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Transcriptional analysis of the histone acetylation and deacetylation in *Candida albicans*

Ph.D Thesis

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Spain, Salamanca, 2009

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CERTIFY:

That the present dissertation entitled “Transcriptional analysis of the histone acetylation and deacetylation in *Candida albicans*”, submitted to apply for Ph.D degree, has been carried out by Ahmad Rashki under my direction in the Department of Microbiology and Genetics, University of Salamanca.

Prof. Dr. Angel Dominguez Olavarri
Professor and Chair

Prof. Dr. Angel Dominguez Olavarri, Head of the Department of Microbiology and Genetics of the University of Salamanca

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That the present dissertation entitled “Transcriptional analysis of the histone acetylation and deacetylation in *Candida albicans*”, submitted to apply for Ph.D degree, has been carried out by Ahmad Rashki under direction Prof. Dr. Angel Dominguez Olavarri in the Department of Microbiology and Genetics, University of Salamanca.

Prof. Dr. Angel Dominguez Olavarri
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The project work has been supported in part by grants:

DGICYT "Identificación de factores de virulencia y nuevas dianas terapéuticas en *Candida albicans* mediante análisis funcional del genoma y del proteoma" (Proyecto BIO2002-02124)

Unión Europea. "Interaction of fungal pathogens with host cells: a post genomic approach" dentro del Progama Marie Curie Research Training Network (MRTN-CT-2003-504148)

DGICYT "Analisis de las interacciones de *Candida albicans* con el hospedador: modelos *"ex vivo"* e *"in vivo"*.
(Proyecto SAF2005-07131)

FIS "Analisis de los mecanismos moleculares de infecciones fungicas mediante aproximaciones genomicas y proteomicas. Establecimiento de modelos animales." (Proyecto PI 050849).

"Glycoshield: surface modulation of the fungal and host response using a genomic approach". Proyectos de investigación en el marco de las redes del Espacio Europeo de Investigación (esquema ERA-NET). GEN2006-27775-C2-E/PAT

Junta de Castilla y León "Histona acetyltransferasas en *Candida albicans* efecto sobre dimorfismo y virulencia. Una aproximación Post-genomica" (SA 141A08)

Read: In the name of thy Lord who createth,
Createth man from a clot.
Read: and thy Lord is the most bounteous,
Who teacheth by the pen,
Teacheth man that which he knew not.

(Holy Qur'an)

Dedicated to my parents, to whom I owe the gift of life.

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Acknowledgements

I would like to express my sincerest appreciation to my advisor, Prof. Dr. Angel Dominguez Olavarri, who believed in me and gave me the chance to start my project in his lab. He trained me to think deeply and thoroughly about research work. He always encouraged me to aim high and work hard to explore the scientific fields. I couldn't have completed this work without his support, advice, guidance, understanding and encouragement. His dedication to science is a great motivation for me to pursue a scientific career.

I appreciate Dr. Maria Carmen Lopez for her helpful advice on molecular biology techniques.

I give special thanks to Dr. Nuria Martin Laso who worked hard to help with my microarray experiments. She was always very patient in helping me, solve problems and giving me suggestions of a beginner.

I am thankful to my colleagues and lab members: Laura Mojardin, Esperanza, Rosa, Ivan, Pilar, Rita, Carlos, Laura, Azul, Ana, Adam and Juan.

I would like to express my deepest gratitude to Professor Carlos Rodriguez Vazquez De Aldana for his friendly assistance in every difficult moment of my work.

I am very grateful to Dr. Alberto Gonzalez-Novo and Dr. Fransisco Conde Perez, for effective and intelligent suggestions.

Many thanks also to Dr. Manuel Sanchez Fernandez for DNA sequencing.

Finally, I thank to my doughters, Mahsa and Rozhan, and my wife. They give me tremendous support and encouragement throughout my graduate study.

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bp	base pair
CFW	Calcofluor white
$CaCl_2$	Calcium Chloride
cDNA	Complementary Deoxyribonucleic acid
CIP	Calf Intestine Alkaline Phosphatase
Cy3	Cyanine Dye 3
Cy5	Cyanine Dye 5
dCTP	Deoxycytidinetriphosphate
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleosidetriphosphate
E. coli	*Escherichia coli*
f.o.i	Frequency of incorporation
GlcNAc	N-acetylglucosamine
h	Hour
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
Kb	Kilobase
LB	Lauria Bertani
M	Molar
min	Minute
ml	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic Acid
NaCl	Sodium Chloride
ng	nanogram
nm	nanometer
OD	Optical Density at specific wavelength
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
Pmol	Picomol
RNA	Ribonucleic Acid
RNAse	Ribonuclease
rpm	revolution per minute
rRNA	Ribosomal Ribonucleic Acid
RT	Room temperature
SDS	Sodium dodecyl sulphate
SSC	Sodium Chloride-Sodium Citrate
TE	Tris, EDTA
TRIS	Tris-(hydroxymethyl)-aminomethane
Ufc	Unit forming colony
UV	Ultra Violet
µg	Microgram
µl	Microlitre
µm	Micromolar
ºC	Degree Centigrade

General biology of *Candida albicans*

Candida albicans is an important human pathogen that displays a remarkable ability to detect changes in the environment and to respond appropriately by changing its cell morphology and physiology [40]. *C. albicans* is a pleomorphism diploid fungus which presents the ability to grow under a variety of morphological forms that include unicellular budding yeast, also known as blastospore, true hypha and pseudohyphal forms. *C. albicans* also can form chlamydospores that is to say; asexual spores which develop over pseudohyphal support cells and appear under unfavourable environmental conditions (Figure 1.1) [254,451].

