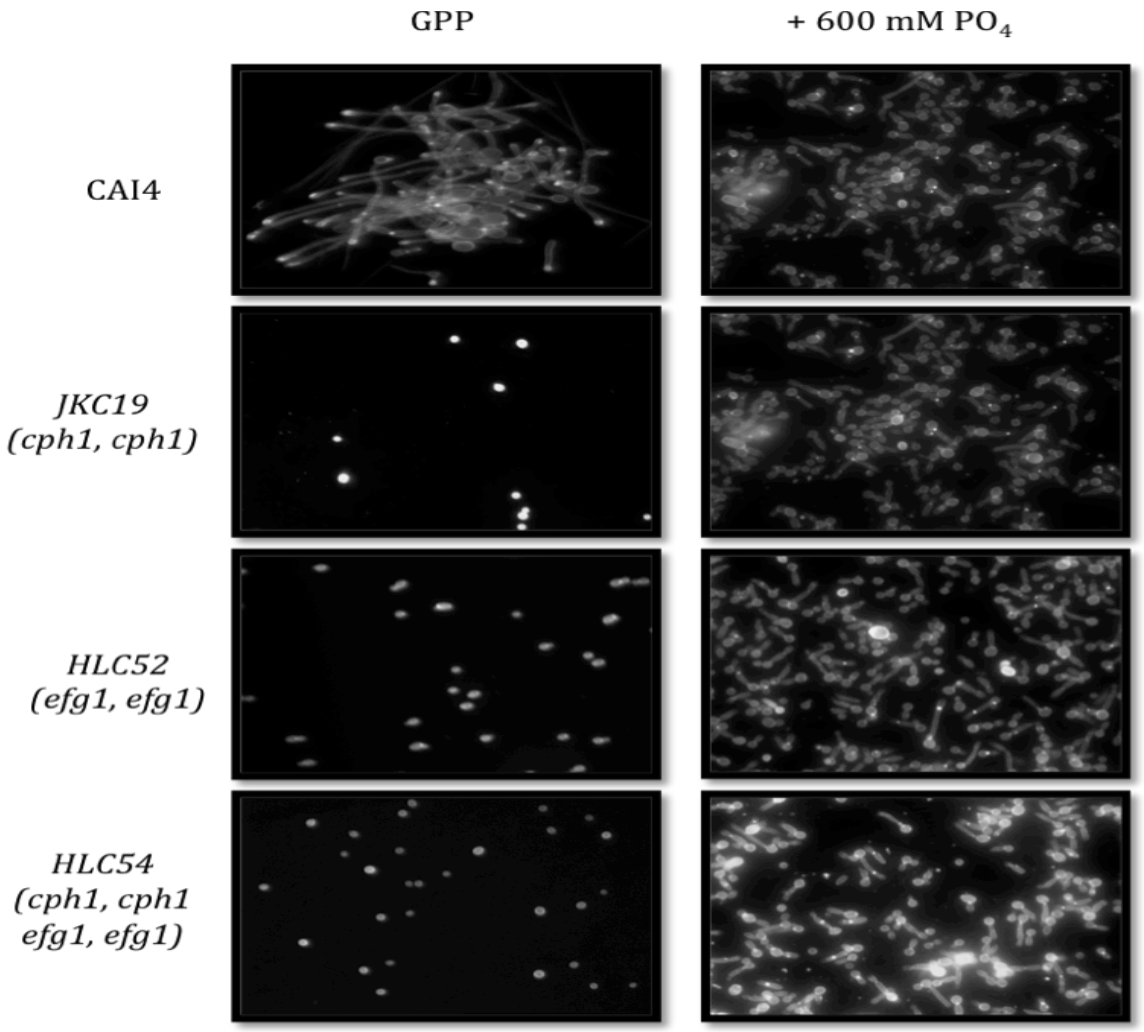


Figure 1.3 High phosphate (600mM) induces both *GCN4* and pseudohyphae

a



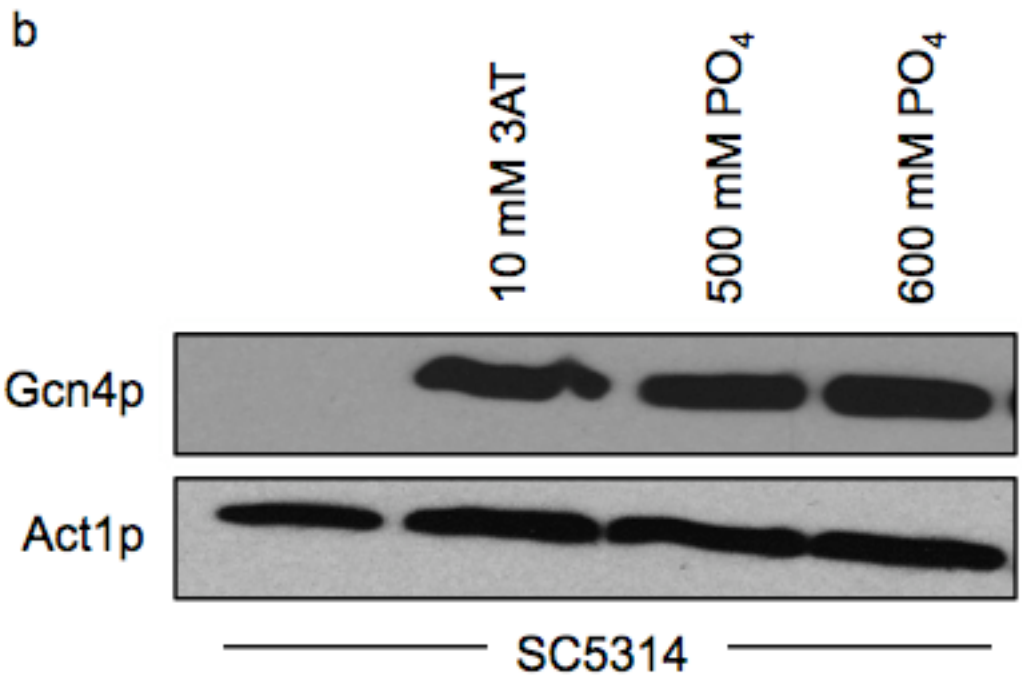
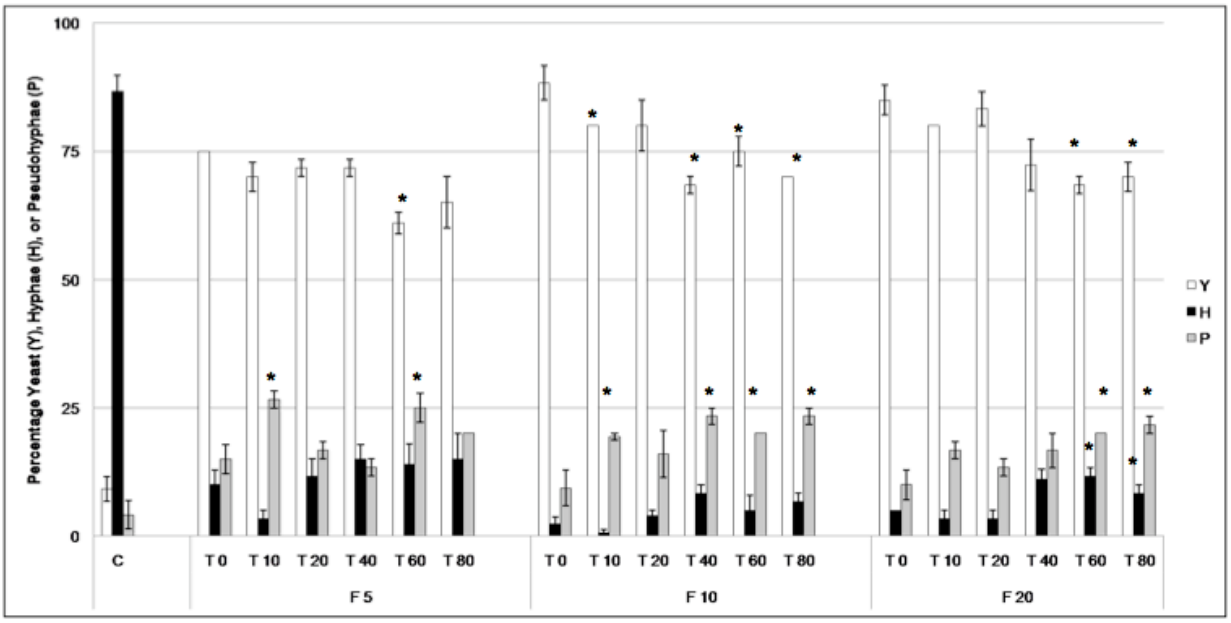

b
10 mM 3AT
500 mM PO4
600 mM PO4
Gcn4p
Act1p
SC5314

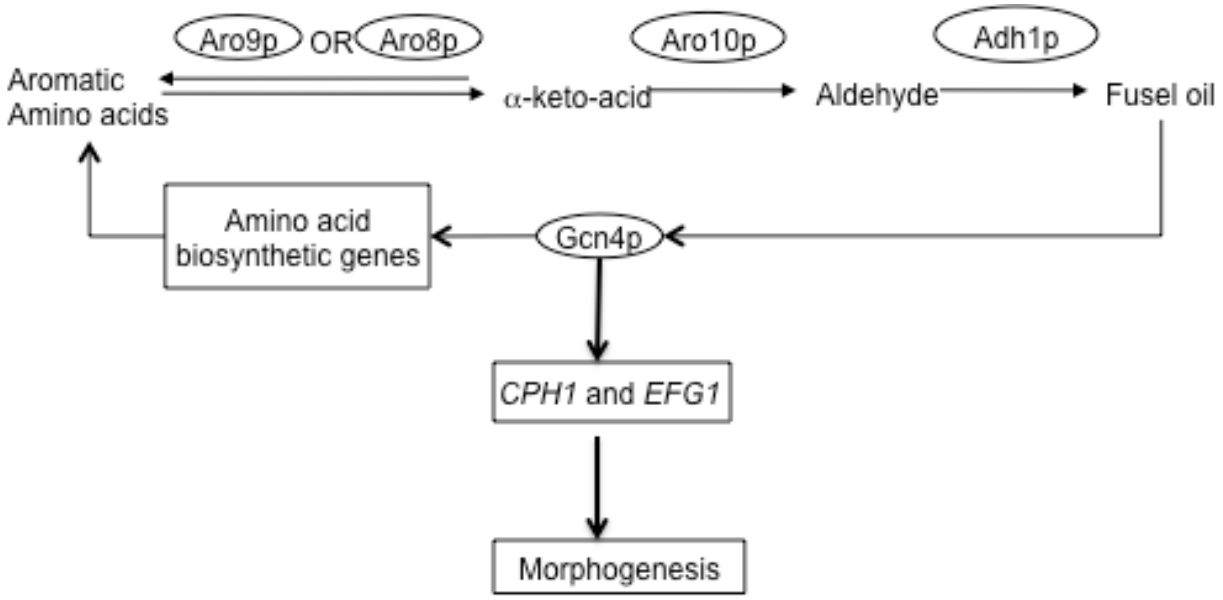
Figure 1.4 Farnesol tyrosol competition in morphogenesis in *C. albicans*



Source: Suman Ghosh, 2009. Physiology, Regulation and Pathogenesis of nitrogen metabolism in the opportunistic fungal pathogen *Candida albicans*.

Figure 1.5 Model for feedback control of amino acid biosynthesis and morphogenesis by fusel alcohols in *C. albicans*

a



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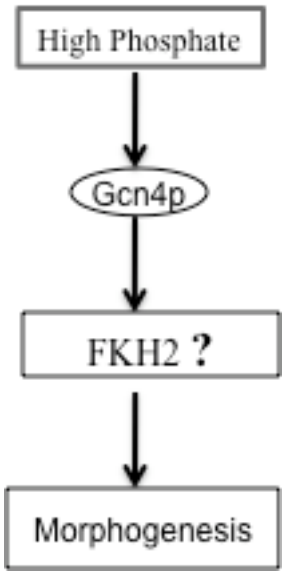

Tables

Table 1.1 Roles of transcription factors in pseudohyphal development

C. albicans strain		Morphology		
		YPD & tryosol[a]	GPP & phosphate	YPD & phosphate
SC5314	wild type	pseudohyphae	pseudohyphae	pseudohyphae
CAI-4	parent	pseudohyphae	pseudohyphae	pseudohyphae
JKC19	cph1/cph1	yeasts	pseudohyphae	pseudohyphae
HLC52	efg1/efg1	yeasts	pseudohyphae	pseudohyphae
HLC54	cph1/cph1 efg1/efg1	yeasts	pseudohyphae	pseudohyphae
CKY230	czf1/czf1	pseudohyphae	pseudohyphae	pseudohyphae
CKY283	czf1/czf1 efg1/efg1	pseudohyphae (20-30%)	pseudohyphae (100%)	pseudohyphae
CR216	cyr1/cyr1	yeasts	yeasts	*

[a]/ Cells grown for 2 hrs at 37°C in designated medium supplemented either with 100μM Tyrosol or to a final phosphate concentration of 600mm. All experiments used an initial cell density of 10^7 cells/ml.

* Did not test this strain in YPD with high phosphate

Table 1.2 Effects of inoculum size and tyrosol on cell morphology in SD medium pH 7.0

Tyrosol (μm)	Inoculum size (cells/ml)[a]		
	10^4	10^5	10^7
0	Y [50-60%] +H [50-40%]	H [60%] +Y [40%]	H [70%] + Ψ [30%]
20	H [90%] +Ψ [5%] + Y[5%]	H [70%] + Ψ [20%] Y [10%]	H [70%] + Ψ [30%]
50	H [60%] +Y [40%]	H [60%] + Ψ [20%] Y [20%]	H [80%] + Ψ [20%]
100	H [70%] + Ψ [20%] + Y [10]	H [60%] + Ψ [30%] Y [10%]	H [70%] + Ψ [30%]
200	H [70%] + Ψ [30%]	H [70%] + Ψ [20%] Y [10%]	H [70%] + Ψ [30%]
500	H [60%] + Ψ [30%]+ [Y [10%]	H [80%] + Ψ [20%]	H [60%] + Ψ [40%]

[a]/ Cells incubated for 2.5 hrs at 37°C in synthetic defined (SD) medium at pH 7.0 using as inocula cells which had been grown in SD pH 4.3 (10).

[b]/ Y = yeasts; H = hyphae; Ψ = pseudohyphae

Table 1.3 Effects of inoculum size and type on cell morphology in SD medium pH 7.0

Tyrosol (μm)	Inoculum size (cells/ml)[a]		
	10^4	10^5	10^7
0	Y [20-30%]+H [20%]+ Ψ [50-60%]	H [50%]+Y [50%]	H [70%H]+ Ψ [10%]+Y[20%]
20	Ψ [60%] + H [20%] + Y [20%]	Ψ [60%] + H [20%] + Y [20%]	Ψ [80%] + H [20%]
50	Ψ [80%] + H [10%]+Y 10%]	Ψ [80%] + H [10%]+Y 10%]	Ψ [80%] + H [20%]
100	Ψ [90%]+ H [10%]	Ψ [90%] + H [10%]	Ψ [100%]
200	Ψ [90%] + H [10%]	Ψ [90%] + H [10%]+	Ψ [100%]
500	Ψ [90%] + H [10%]	Ψ [95%] + Y 5%]	Ψ [100%]

[a]/ Cells incubated for 2.5 hrs at 37°C in synthetic defined (SD) medium at pH 7.0 using as inocula cells which had been grown in YPD pH 7.0.

[b]/ Y = yeasts; H = hyphae; Ψ = pseudohyphae

CHAPTER 2 - Exploring a Novel Modification of Histones In *Candida albicans*

Swetha Tati, Cortney M. Sostrich, Nandakumar Madayiputhiya, Kenneth W. Nickerson

Abstract

Candida albicans is an opportunistic fungal pathogen in humans. It is a polymorphic fungus: it can live as yeast, filamentous, or pseudohyphal forms. Hyphal and pseudohyphal forms of *C.albicans* are pathogenic. *C.albicans* is a natural biotin auxotroph. Biotin is required for cell growth and fatty acid metabolism. Biotin is also used as a cofactor for several carboxylases such as Acetyl-CoA, Pyruvate, and Methylcrotonyl–CoA carboxylase. In addition, we have discovered that biotin is also used to modify histones in *C. albicans.* During this study we observed two histones - H2B and H4 - being biotinylated in *C.albicans*. Residues K8, K11 in histone H4 and K17, K18 and K31 in histone H2B are biotin attachment sites in *C.albicans*. We report roughly equivalent levels of histone biotinylation under several growth conditions including aerobic and anaerobic growth and yeast and hyphal growth. So far the role of histone biotinylation in *C.albicans* is unknown.

Introduction

Candida albicans is a common fungal human pathogen. It is an opportunistic organism, which lives in several morphological forms such as yeast, hyphal, pseudohyphal and chlamydospore. The morphological switch between yeast, hyphal and pseudohyphal forms are important for pathogenicity of *C.albicans. C.albicans* is naturally auxotrophic for biotin. In mammals, five different carboxylases: acetyl-coenzyme A (CoA) carboxylase (I and II isoforms), pyruvate carboxylase, methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase are known to be biotin dependent. Biotin is a coenzyme for five enzymes in yeast as well as the t-RNA binding protein [14] and biotin is required for the growth and metabolism of the organism. The five enzymes in yeast which require biotin are: Acetyl Co-enzyme carboxylase (ACC), both cytoplasmic and mitochondrial forms [18], two isoforms of pyruvate carboxylase (PYC 146KD) which play an important role in gluconeogenisis [21, 2, 20], and a urea-degrading enzyme – urea amidolyase (DUR 1,2) [7]. These five biotin-requiring enzymes all (> 200KD) are essential for growth in yeast. Methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase are absent in yeast and probably in all of the Hemiascomycetes [13]. Biotin is also incorporated into a non-essential 43KDa tRNA binding protein, Arc1p [10]. Biotin protein ligase (BPL) is an essential enzyme that covalently attaches biotin to biotin-requiring enzymes such as the carboxylases. Important metabolic pathways such as gluconeogenisis and fatty acid biosynthesis will be rendered ineffective by the loss of yeast BPL enzyme.