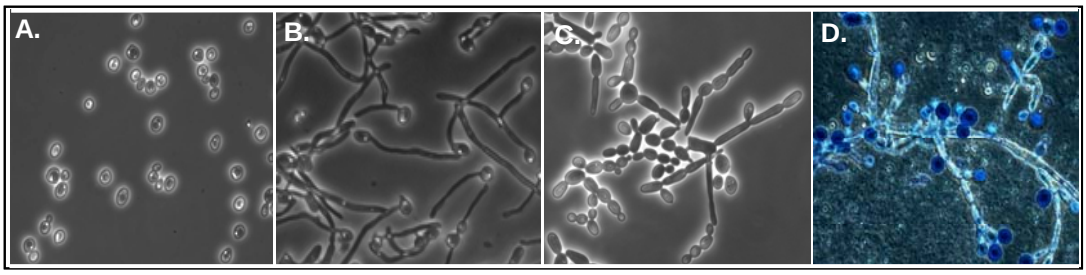

Figure 1.1 Growth of *C. albicans* yeast (**A**), hypha (**B**), pseudohypha (**C**) and chlamydospore (**D**)

Fungi are classified on the basis of their ability to reproduce sexually, asexually or by a combination of both. Thus, *C. albicans* has traditionally been classified in the *Deuteromycota* (fungi imperfecti) since the sexual phase of this fungus is unknown, although recent reports described the presence of mating-type-like orthologs (MTL) in *C. albicans* of both of the *Saccharomyces cerevisiae* mating-type genes (MAT), a and α [213,296]. Therefore, *C. albicans* has been now classified as follows:

Phylum…….*Ascomycota*
Subphylum……..*Ascomycotina*
Class…….*Ascomycetes*
Order……...*Saccharomycetales*
Family…….*Saccharomycetaceae*
Genus…….*Candida*

The genome of *Candida albicans*

The hemiascomycete *C. albicans* is an opportunistic pathogen causing human fungal infections, mainly in immunocompromised hosts. The complete sequence of

the *C. albicans* genome has been determined (*http://www-sequence.stanford.edu/group/candida*) based on a shotgun strategy carried out at the Stanford Genome Technology Center. The total assembly of the haploid genome comprises ~16 Mb (approximately 33% larger than that of *S. cerevisiae*) corresponding to approximately 6244 annotated entries corresponding to 130 tRNA genes and 5917 protein-coding genes that annotated by the European Consortium Galar Fungail (*http://genolist.pasteur.fr/CandidaDB*) [75,96]. This nuclear genome distributed in eight pairs of homologous chromosomes which are numbered from 1 (largest) to 7 (smallest), with the one carrying the ribosomal DNA called R [502]. CandidaDB release 2.0 contains information pertaining to Assembly 19 of the genome of *C. albicans* strain SC5314 [96].

Assembly 19, is distributed over 412 supercontigs, of which 266 constitute a reference haploid genome of 14 855 kb and 146 constitute allelic counterparts of supercontigs included in the reference haploid genome [224]. The reference haploid genome contains 7677 ORFs of 100 codons or longer, and a reduced set of 6419 ORFs has been derived by eliminating the smaller of a pair of ORFs that overlap by more than 50% [224]. Assembly 21 reveals an ancient chromosome fusion, a number of small internal duplications followed by inversions, and a subtelomeric arrangement, including a new gene family, the TLO genes. Correlations of position with relatedness of gene families imply a novel method of dispersion. The sequence of the individual chromosomes of *C. albicans* raises interesting biological questions about gene family creation and dispersion, subtelomere organization and chromosome evolution [482].

Candida albicans as a pathogenic agent

Candida spp. are the fourth leading cause of nosocomial bloodstream infections in the United States, with treatment costs estimated to be more than $2-$4 billion annually [506] and with attributable mortality rates estimated to be between 38% to 49% [168]. *C. albicans* can grow as budding yeast, in a pseudohyphal form or as true hypha depending upon the environmental conditions [451]. Growth is polarized in *C. albicans* hypha, with continuous apical growth throughout the cell cycle and parallel cell walls at the septal junctions. In contrast, the growth of pseudohypha and blastophores is only limited to the apical tip during the initial part of the cell cycle [76]. *C. albicans* pseudohypha has a constriction at the septal junction

between mother and daughter cells. Pseudohypha can be distinguished from true hypha on the basis of their morphological index which quantifies the dimensions of cell compartments. Alternatively they can be distinguished on the basis of the positions of their septal junctions. These lie at the bud neck for pseudohypha, and within the germ tube for emerging hypha [451]. The yeast-hyphal transition is regulated by a complex network of signalling pathways that include evolutionarily conserved MAP kinase and Ras-cAMP modules [122,157,451,501]. The yeast-like form is thought to promote dissemination within the blood stream whereas hyphal development appears to promote invasion [157,451,456]. The link between specific morphotypes and virulence is not firmly established [157]. However, hypha is important for the formation of biofilm, which can provide reservoirs of infection in the host, and morphogenetic variation is likely to be important in responding rapidly to environmental change and to host defence mechanisms [358]. Generally, growth of *C. albicans* in the yeast form is favoured at high cell densities, temperatures below 30ºC and ambient pH below 4. In contrast, the hyphal form is favoured at temperatures above 37ºC, at ambient pH close to 7, at lower cell densities, at high CO_2 concentrations, under some limiting nutrient conditions such as amino acid starvation or hypoxia, or in the presence of serum [120,239,358,472]. The effect of serum is complex but it is proposed to act, in part, by conferring amino acid starvation [472] although other factors are implicated [212,299].

Types of candidiasis

Candidiasis can range from superficial disorders to invasive infections, in general in immunocompromised hosts, and usually are classified into two big groups as follow **superficial candidiasis**, which may involve the epidermal and mucosal surfaces, including those of the oral cavity, pharynx, oesophagus, intestines, urinary bladder, and vagina. Symptomatic mucosal and superficial candidiasis arises in subjects colonized with *Candida* who are predisposed by illness, debility, or a local reduction in host resistance to an overgrowth of their own indigenous flora [358,485], and the different forms of superficial candidiasis are often associated with biofilm formation on surfaces of host tissues or medical devices [415]. In patients with probable immunodeficiency or visible candidiasis of the skin, oropharynx, or esophagus, a *Candida* infection should be considered in case of a gastrostomy site infection [511].

Systemic candidiasis, considerable changes in the relative frequency of systemic infections due to various *Candida* species as well as their *in vitro* susceptibility patterns have been noted in several parts of the world [401]. It is widely known that systemic candidiasis caused by *Candida albicans* strains endangers the lives of hospitalised patients since these pathogens are extremely difficult to defeat by commonly used antifungal agents [417].

Candida albicans may affect the kidneys, liver, spleen, brain, eyes, heart and other tissues the principal risk factors predisposing to deeply invasive candidiasis (Figure 1.2) are protracted courses of broad spectrum antibiotics, cytotoxic chemotherapy, corticosteroids, HIV and vascular catheters [230].