In humans, one of the post-translational modifications of histones is the covalent attachment of the vitamin biotin [8,9] catalyzed by the enzymes Biotinidase and Holo carboxylase synthetase (HCS). The biotin-lysine adduct is referred to as biocytin. The enzyme biotinidase removes biotin from biocytin and makes it available for reuse by other enzymes like Holocarboxylase synthetase, which catalyzes the ATP-dependent attachment of biotin to apocarboxylases or histones via the reactive intermediate bio-5'-AMP. The following sites are biotinylated in human histones: K9, K13, K125, K127 and K129 in histone H2A [4], K4, K9 and K18 in histone H3 [3], and K8 and K12 in histone H4 [17]. Biotinylation of histones plays a role in the regulation of gene expression [16], cell proliferation [19,12], and the cellular response to DNA damage [11, 16]. In this study we report biotinylation of histones in *C.albicans*, which is a natural auxotroph for biotin. We found biotinylation of Histones H2B and H4. The sites that are biotinylated in *C.albicans* are K17, K18, and K31 in histone H2B as well as K8 and K11 in histone H4. We also show the stimulatory role of biotin in the kinetics of germ tube formation in *C.albicans* by conducting germ tube formation assays with varying concentrations of biotin including biotin-starved cells of *C.albicans*.

Methods and Materials

Strains, media and growth condition

The clinical isolate of *C. albicans* SC5314 was provided by Dr. Alexander Johnson of University of California at San Francisco.

YPD medium (10g of yeast extract, 5g of peptone and 20g of glucose per liter) at 30°C was used for growth and maintenance of *C.albicans*.

Germ tube formation assays were performed using freshly, YPD grown, stationary phase cells in 25ml flasks with 5ml of pre-warmed (37°C) GPP medium (14) with and with out filter sterilized biotin (Stock: 12μM) to give a final cell density of 10^7 cells/ml. The flasks were shaken on a New Brunswick Scientific G2 shaker at 37°C and 225rpm for 3hrs-4hrs and examined for the percentage of Germ Tube Formation (GTF) by phase contrast microscopy at various times. At time zero, the inoculated cells were > 98% undifferentiated with 0% germ tubes and 0 to 2% yeasts.

Anaerobic cells were grown using a Gaspak jar in YPD medium at 30°C.

Biotin-starved cells were grown in defined medium such as GS (1.5% glucose, 2gms KH_2PO_4, 1gm $(NH_4)_2SO_4$, 0.1gm $MgSO_4$.7H2O, 50mg of $CaCl_2.2H_2O$ per liter of double distilled water, pH 5.6), mGS (1.5% glucose, 1% peptone 2gms KH_2PO_4, 1gm $(NH_4)_2SO_4$, 0.1gm $MgSO_4$.7H2O, 50mg of Cacl2.2H2O per liter of double distilled water, pH 5.6), GSU (1.5% glucose, 2gm KH_2PO4, 0.1% of filter sterilized Urea, 0.1gm $MgSO_4.7H_2O$, 50mgs of $CaCl_2.2H_2O$ per liter of double distilled water, pH 5.6).

Histone extraction

Cells were grown in 10ml of YPD medium overnight. 1X 10^7cells from the overnight cultures were taken and inoculated into a 100ml YPD medium grown up to mid-log phase. {This protocol is a modified version of histone extraction method suggested by Abcam Inc. Cambridge, MA, USA}. The cells were harvested at 5000rpm for 5 minutes. Cell pellets (0.5gm) were treated with 1ml of spheroplasting buffer [1M Sorbitol, 2.5mM Tris-HCL, 100mM Di-thio-tritol (DTT), 100mM PMSF (phenylmethanesulfonylfluoride), 25mM EDTA, 0.01%(v/v) β-mercaptoethanol] and incubated at room temperature for 1 hour. The cells were then centrifuged at 5000rpm, 4°C for 10mins. Yeast cell-wall-degrading enzymes were prepared in spheroplasting buffer 2.5mg/1ml of Zymolyase (20T) and Lyticase 7.5mg/1ml. Cell pellet was treated with 1ml of spheroplasting buffer with enzymes for 3hrs-4hrs at 30°C with gentle shaking. Spheroplasting buffer treated cells were centrifuged at 3000rpm for 10mins at 4°C. Spheroplasts were treated with 2ml of Triton Extraction Buffer (TEB: PBS containing 0.5% Triton X 100 (v/v), 2mM phenylmethylsulfonyl fluoride (PMSF), 0.02% (w/v) NaN3) on ice for 10 minutes with gentle stirring. To remove and discard the supernatant, cells were centrifuged at 3000rpm for 10 minutes. Cells were then washed and centrifuged at 3000rpm for 10 minutes in half the volume of TEB. Pellet was resuspended with 0.2N HCL. Histones were extracted overnight at 4°C with gentle shaking**.** Crude histones were collected by centrifuging at maximum speed, 4°C for 10 minutes. Protein content was determined using Bradford assay. Crude histones were also extracted using extraction buffer in place of 0.2N HCL from an Active-Motif mini histone extraction kit (Active- motif. Inc. CA -92008), the crude histone extract was

comparable from both the Acid histone extraction and Active-motif kit methods as judged by western blot [Fig. SF-2].

Histones were separated using SDS PAGE (4-20% BioRad TGX gels), and as stained with commasive blue R-250.

In–gel Trypsin Digestion was performed using Dr. Lauren Jensen and John Lapek protocol. In addition to trypsin, the enzyme Endopeptidase glu C was used to treat histone peptide in a ratio of (3(trypsin): 1(glu C)) and incubated for 3 hours at 30ºC to activate gluC and then overnight at 37ºC to activate trypsin.

2D LC Method

Instrumentation and Chromatographic conditions

Fully automated on-line one-dimensional LC/MS/MS was performed with an ultimate 3000 Dionex MDLC system (Dionex Corporation, USA) integrated with a nanospray source and LCQ Fleet Ion Trap mass spectrometer. (Thermofinnigan, USA).

The method was included with on–line sample pre concentration and desalting using a monolithic C 18-trap column (Pep Map, 300 um I.D, 5 um, 100A, 1mm monolithic C18 column). Loading of the sample on the monolithic trap column was conducted using a micro pump at a flow rate of 300 nl/min. The desalted peptides were then eluted and separated on a C 18 Pep Map, 75 um I.DX15 cm, 3 um, 100A column applying an acetonitrile gradient (ACN plus 0.1% formic acid, 90 minute gradient including 25 minutes re-equilibration at a flow rate of 300 nl/min and introduced into mass spectrometer using the nano spray source. The LCQ Fleet mass spectrometers were operated with the following parameters: nano spray voltage (2.0) kV), heated capillary temperature 200C, full scan m/z range) 400-2000). The LCQ was operated in data

dependent mode with 4 ms/ms spectra for every full scan, 5 micros can averaged for full scans and ms/ms scans, 3 m/z isolation width for ms/ms isolations and 35% collision energy for collision induced dissociation. Dynamic exclusion was enabled with exclusion duration of 1 min.

The acquired MS/MS raw data was searched against *C.albicans* protein sequences (NCBI) using MASCOT (Matrix Sciences, UK) bioinformatics software to identify the proteins.

Western blot analysis was performed with total crude histone extract by taking total cell lysate, prepared as described by Atkin et al [1]. The histones were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, BioRad TGX 4-20% gels), followed by western blotting. The western blots were probed with anti-biotin HRP conjugate antibody [Sigma]. The primary antibodies were detected with HRP-conjugated secondary antibodies using the ECL Western Blotting Substrate (Pierce Biotechnology) on Kodak Biomax XAR film.