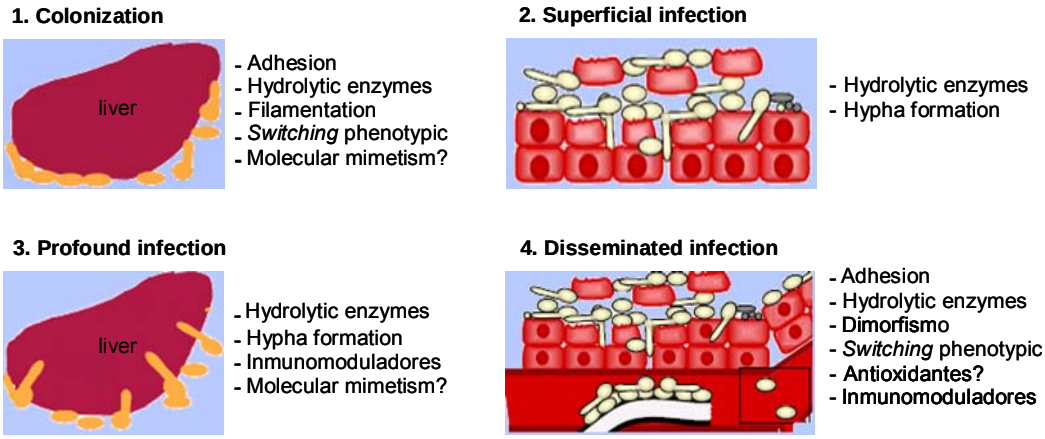

Figure 1.2 Schematic diagrams illustrating the contribution of the virulence attributes to *C. albicans* pathogenicity. *C. albicans* commonly colonizes the epithelial surface (stage 1) and causes superficial infections (stage 2), but under conditions when the host is compromised, the fungus establishes deep-seated infections (stage 3) by penetrating further into the epithelial tissue. Occasionally, *C. albicans* causes disseminated infections (stage 4), which allow the fungus to colonize and infect other host tissues and can be fatal.

Treatment of candidiasis

Invasive fungal infections are an important cause of morbidity and mortality, particularly in patients with underlying risk factors e.g. neutropenia, cancer chemotherapy, transplantation and AIDS [74]. *C. albicans* is a eukaryotic organism and therefore share many of its biological processes with human cells; most antifungal drugs cause deleterious side effects and, at the doses used, are fungistatic rather than fungicidal. For that reason, an important goal in antifungal drug discovery is the identification of new suitable fungal targets with no mammalian homologies. Some of the antifungal agents currently available are classified, according with their specific mode of action as follows:

- **Amphotericin B**. Although our antifungal armamentarium has been enlarged

recently with new azoles (voriconazole and posaconazole) and echinocandins (caspofungin, micafungin, anidulafungin), the polyenes still have an important role in antifungal strategies because of their extended antifungal spectrum and rarity of mycological resistance [264]. This antifungal agent acts by binding to ergosterol, the principal sterol in fungal membranes, thereby perturbing membrane function to the point of causing leakage of cellular contents. Despite of the conformational difference between cholesterol and ergosterol, and the major binding affinity of Amphotericin B for this last one, this antifungal agent has potential toxicity for mammalian cells, so a variety of reformulated versions of the agent have been introduced in the last years, to overcome Amphotericin B nephrotoxicity by slowing the rate at which the compound is delivered to the kidneys [116].

- **Azoles**. The azole antifungal drugs are used to treat infections caused by *Candida albicans* and other fungi. These drugs interfere with the biosynthesis of ergosterol, the major sterol in fungal cells, by inhibiting an ergosterol biosynthetic enzyme, lanosterol 14 alpha demethylase, encoded by the *ERG11* gene [359]. Fluconazole is effective for the prevention and treatment of candidiasis but its inactivity against fungi and increasing resistance are limiting factors. Newer azoles, particularly voriconazole and posaconazole, have an enhanced spectrum of activity that includes *Candida* species, *Aspergillus* species, *Cryptococcus* species, dimorphic fungi, *Fusarium* species, and for posaconazole, *Zygomycetes*. Recent data suggest that these agents are highly effective in a variety of clinical settings [74].

- **Other sterol synthesis inhibitors**. The increased incidence of invasive mycoses and the emerging problem of antifungal drug resistance have encouraged the search for new antifungal agents or effective combinations of existing drugs. Infections due to *Candida albicans* are usually treated with azole antifungals such as Fluconazole, Ketoconazole or Itraconazole. Whilst azoles may have little or no toxicity, they generally offer rather poor fungicidal activity. Even in the absence of resistance, treatment failures or recurrent infections are not uncommon, especially in immunocompromised individuals. Non-classical antifolate pyrimethamine shows synergy with azole antifungal compounds and interferes with the ergosterol biosynthesis pathway in *C. albicans.* By disturbing folate metabolism in this fungus, pyrimethamine can inhibit ergosterol production [344]. There are two other classes of antifungal agents that acts at this same pathway, allylamines, which act by

inhibition of squalene epoxidase, with fungicidal consequences in many filamentous fungi but not so much effective in pathogenic yeasts, and morpholines, affecting two different enzymes in the ergosterol pathway, Erg24p and Erg2p.

- **Equinocandins.** Echinocandins have good activity against *Candida* species and *Aspergillus* species but their spectrum generally does not include *Fusarium*, *Cryptococcus*, *Trichosporon*, *Zygomycetes* and *Dematiaceous* fungi. Their target is the complex of proteins responsible for synthesis of cell wall β-1,3 glucan polysaccharides, and the component to which equinocandins bind is Fsk1p, although the complete mechanism of the action still remains unknown [481,513].

Virulence factors

The virulence of *Candida* species depends on many environmental conditions including extracellular pH concentration of alkali metal cations [246], expression of host recognition molecules or adhesins [138,422,540], cell wall organization and growth rate [534] and expression of tissue invasion-facilitating enzymes, such as phospholipases and secreted aspartyl proteinases [127,147,242,337,406].

Adhesins

The fungal cell surface contributes to pathogenesis by mediating interactions with host cells and eliciting host immune responses [343]. The virulence factors expressed or required by *Candida* species, and in particular by *C. albicans*, vary depending on the type of the infection (e.g. mucosal or systemic), the site and stage of infection, and the nature of the host response [123]. One of the important virulence attributes is adhesion. Most studies focus on the role of two well-characterized adhesins (ALS family and Hwp1p) in morphogenesis, pathogenicity, and phenotype switching of *C. albicans* [454].

ALS family. The ALS gene family of *C. albicans* encodes large cell surface glycoproteins with a three domain structure that are implicated in the process of adhesin to host surfaces [200].

Hwp1p. This protein encodes an outer mannoprotein, with a cell surface-exposed NH_2-terminal domain and COOH-terminal features conferring covalent integration into cell wall β-glucan. It is conditionally required for hyphal formation: the ability to form hypha on solid media is severely reduced in an *HWP1* heterozygous mutant

and eliminated in the null mutant. In the presence of serum, colonies of the null mutant produce peripheral hypha but at reduced levels compared to the wild type. This protein encodes an outer surface mannoprotein that is believed to be oriented with its amino-terminal domain surface-exposed and the carboxyl terminus, most probably covalently integrated with cell wall β-glucan. The amino-terminal sequence of Hwp1p was found to resemble mammalian transglutaminase substrates suggesting that Hwp1p is involved in the formation of stable complex between germ tubes (Figure 1.3) (initial projections observed when *Candida* switches from yeast form to hyphal growth) [443].

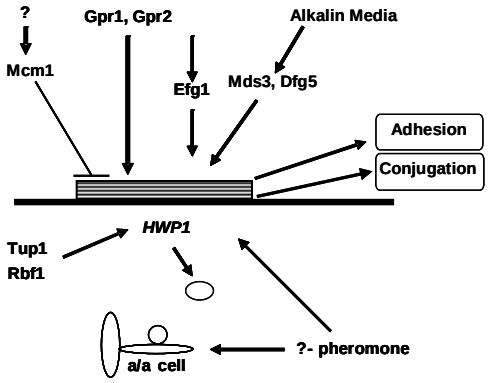

Figure 1.3 Transcriptional and functional regulation of *HWP1* [34]

HWP1 is expressed during hyphal development [235,444]. Production of Hwp1p has been confirmed in the mouse model of systemic infection by immunohistochemistry [444]. More recently *HWP1* expression has been observed in samples from human oral and vaginal infections by antibody and RT-PCR-based approaches [92,338]. These authors suggest that Hwp1p is important both for benign and invasive interactions of *C. albicans* with humans, and is also required for biofilm formation [355].

Extracellular hydrolytic enzymes

Extracellular enzymes secreted by *Candida albicans* are claimed to be virulence factors responsible for penetration of the yeast into host cells [527]. The secreted aspartyl proteinases (SAP) and phospholipases (PL) are two rather large families of *C. albicans* enzymes, some of which have been associated with virulence [53]. Secreted aspartic proteases (Saps) contribute to the virulence of *C. albicans*, a major fungal pathogen of humans [97]. The differential expression of specific

virulence factors at different stages of an infection could be the reason why *C. albicans* not only has single genes for extracellular hydrolytic enzymes, but gene families. Both secreted aspartate proteinases (Saps) and secreted lipases (Lips) from *C. albicans* are encoded by at least 10 different genes. This high number of similar genes might provide it *C. albicans* with the ability to secrete a specific and appropriate enzymatic response at distinct stages of an infection [447].

Phenotypic switching

The basic mechanism of phenotypic switching is not clear, although is known that *C. albicans* can switch among different phenotypes (smooth, rough, star, stippled, hat, irregular wrinkle and fuzzy) at high frequency [430,435-438]. Of all the switch phenotypes described, the most studied is the white opaque system in strain WO-1, in which smooth, white colonies, switch to flat and grey colonies (opaque) (Figure 1.4). The role of phenotypic switching in the virulence of this organism remains to be elucidated, although it has been observed that opaque cells colonize the skin in a cutaneous model more than white-phase cells, but are less virulent in a systemic animal model [257,437].

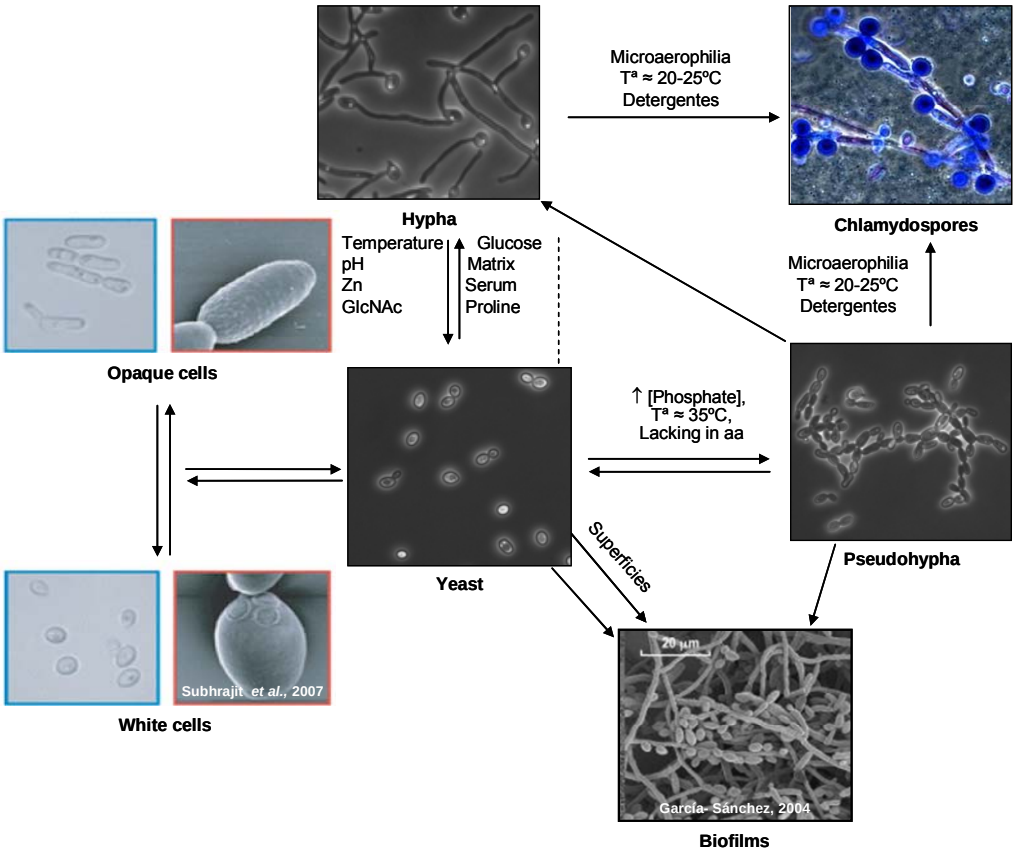

Figure 1.4 Morphological forms of *C. albicans*

Biofilm formation

The ability to adhere to surfaces and develop as a multicellular community is an adaptation used by most microorganisms to survive in changing environments. Biofilm formation proceeds through distinct developmental phases and impacts not only medicine but also industry and evolution. In organisms such as the opportunistic pathogen *C. albicans,* the ability to grow as biofilms is also an important mechanism for persistence, facilitating its growth on different tissues and a broad range of abiotic surfaces used in medical devices [334]. Biofilm formation is a major virulence attribute of *Candida* pathogenicity which contributes to higher antifungal resistance [413]. *Candida* species, particularly *C. albicans*, readily form biofilms, consortia of cells that coexist as an organized community with a complex three-dimensional architecture that is enveloped within an exopolysaccharide matrix, attached to a solid substratum [82,83,109]. Mature biofilms are characterized by the production of a thick extracellular matrix and an altered resistance phenotype to common antifungal agents [82,83]. The basis of the characteristic drug resistance of cells growing in biofilms is a source of much speculation, although recent studies suggest multiple factors involved in this process [19,409].