Results

Biotin increases germ tube kinetics in C.albicans

We studied the morphology of *C.albicans* by varying biotin concentrations. Resting phase cells which had been grown in YPD were inoculated to in pre-warmed GPP media with 0, 100nM, 400nM, and 1μM filter-sterilized biotin at 37°C. Every half an hour, cell morphology was evaluated by microscopy [Fig 2.1]. We found that high amounts of biotin (400nM**,** 1μM) gave rapid germ tube formation. Cells were able to reach 100 % germ tubes within 2 to 2 and half hours. Under normal conditions, 100nM of biotin took 4 hrs to reach 100% germ tubes in GPP medium [Fig. 2.1]. However, when germ tube formation was triggered by GlcNAc medium (2.5mM N-Acetyl Glucosamine, 12.5mM imidazole, 3mM MgSO4), varying the biotin concentrations did not show significant change in the germ tube kinetics [Fig.2-2]. Thus, biotin was required for germ tube formation under growing conditions [Fig. 2.1] but not under non-growing, cell differentiation [Fig.2.2]

Histones are biotinylated in Candida albicans

Candida albicans is a natural biotin auxotroph. Thus, we next wanted to address whether *C.albicans* is using biotin to modify its histones and if so to identify them. For this purpose, we isolated histones from the wild type strain of *C.albicans* SC5314 grown at four different growth conditions: a rich medium (YPD), a defined medium (GSB), mGSB (GSB with 0.1% peptone), and GPP. Biotin (the B in GSB) is present at 100nM in both GSB and mGSB. Histones were also isolated from SC5314 cells grown in YPD medium as yeasts (30°C) and hyphae (37°C), as well as both aerobically and

anaerobically (30°C). Western blot analyses were then performed with the crude histone extracts using anti biotin HRP conjugated antibody. These studies revealed that histones from all the crude histone preparations showed broad bands between 10 and 15kDa. Thus, *C.albicans* histones were biotinylated at all growth conditions tested [Fig. 2.6].

Histone H2A and H4 were biotinylated in Candida albicans

To find out which histones (H1, H2A, H2B, H3 or H4) are biotinylated and the sites of biotinylation in *Candida albicans,* we performed mass spectrometry (LC/MS/MS). Crude histone lysates (H1: 18471Da, H2A: 13,824Da, H2B: 14,090Da, H3: 15,344Da and H4: 11,621Da) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), followed by in-gel trypsin and endoproteinase Glu-C digestion of proteins. Trypsin cuts the peptide bond specifically at the carboxyl side of the basic amino acids arginine and lysine. Endoproteinase Glu-C, also called proteinase V8, specifically cuts at aspartic acid and glutamic acid. The digested peptides were run through the LC/MS/MS. We found two histones to be biotinylated: H2B and H4. In particular K17, K18 and K31 in H2B and K8 and K11 in H4 were detected as biotin attachment sites with a single biotin molecule [Fig 2.3, Fig 2.4]. We did not find any biotinylated attachment sites in H2A [Fig 2.5]

Germ Tube formation assays with biotin-starved SC5314 cells

Candida albicans strain SC5314 cells were grown in media without biotin such as GS, or mGS. Germ tube assays were performed with GS and mGS grown cells in GPP and N-Acetyl Glucosamine with and without varying amounts (100nM, 400nM) of biotin. After 2 hours of incubation, the cultures with added of biotin showed higher percentages of

germ tube formation (> 85%) [Table 2.1, Table 2.2]. Cells in media without biotin showed fewer germ tubes (1-5%) in GS than in mGS grown cells (>15%) [Table 1, Table 2]. The peptone in mGS might be contaminated with biotin.

Biotinylation of histones with biotin-starved cells

Crude histones were extracted from *C. albicans* SC5314 cells grown in defined media GS, mGS and GSU, without biotin. Western blot analysis was performed with crude histone extract using anti-biotin peroxidase antibody. Results from this experiment showed that there was no biotinylation of histones with any of the biotin-starved cells. Similarly, histones were extracted from wild type *Saccharomyces cerevisiae* strain BHY10 and then blotted against anti-biotin antibody were no detectable bands [Fig. 2.7]. This negative result for *Saccharomyces cerevisiae* agrees with prior negative results obtained by Profs Zemplini and Bi.

Discussion

In this report we demonstrate the role of biotin in germ tube kinetics of *Candida albicans*. Concentrations of biotin of at least 400nM, compared to standard biotin concentration of 100nM in GSB, 100nM in GPP, – accelerated germ tube formation in growth medium (GPP), but not in a non-growth medium (GlcNAc). The higher the concentration of biotin, the faster the formation of germ tubes in *C.albicans* (4μM in Lee's medium). The high concentration of biotin in Lee's medium (4μM) likely contributes to its ability to stimulate germ tube formation. *C.albicans* is a natural biotin auxotroph; it has to get biotin from the host or the environment**.** Biotin availability in the host depends on the type of food they consume. The other source for biotin is intestinal bacteria that produce an excess of the daily requirement for humans.

We show a novel post-translational histone modification in the form of biotinylation. Histones H2B and H4 are biotinylated in *C.albicans.* Biotin targets ε-amine groups of lysine side chains of protein molecules. Lysines at K8 and K11 in histone H4 as well as K17, K18, and K31 in histone H2B are the sites of biotinylation in histones in *C.albicans* according to our MS data. We also examined peptides from histone H2A along with H2B and H4. However, H2A did not have any biotin attachment to lysines. This absence of H2A biotinylation emphasizes the specificity of the H2B and H4 biotinylation. Similarly, it is unlikely that histone H1 is biotinylated because the broad western band (Fig.2.7) covers only 11-15KDa whereas histone H1 is 18.47KDa. Biotin-starved *C.albicans* cells (GS, mGS, GSU) did not show the presence biotinylated histones. This absence was especially dramatic for cells grown in GSU, which has urea

as a nitrogen source; thus, needing the biotin containing enzyme urea amidolyase. So, the cell has to make a choice of biotinylating either histones or urea amidolyase. We did not detect any biotin containing histones in these cells. SC5314 cells grown in media containing biotin and urea as a nitrogen source (GSUB) showed biotin containing histones (Fig.2.7).

However, there is no homologue for the holocarboxylase synthetase gene in *C.albicans*. All it has is the Biotin protein ligase (BPL) enzyme, which is involved in the biotinylation of carboxylases. BPL is the only enzyme present in yeast to modify proteins via biotinylation. So, it appears that BPL is the only possible enzyme for the post-translational modification of histones in *C.albicans*.

Studies thus far in higher eukaryotes like humans and Drosophila show that histones are biotinylated. It is known that enrichment of biotinylated histones occurs in transcriptionally silent regions of chromatin in chicken erythrocytes [14]. According to Dr. Janos Zempleni (in USDA website: Regulation of biotinylation of histones in *S. cerevisiae*) and based on our data on strain BHY10, biotinylation of histones was not seen in *S. cerevisiae* - a close relative of *C.albicans*. However, the BPL enzyme in *C. albicans* is only 59% similar to that in *S. cerevisiae* based on protein sequence alignment from the Waterman-Eggert local alignment (Mobyle@Pasteur) program. This large divergence between the *C.albicans* and *Saccharomyces cerevisiae* BPL sequences occurs despite the fact that BPL is considered to be a highly conserved enzyme [10]. However, that large divergence might be a reason we observed biotinylation of histones in *C.albicans* but not in *S. cerevisiae*. Further study of these modifications is required to find significance of biotinylation of histones in *C. albicans*.

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Figure Legends

Figure 2.1. Biotin increases the germ tube formation kinetics in *C.albicans.* The percentage germ tube bioassay was conducted in GPP (pH 6.5) at 37°C with 100nM, 400nM, and 1µM of biotin. The percentage of germ tubes was observed every half an hour. Inoculum was grown in rich YPD medium until they reach stationary phase.

Figure 2.2. The percentage germ tube bioassay was conducted in GlcNAc medium at 37°C without biotin and with biotin concentration of 100nM, 400nM, and 1µM. The percentage of germ tubes was observed every half an hour. Inoculum was grown in rich YPD medium until they reach stationary phase.

Figure 2.3. MS/MS Fragmentation of biotinylated protein **MAPKAEKKPASKAP** found in CAWT_00976, l CAWG_00976 l *C. albicans* WO1 histone H2B.2 (131aa). Crude histone extracts of SC5314 cells grown in rich YPD medium were loaded in 4-20% gradient BioRad TGX gels. Protein bands between 10 and 15KDa were cut and processed using In-gel extraction protocol for LC/MS/MS.

Figure 2.4. MS/MS Fragmentation of **MSGTGRGKGGKGLGKGGAKR** found in CAWT_00976, l CAWG_00976 l *C. albicans* WO1 histone H4 (106aa). Crude histone extracts of SC5314 cells grown in rich YPD medium were loaded in 4-20% gradient BioRad TGX gels. Protein bands between 10 and 15KDa were cut and processed using In-gel extraction protocol for LC/MS/MS.

Figure 2.5. MS/MS Fragmentation of **VTIAQGGVLPNIHQSLLPAK** non-biotinylated protein histone H2A found in CAWT_00976, l CAWG_00976 l *C. albicans* WO1. Crude histone extracts of SC5314 cells grown in rich YPD medium were loaded in 4-20% gradient BioRad TGX gels. Protein bands between 10 and 15KDa were cut and processed using In-gel extraction protocol for LC/MS/MS.

Figure 2.6 Western blots performed with histones from a wild type *C.albicans* strain SC5314 grown at 30ºC, 37ºC aerobically and anaerobically in YPD medium. SC5314 was also grown in different defined medium, GSB (Glucose Salts and Biotin), mGSB (modified Glucose salt, Biotin and peptone) and defined media GPP.

Figure 2.7: Western blot performed with histones from SC5314 cells grown in a defined medium without biotin, GS, mGS, GSU, GSUB and in a rich medium YPD**.** Wild type strain of *Saccharomyces cerevisiae* BHY10 was grown in YPD medium.

Figures

Figure 2.1 Biotin increases germ tube kinetics in *C. albicans*

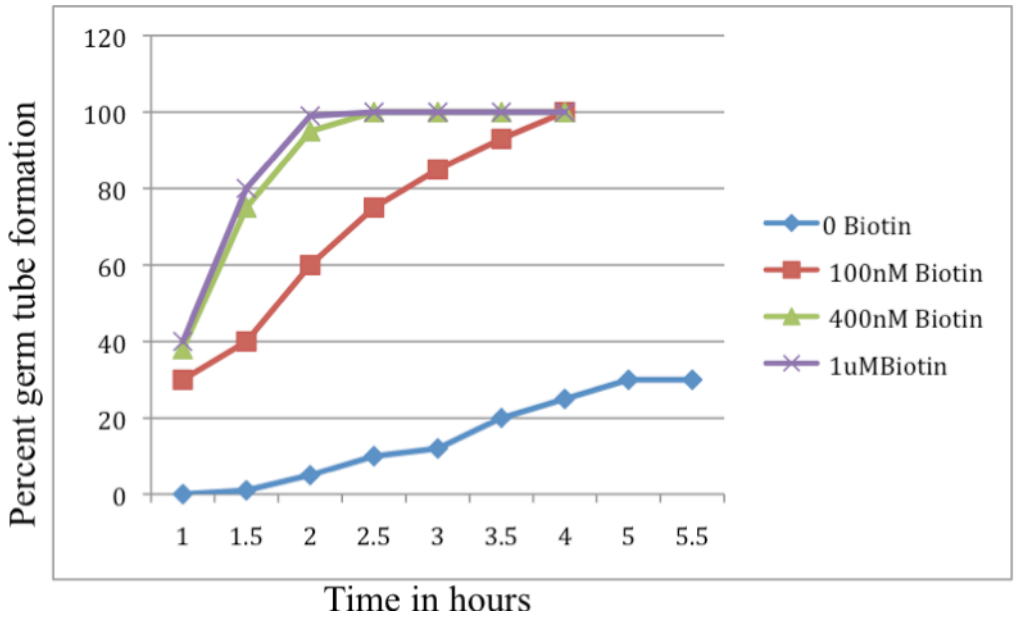


Figure 2.2 Germ tube kinetics of *C. albicans* in GlcNAc with biotin

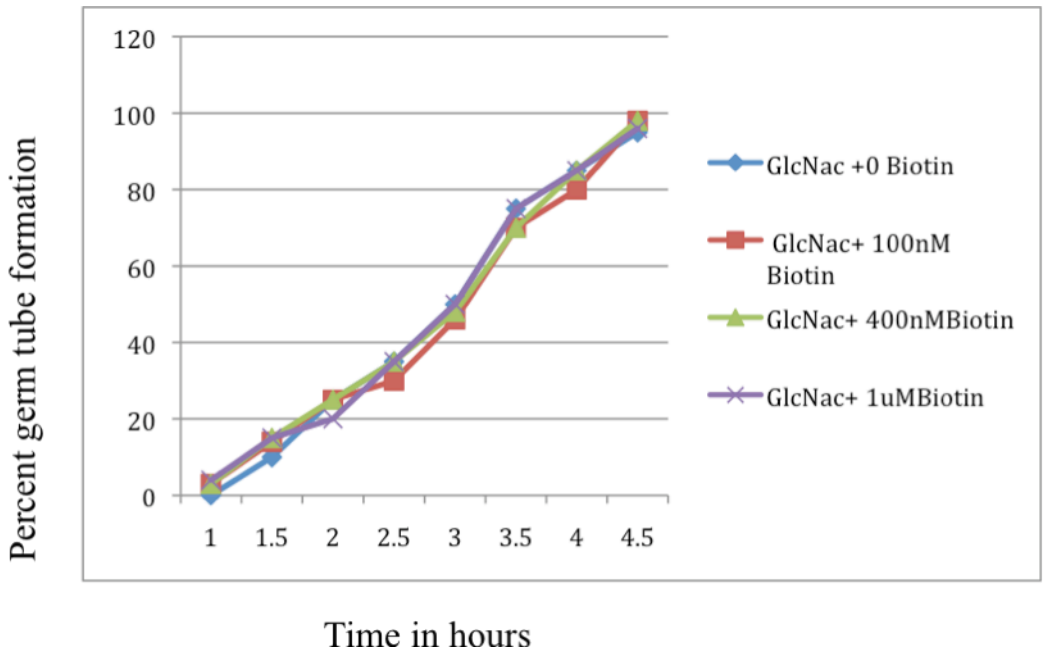


Figure 2.3 Biotinylation of Histone H2B

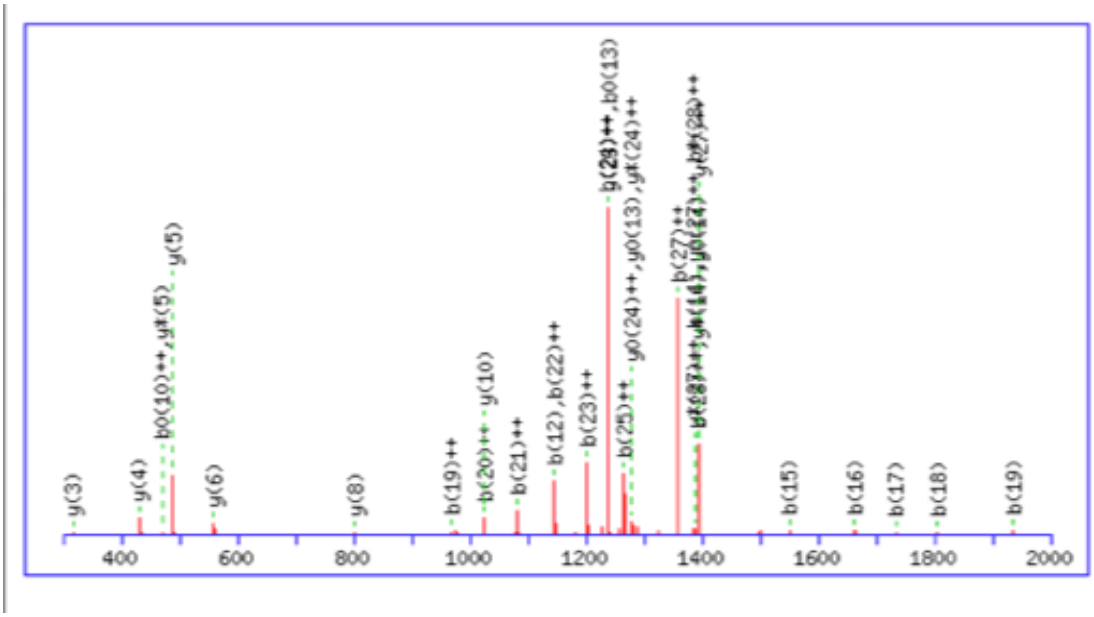
Protein View

Match to: **CAWT_02708** Score: **46**
| CAWG_02708 | Candida albicans WO1 histone H2B.2 (131 aa)

Nominal mass (M_r): **14082**; Calculated pI value: **10.10**
NCBI BLAST search of CAWT_02708 against nr
Unformatted sequence string for pasting into other applications

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Biotin (K)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **37%**
Matched peptides shown in **Bold Red**

1 M**APKAEKKPA SK**APAEKKPA AKKTASTDGA KKRTKARKET YSSYIYKVLK
51 **QTHPDTGISQ K**AMSIMNSFV NDIFERIATE ASKLAAYNKK STISAREIQT
101 AVR**LILPGEL AKHAVSEGTR AVTKYSSASS**



Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence
8 - 17	418.3200	1251.9382	1251.6645	0.2737	1	K.KPASKAPAEK.K Biotin (K) (Ions score 12)
13 - 17	515.5000	514.4927	514.2751	0.2176	0	K.APAEK.K (Ions score 8)
13 - 17	515.5300	514.5227	514.2751	0.2476	0	K.APAEK.K (Ions score 1)
18 - 23	867.4000	866.3927	867.5000	-1.1073	1	K.KPAAKK.T Biotin (K) (Ions score 12)
24 - 31	488.7700	975.5254	975.4331	0.0923	0	K.TASTDGAK.K Biotin (K) (Ions score 9)

Peptide View

MS/MS Fragmentation of **KPAAKK**
Found in **CAWT_02708**, | CAWG_02708 | Candida albicans WO1 histone H2B.2 (131 aa)

Monoisotopic mass of neutral peptide Mr(calc): 867.5000
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
K1 : Biotin (K)
Ions Score: 12 **Expect:** 3.7
Matches (Bold Red): 2/20 fragment ions using 5 most intense peaks