Hyphal-specific gene transcription

A variety of factors, including temperature, amino acids, pH changes and serum can induce the yeast-hypha transition in *C. albicans* [121,158,312]. The contribution of a range of signalling pathways to morphogenesis has been investigated, suggesting that the yeast-hypha transition is the subject of both positive and negative regulation [158,500]. Several genes have been identified whose expression is induced during hyphal induction. Such genes are listed next, and most of them encode either cell-wall proteins or secreted proteins:

- *ECE1*: expression of this gene was not detected when *C. albicans* grew as a budding yeast cell but was observed within 30 min after cells had been induced to form hypha [32].

- *HYR1*: encodes a non-essential cell wall glycoprotein and null mutant for this gene also displays a normal hyphal development [18].

- *RBT1* and *RBT4*: these two genes repressed by *TUP1* encode a cell-wall protein

and a secreted protein, respectively [37].

Regulation of central metabolic pathways in a fungal pathogen

To grow, a microbe must assimilate carbon. Pathogens such as *C. albicans* , which can thrive within diverse niches such as the skin, mucous membranes, blood and internal organs of its human host and in biofilms [51,358], must display sufficient metabolic flexibility to assimilate the available nutrients in these niches. Data from several transcript profiling studies are consistent with this notion [41]. For example, amino acid biosynthetic genes are up-regulated in *C. albicans* cells growing in yeast-hypha transition (our data in Chapter II). Also, following exposure to human neutrophils or cultured macrophages, *C. albicans* populations up-regulate amino acid biosynthetic genes and display a shift from fermentative to non-fermentative metabolism [134,286,393]. This includes the down-regulation of glycolytic genes and the activation of glyoxylate cycle genes (*ICL1* and *MLS1*), which facilitate the assimilation of two-carbon compounds in concert with gluconeogenic genes (*PCK1* and *FBP1*). Further evidence for the activation of glyoxylate cycle genes following exposure to macrophages has been obtained by differential display reverse transcription polymerase chain reaction (PCR) [287,373]. It has been reported that *C. albicans icl1/icl1* mutant display attenuated virulence in the mouse model of systemic candidiasis. This has led to the suggestion that the glyoxylate cycle is required for fungal virulence [287,288]. However in *S. cerevisiae*, gluconeogenic and glyoxylate cycle genes are exquisitely sensitive to glucose [526]. These genes are repressed by glucose at concentrations as low as 0.01%, which are well below those present in the bloodstream. If *C. albicans* gluconeogenic and glyoxylate cycle genes are regulated in an analogous fashion to those in *S. cerevisiae*, which is often presumed to be the case, one would expect them to be repressed during systemic infections. How then is the glyoxylate cycle is required for the establishment of systemic *C. albicans* infections? To address this apparent paradox, it has been revisited the role of the glyoxylate cycle and glycolytic and gluconeogenic pathways in *C. albicans* virulence. Most of the enzymes on the glycolytic pathway catalyse reversible reactions that also contribute to gluconeogenesis. However, two steps in glycolysis are essentially irreversible and these are catalysed by the glycolysis-specific enzymes, phosphofructokinase and pyruvate kinase. *PFK2* encodes one of two phosphofructokinase subunits, and

PYK1 encodes pyruvate kinase. *PCK1* encodes the gluconeogenenic specific enzyme, phosphoenolpyruvate carboxykinase. *ICL1* encodes the glyoxylate cycle enzyme, isocitrate lyase (Figure 1.5). This view arises from both a general metabolism perspective and the identification of the precise metabolic points that distinguish both morphological phases. Some drug targets and genes related to virulence were also detected in several metabolic pathways, and the majority of genes involved in signal transduction pathways possibly participating in cell differentiation and infection are annotated in *C. albicans.*

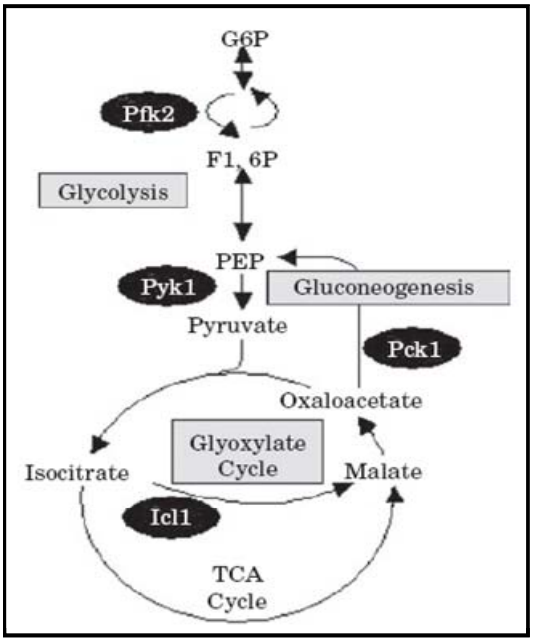

Figure 1.5 Cartoon of central carbon metabolism

Virulence phenotypes and the choice of animal model

In *C. albicans*, attenuation of mouse lethality in mutant fungal strains has been the most common approach to elucidating the roles of specific genes in virulence. It is clear, however, that expression of virulence by a fungus can depend on the particular host niche that is being affected. It is therefore possible to miss virulence factors when only one model for virulence is tested. In *C. albicans,* disruption of the *GCN5* (a histone acetyltransferase) gene led to attenuated lethality when injected intra-peritonealy into mice (our data in chapter III), whereas disruption of the related *HDA1* (a histone deacetylase encode) gene had no effect on lethality [307]. Similarly, in *C. neoformans,* a mutant with a disrupted *URE1* urease gene showed normal virulence when injected intracisternally into rabbits, but showed attenuated

lethality in a mouse infected intravenously [77]. Extensive studies of the family of secreted proteinase enzymes (SAP gene products) in *C. albicans* have also indicated differential expression of the individual SAP genes according to the stage and type of infection under investigation [407]. Clearly, there is no substitute for thorough investigation of virulence phenotypes in as many models of infection as possible *in vivo* and *ex vivo,* particularly when single-gene disruptants are under scrutiny, if the true role of virulence molecules in the infection process is to be fully determined.

The actin cytoskeleton is required for the dimorphic switch in *Candida albicans*

During hyphal development, cell-surface expansion is highly restricted to the apical tip of hyphal filaments. This polarized apical growth requires the actin cytoskeleton. *C. albicans* yeast cells display a temporal change in the organization of the actin cytoskeleton during cell cycle progression like that in *S. cerevisiae* cells, whereas the actin cytoskeleton is polarized at the tip of all hyphal cells during filamentation [12]. In *S. cerevisiae* and other organisms, the small GTPase of the Rho subfamily Cdc42p is known to be critical for establishing a polarized actin cytoskeleton in response to extracellular stimuli. In the filamentous ascomycete *Ashbya gossypii*, three Rho-GTPases control distinct steps during polarized hyphal growth with Cdc42p being required for the establishment of actin polarization [499]. *C. albicans* Cdc42p is also essential for cell morphogenesis [193] as down-regulated in our hypha defect mutant cells, is probably responsible for establishing the polarized actin cytoskeleton during hyphal induction.

Transcriptional control and chromatin remodelling proteins

The organization of the eukaryotic chromatin has a major impact on all nuclear process involving DNA substrates. Gene expression is affected by the positioning of individual nucleosomes relative to regulatory sequence elements, by the folding of the nucleosomal fiber into high-order-structures and by the compartmentalization of function domains within the nucleus. Because site-specific acetylation of nucleosomal histones influence all three aspects of chromatin organization, it is central to the switch between pemissive and repressive chromatin structure. The targeting of enzymes that modulate the histone acetylation status of chromatin, in

synergy with the effects mediated by other chromatin remodelling factores, is central to gene regulation.

Chromatin structure and nucleosomes

Sience the discovery of the basic principles of chromatin organization, which is involves in the wraping of DNA around histone octamers to form nucleosomes and the folding of the nucleosomal fiber in to higher-order-structures, the question of how such extensive packaging can be compatible with reactions that be involve `reading` the DNA has stimulated extensive reaserch. The fundamental unit of chromatin is the nucleosome, which consists of 147 base pairs of DNA wrapped 1.6 times around a core of eight histones (two molecules of H2A, H2B, H3 and H4) (Figure 1.6 A, B) [284,290]. Chromatin structure is central for the regulation of gene expression [471], and defined as a complex of DNA, histones, and non-histone proteins from which eukaryotic chromosomes are formed.

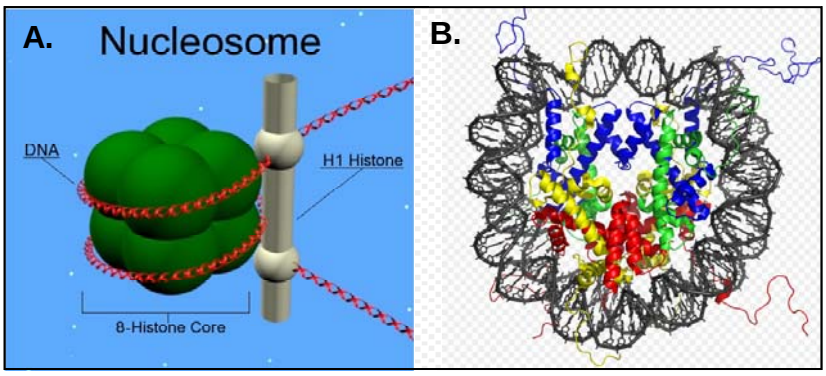

Figure 1.6 (**A**) Nucleosome structure. (**B**) The crystal structure of the nucleosome core particle consisting of H2A, H2B, H3 and H4

The core histones are small proteins with a basic charge that share the same basic structure: a globular C-terminal domain critical to nucleosome formation and a flexible N-terminal tail that protrudes from the nucleosome core [404].

It soon became apparent many aspects of chromatin structure could be explained by interaction between nuclesomal histones and DNA, neighboring nucleosomes and non-histone proteins. Most of these interactions involve the N-terminal tails of the core histones, which reach out from the rather compact nuclesomal core particle. The flexible N-terminal tails are enriched with basic residues which are theorized to interact with the negatively charged backbone of DNA [10]. The four canonical histone proteins are composed of a structured central (globular) domain

that is in close contact with the DNA and a less well-structured amino-terminal tail domain. Histones are also distinguished by the fact that they undergo abundant post-translational modifications to both the globular and tail domains. In the presence of linker histones or heterochromatin associated-proteins, nucleosomes can be condensed into 30 nm diameter fibers and high-ordered assemblies whose structures are poorly understood. These hierarchical structures form a repressive environment that inhibits the activities of enzymes that require direct access to the DNA template, such as in transcription, DNA repair, replication and recombination. Chromatin condensation can be modulated through a variety of mechanisms, including covalent modifications of DNA and histones. While DNA undergoes methylation of its cytosine bases within CpG repeats, histones are subject to a myriad of modifications in their random coil N-terminal tails, and to a lesser extent within their C-terminal tails and globular domains [245]. The 1960s and 1970s witnessed the discovery of a multitude of histone modifications including lysine acetylation [9], lysine methylation [9,335], serine phosphorylation [238], ADP ribosylation [352] and ubiquitination [152,214]. Although data from many laboratories furnished circumstantial evidence linking modification states to the transcriptional status of genes, the exact biological functions of histone modifications remained enigmatic for several decades.

In 1996, two groups reported the discovery of histone modifying enzymes that were related by sequence homology to previously identified transcriptional regulators in *S. cerevisiae*. Utilizing an affinity matrix, Schreiber et al [305,411] isolated a mammalian histone deacetylase (HDAC) that harbors 60% sequence identity with the yeast transcriptional repressor Rpd3p [462]. Concurrently, Allis et al [11] purified a histone acetyltransferase (HAT) from *Tetrahymena thermophila* that is highly homologous to the yeast transcriptional adaptor Gcn5p [45]. The identification of these enzymes represented a milestone in understanding the biological functions underlying histone modifications because they furnished the first direct evidence unequivocally linking histone modification states and transcriptional regulation. The discovery of Gcn5p and Rpd3p presaged the identification and characterization of other families of HATs and HDACs as well as other classes of histone modifying enzymes, including kinases [405,469], lysine and arginine-specific methyltransferases [89,380], arginine deiminases [78,496], ubiquitinases [387], deubiquitinases [119,187], and lysine- and arginine-specific

demethylases (HDMs) [84,473]. Some studies had implicated many of these proteins in transcription regulation or other genomic functions, further underscoring the correlation between histone modifications and chromatin-dependent processes. Collectively, these efforts have transformed our understanding of the role of chromatin modifications in governing eukaryotic gene expression and other DNA-dependent functions. Following their discovery, structural and biochemical studies have played a central role in defining the molecular basis for the substrate specificities and catalytic mechanisms of histone modifying enzymes. This paragraph of chapter summarizes current advances in understanding these enzymes, focusing on mechanisms of HATs (*GCN5*) and HDACs (*HDA1*).