#	b	b*	Seq.	y	y*	#
1	355.1798	338.1533	K			6
2	**452.2326**	435.2060	P	514.3348	497.3082	5
3	523.2697	506.2432	A	417.2820	400.2554	4
4	**594.3068**	577.2803	A	346.2449	329.2183	3
5	722.4018	705.3752	K	275.2078	258.1812	2
6			K	147.1128	130.0863	1

Peptide sequence from N-terminus (b) to C-terminus (y): K-unmodified molecular weight is 147.11. K-modified with biotin is 373.1904-147.11 = 226.08 (biotin molecular weight)

Peptide View

MS/MS Fragmentation of **TASTDGAK**

Found in **CAWT_02708**, | CAWG_02708 | Candida albicans WO1 histone H2B.2 (131 aa)

Monoisotopic mass of neutral peptide Mr(calc): 975.4331
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
K8 : Biotin (K)
Ions Score: 9 **Expect:** 11
Matches (Bold Red): 4/64 fragment ions using 18 most intense peaks

#	b	b^{++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	102.0550	51.5311	84.0444	42.5258	T							8
2	173.0921	87.0497	155.0815	78.0444	A	**875.3927**	438.2000	858.3662	429.6867	857.3822	429.1947	7
3	260.1241	130.5657	242.1135	121.5604	S	**804.3556**	402.6814	787.3291	394.1682	786.3451	393.6762	6
4	361.1718	181.0895	343.1612	**172.0842**	T	**717.3236**	359.1654	700.2970	350.6522	699.3130	350.1602	5
5	476.1987	238.6030	458.1882	229.5977	D	616.2759	308.6416	599.2494	300.1283	598.2654	299.6363	4
6	533.2202	267.1137	515.2096	258.1084	G	501.2490	251.1281	484.2224	242.6148			3
7	604.2573	302.6323	586.2467	293.6270	A	444.2275	222.6174	427.2010	214.1041			2
8					K	373.1904	187.0988	356.1638	178.5856			1

Peptide sequence from N-terminus (b) to C-terminus (y): K-unmodified molecular weight is 147.11. K-modified with biotin is 373.1904-147.11 = 226.08 (biotin molecular weight)

Peptide View

MS/MS Fragmentation of **KPASKAPAEK**
Found in **CAWT_02708**, | CAWG_02708 | *C. albicans* WO1 histone H2B.2 (131 aa)

Monoisotopic mass of neutral peptide Mr(calc): 1251.6645
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
K10 : Biotin (K)
Ions Score: 12 **Expect:** 5.3
Matches (Bold Red): 16/100 fragment ions using 64 most intense peaks

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	**129.1022**	65.0548	112.0757	56.5415			K							10
2	**226.1550**	113.5811	209.1285	105.0679			P	1124.5768	562.7921	1107.5503	554.2788	1106.5663	553.7868	9
3	**297.1921**	149.0997	**280.1656**	**140.5864**			A	1027.5241	514.2657	1010.4975	505.7524	1009.5135	505.2604	8
4	**384.2241**	192.6157	367.1976	184.1024	366.2136	183.6104	S	956.4870	478.7471	939.4604	470.2338	938.4764	469.7418	7
5	**512.3191**	256.6632	495.2926	248.1499	494.3085	247.6579	K	869.4549	435.2311	852.4284	426.7178	851.4444	426.2258	6
6	583.3562	292.1817	566.3297	283.6685	565.3457	283.1765	A	741.3600	371.1836	**724.3334**	362.6704	**723.3494**	362.1783	5
7	680.4090	340.7081	663.3824	332.1949	662.3984	331.7028	P	670.3229	**335.6651**	653.2963	327.1518	652.3123	326.6598	4
8	**751.4461**	376.2267	734.4196	367.7134	733.4355	367.2214	A	**573.2701**	287.1387	556.2436	278.6254	555.2595	278.1334	3
9	**880.4887**	440.7480	863.4621	432.2347	862.4781	431.7427	E	**502.2330**	251.6201	485.2064	243.1069	484.2224	242.6148	2
10							K	373.1904	**187.0988**	**356.1638**	178.5856			1

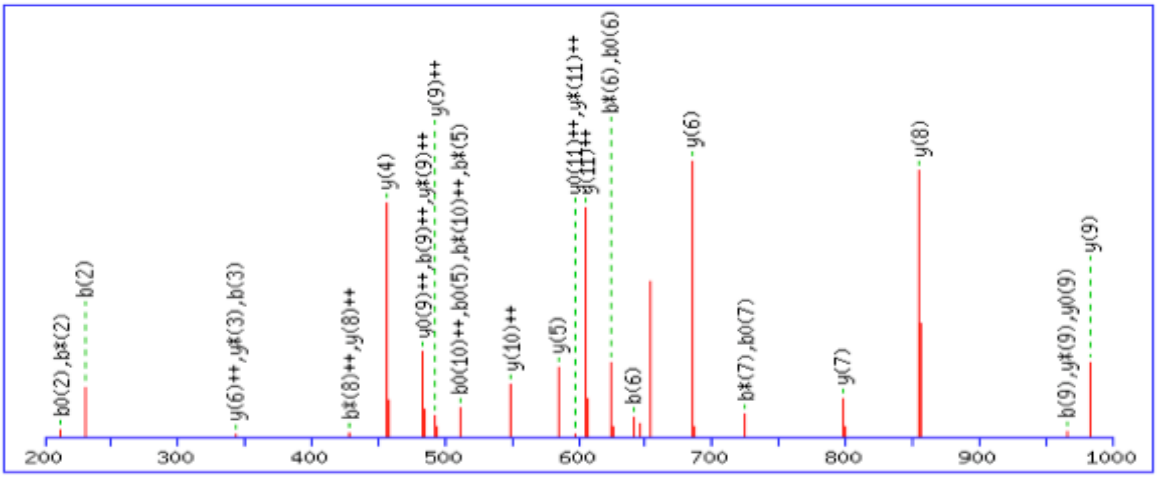
Peptide sequence from N-terminus (b) to C-terminus (y): K-unmodified molecular weight is 147.11. K-modified with biotin is 373.1904-147.11 = 226.08 (biotin molecular weight)

Figure 2.4 Biotinylation of histone H4

Protein View

Match to: **CAWT_00969** Score: **244**
| CAWG_00969 | Candida albicans WO1 histone H4 (106 aa)
Nominal mass (M_r): **11614**; Calculated pI value: **11.36**
NCBI BLAST search of CAWT 00969 against nr
Unformatted sequence string for pasting into other applications
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Biotin (K)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **51%**
Matched peptides shown in **Bold Red**

1 **MSGTGRGKGG KGLGKGGAKR** HRKILR**DNIQ GITKPAIR**RL
ARR**GGVKR**IS
51 ALIYEEVRVV LKQFLENVIR **DAVTYTEHAK** RKTVTSLDVV
YALKRQGR**TL**
101 **YGFGG**4



Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
1 - 11	518.9300	1035.8454	1034.5291	1.3163	2	-.MSGTGRGKGGK.G	(Ions score 18)
1 - 11	631.2900	1260.5654	1260.6067	-0.0413	2	-.MSGTGRGKGGK.G	Biotin (K) (Ions score 11)
1 - 11	631.6500	1261.2854	1260.6067	0.6787	2	-.MSGTGRGKGGK.G	Biotin (K) (Ions score 10)

Peptide View

MS/MS Fragmentation of **MSGTGRGKGGK**
Found in **CAWT_00969**, | CAWG_00969 | Candida albicans WO1 histone H4 (106 aa)

Monoisotopic mass of neutral peptide Mr(calc): 1260.6067
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
K8 : Biotin (K)
Ions Score: 10 **Expect:** 8.7
Matches (Bold Red): 30/94 fragment ions using 100 most intense peaks