Histone acetylation and transcriptional control

Activation of transcription in eukaryotes often requires modification of the chromatin template. Among the well-characterized chromatin-modifying enzymes, histone acetyltransferases (HATs) are known to regulate gene expression by adding acetyl groups to lysine residues within the N-terminal tails of histones [392].

Transcriptional activators often require histone acetyltransferases (HATs) for full activity. The common explanation is that activators directly recruit HATs to gene promoters to locally hyperacetylate histones and thereby facilitate transcription complex formation [216]. A number of HAT enzymes have been isolated from various organisms [57]. The identification of new HATs has resulted in other important findings. It has been demonstrated that HATs are evolutionarily conserved from yeast to humans, that HATs generally contain multiple subunits [236], and that the functions of the catalytic subunit depend largely on the context of the other subunits in those complex [480]. Furthermore, recent work on HAT complex has resulted in their categorization on the basis of their catalytic domains. The picture is complicated by the observation that some HAT enzymes can modify different histone substrates and that some HAT enzymes also acetylate an ever growing number of non-histone substrates [523]. In addition, histone acetylation is a dynamic reversible process. The balance of histone acetylation is important for proper cellular function and the cell has evolved enzymes that catalyse the removal of acetyl groups, termed histone deacetylases (HDACs) [118]. HATs are a diverse set of enzymes; the multiprotein complexes in which they reside also vary. Different HAT complex are composed of various unique subunits. The combinations of these

subunits contribute to the unique features of each HAT complex. For example, some subunits have domains that cooperate to recruit the HAT to the appropriate location in the genome; these include bromodomains, chromodomains, WD40 repeats, Tudor domains and PHD fingers. It has been discussed various histone substrates of HAT complex and the various HAT subunits, and how protein domains read and interpret the 'histone code' that is, the combination of post-translational marks on the histones [269].

HATs serve a multiplicity of functions

As mentioned a number of different HAT complex exist in yeast and other organisms, and these HAT complex are composed of assorted proteins with various chromatin-binding domains that influence HAT recruitment. What is the reason for such complexity? In large part, the answer lies in the fact that HATs serve a multiplicity of functions. First, HAT complex modify histones in relation to the state and/or function of the DNA that the histones are compacting. As discussed above, HAT enzymes take advantage of their associated proteins as well as cellular signals for their recruitment to distinct regions of the genome. In turn, these associations allow them to carry out specialized functions, which the enzyme would not be capable of on its own [256,452]. Such specialized functions of HATs include roles in processes such as genome stability and DNA repair. Second, HAT enzymes do not only modify histones, but function to acetylate an increasing number of non-histone substrates [523]. For example, many HATs also function as transcription factor acetyltransferases (FATs), which means that these same enzymes can acetylate nonhistone substrates to regulate their activities in the cell [244,523]. Recently, much research has gone into deciphering specific functions of HATs and the mechanisms by which they carry out these functions. As is the case with the function of many proteins, there are a number of factors that can influence the manner in which they function. The same is true for HATs. HATs carry out a broad range of functions and the regulation of these functions is in many cases vital for maintaining the integrity of the organism.

Histone acetyltransferases *GCN5*

The yeast transcriptional coactivator *GCN5*, a histone acetyltransferase (HAT), is part of a large multimeric complex that is required for chromatin remodeling and

transcriptional activation [125]. In the yeast *S. cerevisiae*, one of the best known HATs is Gcn5p, which is the catalytic subunit of the SAGA complex that acetylates primarily histones H3 and H2B [453,535]. Work in recent years has provided overwhelming evidence that this HAT-containing complex functions as a coactivator and directly participates in the transcription process by being specifically recruited by activators to the promoter of many genes [392]. Recent genome-wide location studies suggest that Gcn5-containing complex may actually be recruited by regulatory proteins to the upstream activating sequence (UAS) of most active genes [385].

In budding yeast the histone acetyltransferase Gcn5p is involved in cell cycle progression, whereas its absence induces several mitotic defects, including inefficient nuclear division, chromosome loss, delayed G2 progression, and spindle elongation. The fidelity of chromosome segregation is finely regulated by the close interplay between the centromere and the kinetochore, a protein complex hierarchically assembled in the centromeric DNA region, while disruption of *GCN5* in mutants of inner components results in sick phenotype. In these synthetic interactions involving the ADA complex lay the genetic basis for the critical role of Gcn5p in kinetochore assembly and function. Gcn5p have been physically linked to the centromere, where it affects the structure of the variant centromeric nucleosome [488]. Gcn5p, a major HAT in yeast, has diminished histone acetyltransferase activity in particular mutants, providing a plausible explanation for reduction of cellular acetylation levels *in vivo*.

C. albicans histone acetyltransferase *GCN5*

The yeast transcriptional coactivator *GCN5* is part of large multimeric complex that are required for chromatin remodeling and transcriptional activation. Like other eukaryotes, the *C. albicans* DNA is organized into nucleosomes and the genome encodes components of chromatin-remodeling complex. *GCN5* is conserved in *Candida* species and that the most homologous regions are within the HAT domain and the bromodomain (Figure 1.7 and 1.8).

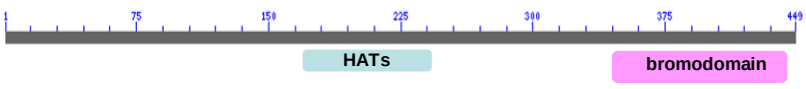

Figure 1.7 Diagram representing the Hat and Bromodomain of *C. albicans GCN5*

Many yeast genes encode histone acetytransferase and histone deacetylase proteins in *C. albicans* and their relative phenotype are totally unknown. Thus, our laboratory have performed a phenotypic analysis of yeast strain carrying deletions of *GCN5* and other genes encoding members of the HATs and HDACs family of *C. albicans.* The *C. albicans* genome contains six ORFs that potentially encode histone deacetylases proteins containing the HDACs domine and five histone acetyltransferases. A search of the NCBI database (*http://www.ncbi.nlm.nih.gov/BLAST/*) indicates that a *Candida albicans* protein named *Gcn5* (orf19.705) shares the highest similarity with *Pichia guilliermondii* ATCC 6260 (81%), *Pichia stipitis* (78%), *Lodderomyces elongisporus* (70%), *Debaryomyces hansenii* (73%) and *Saccharomyces cerevisiae* (70%)(Figure 1.8). The GeneBank accession number for the *C. albicans GCN5* nucleotide sequence is NW_139602.