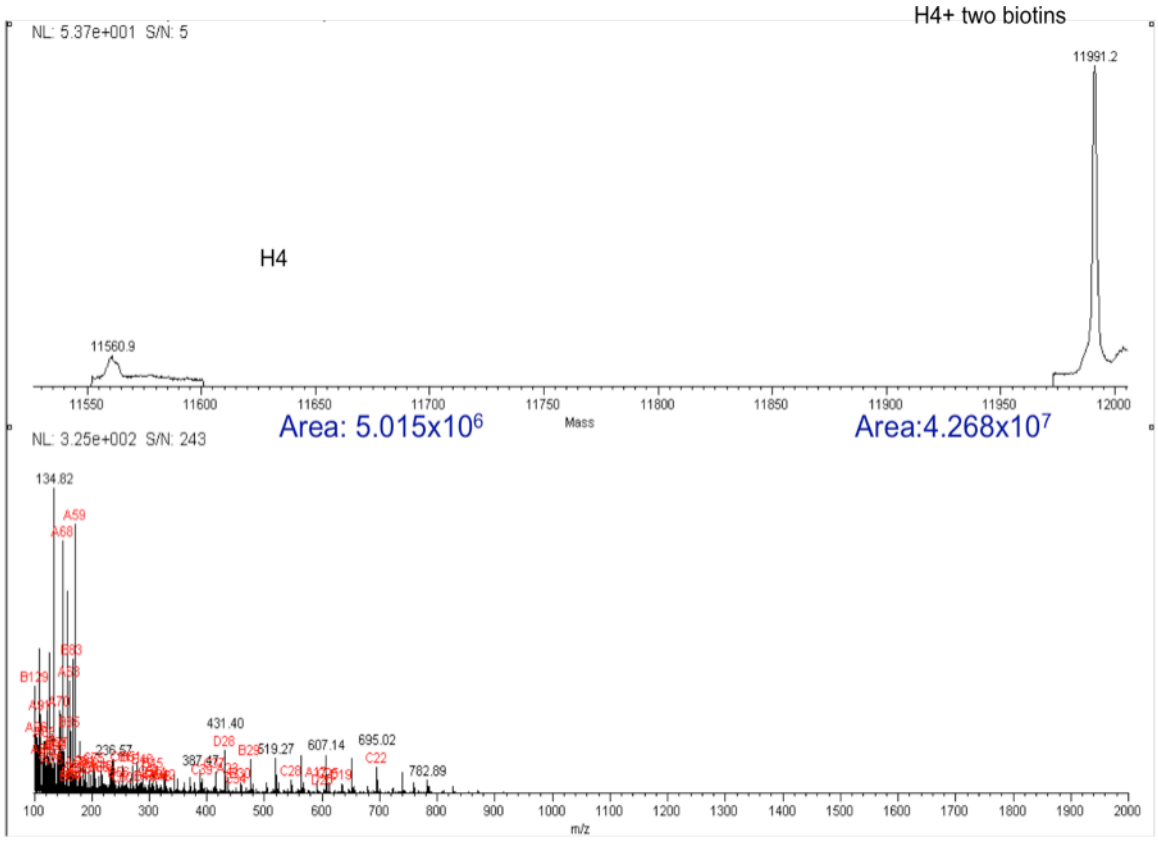
#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	132.0478	66.5275					M							11
2	**219.0798**	110.0435			201.0692	101.0382	S	1130.5735	565.7904	**1113.5469**	**557.2771**	**1112.5629**	**556.7851**	10
3	276.1013	138.5543			258.0907	129.5490	G	**1043.5415**	**522.2744**	1026.5149	513.7611	1025.5309	513.2691	9
4	377.1489	189.0781			359.1384	180.0728	T	**986.5200**	493.7636	**969.4935**	485.2504	**968.5094**	484.7584	8
5	434.1704	217.5888			416.1598	208.5836	G	**885.4723**	**443.2398**	**868.4458**	434.7265			7
6	590.2715	295.6394	573.2450	287.1261	572.2609	286.6341	R	**828.4509**	414.7291	811.4243	406.2158			6
7	647.2930	324.1501	630.2664	**315.6368**	629.2824	**315.1448**	G	672.3497	336.6785	655.3232	**328.1652**			5
8	1001.4655	501.2364	**984.4390**	492.7231	983.4550	492.2311	K	**615.3283**	308.1678	**598.3017**	299.6545			4
9	1058.4870	529.7471	**1041.4604**	521.2339	1040.4764	520.7419	G	**261.1557**	131.0815	244.1292	122.5682			3
10	**1115.5085**	**558.2579**	**1098.4819**	**549.7446**	**1097.4979**	**549.2526**	G	**204.1343**	102.5708	**187.1077**	94.0575			2
11							K	147.1128	74.0600	130.0863	65.5468			1

Peptide sequence from N-terminus (b) to C-terminus (y): K-unmodified molecular weight is 147.11. K-modified with biotin is 373.1904-147.11 = 226.08 (biotin molecular weight)

Monoisotopic mass of neutral peptide Mr(calc): 1260.6067
Fixed modifications: Carbamidomethyl (C)
Variable modifications:
K11 : Biotin (K)
Ions Score: 11 **Expect:** 6.7
Matches (Bold Red): 27/94 fragment ions using 91 most intense peaks

#	b	b^{++}	b*	b^{*++}	b^{0}	b^{0++}	Seq.	y	y^{++}	y*	y^{*++}	y^{0}	y^{0++}	#
1	132.0478	66.5275					M							11
2	**219.0798**	110.0435			201.0692	101.0382	S	1130.5735	565.7904	**1113.5469**	**557.2771**	**1112.5629**	**556.7851**	10
3	276.1013	138.5543			258.0907	129.5490	G	**1043.5415**	522.2744	1026.5149	513.7611	1025.5309	513.2691	9
4	377.1489	189.0781			359.1384	180.0728	T	**986.5200**	**493.7636**	**969.4935**	485.2504	**968.5094**	484.7584	8
5	434.1704	217.5888			416.1598	208.5836	G	**885.4723**	443.2398	**868.4458**	434.7265			7
6	590.2715	295.6394	573.2450	287.1261	572.2609	286.6341	R	**828.4509**	414.7291	811.4243	406.2158			6
7	647.2930	324.1501	630.2664	315.6368	629.2824	315.1448	G	672.3498	336.6785	655.3232	**328.1652**			5
8	775.3879	**388.1976**	758.3614	**379.6843**	757.3774	**379.1923**	K	**615.3283**	308.1678	**598.3017**	**299.6545**			4
9	832.4094	416.7083	**815.3828**	408.1951	**814.3988**	407.7031	G	487.2333	244.1203	470.2068	**235.6070**			3
10	889.4309	445.2191	872.4043	**436.7058**	**871.4203**	436.2138	G	**430.2119**	215.6096	413.1853	207.0963			2
11							K	**373.1904**	187.0988	356.1638	178.5856			1

Peptide sequence from N-terminus (b) to C-terminus (y): K-unmodified molecular weight is 147.11. K-modified with biotin is 373.1904-147.11 = 226.08 (biotin molecular weight)



According to this data, 85% histones are biotinylated.

Fig: Reverse phase UPLC chromatograph (C18 column) using TOF-MS.

Intact histone H4 (11560.9 Da) mass by deconvoluting using Megtran program showing histone H4 with and with two biotins (Molecular weight: 226) on it is showing 11991.2 Da.

Figure 2.5 Non biotinylated histone H2A

Protein View

Match to: **CAWT_00976** Score: **95**
| **CAWG_00976** | **Candida albicans WO1 histone H2A.2 (132 aa)**
Found in search of C:\Documents and Settings\rbc\Desktop\april2010\Swetha2_100626180648.mgf

Nominal mass (M_r): **13816**; Calculated pI value: **10.24**
NCBI BLAST search of CAWT_00976 against nr
Unformatted sequence string for pasting into other applications

Fixed modifications: Carbamidomethyl (C)
Variable modifications: Biotin (K)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: **30%**

Matched peptides shown in **Bold Red**

```
  1 MSGGKGKAGS SEKASTSRSA KAGLTFPVGR VHRLLRKGNY AQRIGSGAPV
 51 YLTSVLEYLA AEILELAGNA ARDNKKSRII PRHLQLAIRN DEELNKLLGD
101 VTIAQGGVLP NIHQSLLPAK KAKAGAASQE L
```

Show predicted peptides also

Sort Peptides By (•) Residue Number ( ) Increasing Mass ( ) Decreasing Mass

Start - End	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Sequence	
22 - 30	459.5100	917.0054	916.5131	0.4924	0	K.AGLTFPVGR.V	(Ions score 52)
22 - 30	459.7800	917.5454	916.5131	1.0324	0	K.AGLTFPVGR.V	(Ions score 47)
22 - 30	459.8500	917.6854	916.5131	1.1724	0	K.AGLTFPVGR.V	(Ions score 60)
37 - 43	418.2900	834.5654	835.4300	-0.8646	1	R.KGNYAQR.I	(Ions score 8)
97 - 120	818.6900	2453.0482	2453.4006	-0.3524	0	K.LLGDVTIAQGGVLPNIHQSLLPAK.K	(Ions score 6)

Figure 2.6 Biotinylated histones from *C.albicans*

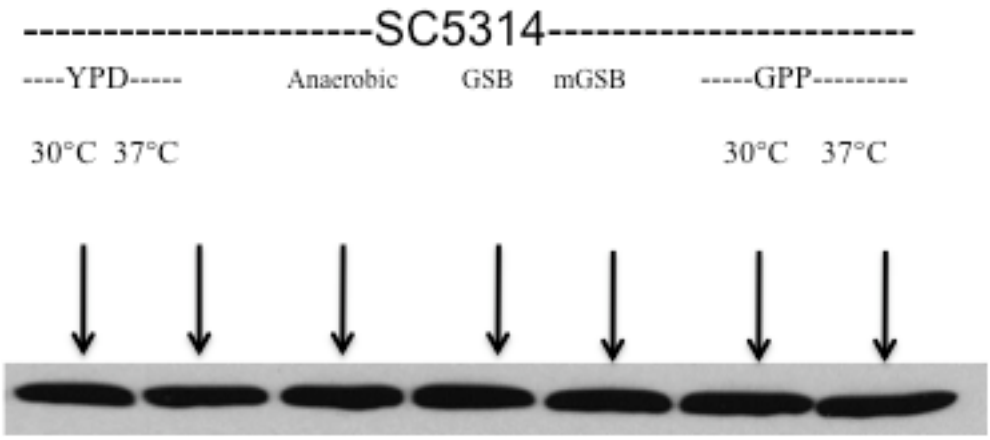


Figure 2.7 Biotinylated histones from biotin starved cells of *C.albicans*

Tables

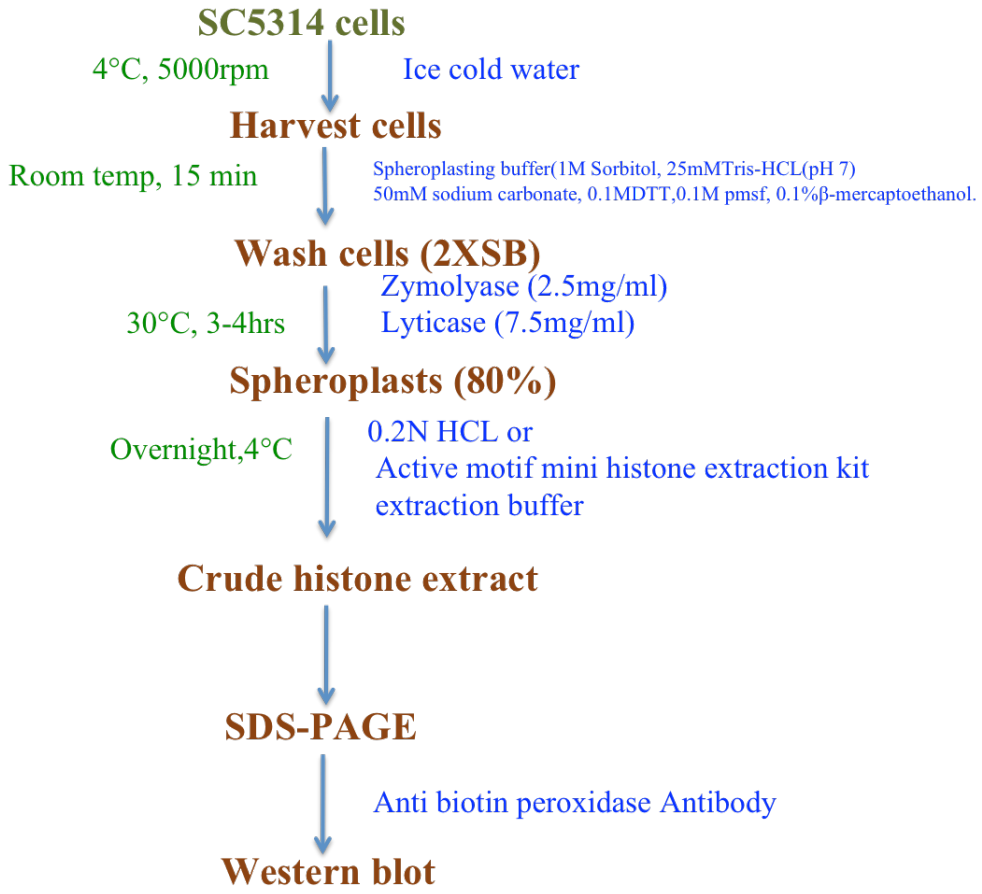
Table 2.1 Germ tube formation assays with GS grown cells

Media	% Hyphae		
	at 1:30hrs	at 2:30hrs	at 4:00hrs
GPP (w/0 biotin)	1	10	20
GPP (100nM biotin)	30	90	100
GPP (400nM biotin)	50	100	100
GlcNAc	1	1	1
GlcNAc (100nM biotin)	20	50	85
GlcNAc (400nM biotin)	50	70	90

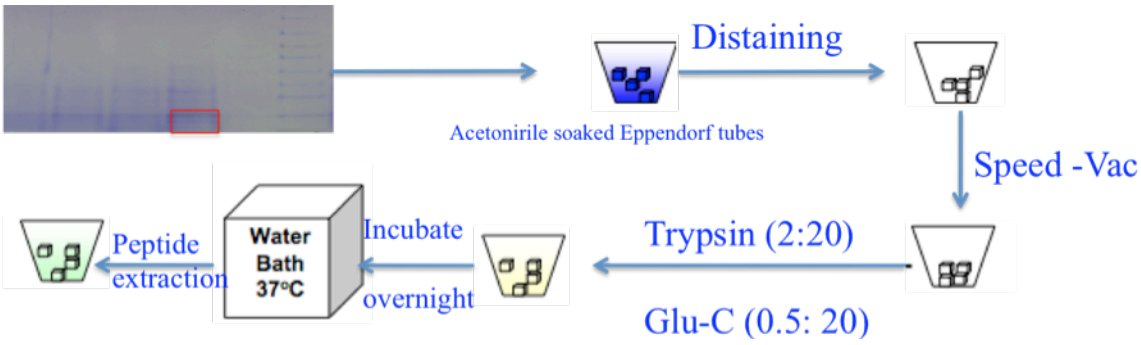
Table 2.2 Germ tube formation assays with mGS grown cells

Media	% Hyphae		
	at 1:30hrs	at 2:30hrs	at 4:00hrs
GPP (w/0 biotin)	20	50	60
GPP (100nM biotin)	25	60	100
GPP (400nM biotin)	30	100	100
GlcNAc	1	5	5
GlcNAc (100nM biotin)	5	25	70
GlcNAc (400nM biotin)	5	30	90

Protocol: Crude histone extraction:

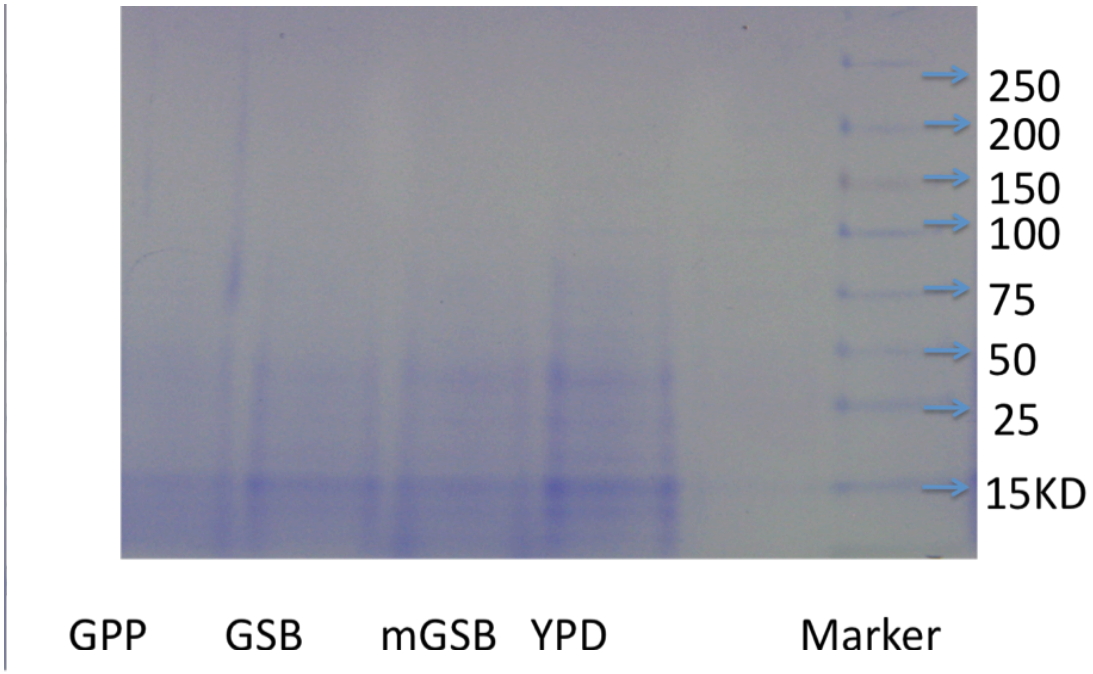


Protocol: In-gel digestion:-



Details of this illustration are provided in the Methods section.

Supplementary Figure 1: Crude histone extracts from *C. albicans* strain SC5314 grown rich (YPD) and defined (GPP, GSB, mGSB) medium. Histones were run through SDS PAGE and stained with commasive-blue.



Supplementary Figure 2: Histone extraction using different methods

Histones extracted from *C. albicans* strain SC5314 using 0.2NHCL and Extraction buffer from Active-motif. Histones were run through SDS-PAGE and detected band using Anti-biotin peroxidase antibody in western blotting.

Supplementary Figure 3:

Crude histones were extracted from biotin-starved *C. albicans* strain SC5314 cells (GS, mGS, GSU) and YPD, GSUB. We also extracted histones from *Saccharomyces cerevisiae* strain BHY10. These histones were run through SDS- PAGE to conform our results from western blot to the same cells.