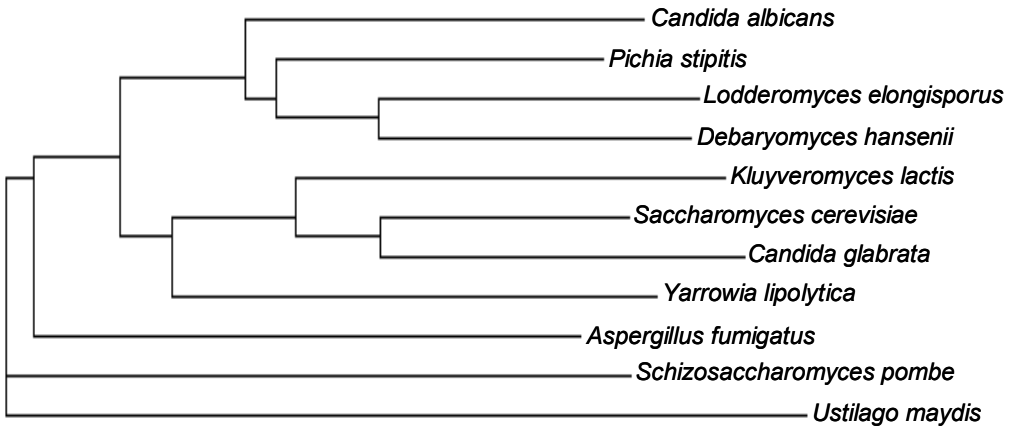

Figure 1.8 Dendograms representing the phylogenetic relationships of *C. albicans GCN5*

Yeast Gcn5p, the ancestor of the histone acetyltransferase (HAT) family, marks histone H3 and H4 tails with an ε-acetyl group on specific lysines [392,516]. Gcn5p does not act on its own but rather as the catalytic subunit of two separate and conserved HAT complexes named ADA and SAGA (Spt-Ada-Gcn5-acetyltransferase). Specifically, Spt proteins are exclusively present in SAGA, while Spt20p is necessary for maintaining the integrity and function of the whole complex [517]. Histone acetylation by Gcn5p is implicated in the displacement of nucleosomes from promoters during transcriptional activation and also in aiding the recruitment of TATA binding protein, RNA polymerase II and coactivators [154]. Acetylation therefore facilitates the formation of an accessible “open” chromatin structure corresponding to the transcribing genome [99,251,449]. During cell

division, chromatin remodeling expands to wide chromosomal regions, producing long waves of compaction and decondensation over the whole genome at each cell division [93,489]. Disturbing the HAT/histone deacetylase balance therefore alters protein activities on a cellular scale, which leads to various diseases, including cancer. Several reports have highlighted the involvement of Gcn5p alone or in combination with other HATs such as Sas3p in the cell cycle [199,223] and in the transcriptional regulation of a set of genes required at the end of telophase [247]. In mammalian cells, loss of the homologue Gcn512p is lethal during embryogenesis, induces a high level of apoptosis [519], and affects G2/M transition in null mouse embryonic stem cells [277]. In yeast, the specialized centromeric nucleosome, which contains the histone H3 variant Cse4p [318], is necessary for the assembly and interactions of inner kinetochore components at the centromere [72] and for the correct attachment of the chromosomes to the spindle in metaphase. Mutations in the centromeric/kinetochore components or epigenetic modifications of this structure may lead to chromosome missegregation and G2/M delay. In yeast Gcn5p is, in fact, physically linked to the centromere, where it affects the structure of the variant centromeric nucleosome. It offers a key insight into a Gcn5p-dependent epigenetic regulation at centromere/kinetochore in mitosis [488].

HAT's and their biological functions

In eukaryotes, chromatin reorganization is essential for transcription, and histone acetylation has been directly implicated in this process owing to the discovery that transcriptional activators associate with histone acetyltransferases (HATs) and transcriptional repressors recruit histone deacetylases. Although the precise molecular underpinnings are still being defined, recent evidence suggests that histone acetyltransferases also play important roles in histone-modulated processes such as DNA replication, recombination and repair. In addition, acetylation of transcriptional cofactors and other proteins is an efficient means of regulating a diverse range of molecular interactions [90,448]. Moreover, acetylation (Ac) of histones has been widely demonstrated as an important post-translational modification that can regulate many DNA-based processes including gene expression [252,449]. There are numerous lysines (K) in both the amino termini and the globular domains of the core histones including K_9, K_{14}, K_{18}, K_{23}, K_{27} and K_{56} in histone H3 and K_5, K_8, K_{12} and K_{16} in H4 as well as other lysines in histones

H2A and H2B. These lysines can be acetylated or deacetylated by various histone acetyltransferases (HATs) or deacetylases (HDACs) that show specificity toward histone subtypes as well as individual residues within a given histone. For instance, Gcn5p, a member of the Spt-Ada-Gcn5-acetyltransferase (SAGA) complex in *S. cerevisiae*, is the main HAT for lysine residues in the amino terminus of histone [160,161,251] whereas Rtt109p specifically acetylates K_{56}, a lysine in the globular domain of H3 [174]. Moreover, it is apparent that other DNA-based processes such as replication, recombination and repair require chromatin remodeling and that histone acetylation may play a key role. HAT's can be classified with respect to their intracellular location and substrate specificity as either nuclear A-type (HAT A) or cytoplasmic B-type (HAT B). A-type HAT's are involved in the post-synthetic acetylation of all four nucleosomal core histones and have long been thought to promote transcription related acetylation although their involvement in other processes such as DNA repair and replication is also likely [249]. Conversely B-type HAT's are believed to have a housekeeping role in the cell, acetylating newly synthesised free histones (primarily histone H4 at lysines 5 and 12) in the cytoplasm for transport into the nucleus, where they may be deacetylated and incorporated into chromatin [11,241,394]. It is important to note that HATs function in several other processes that are becoming clear, including DNA replication and recombination. The study of HATs is a broad and complex field that is still expanding, and although our knowledge of these important proteins and complex has increased substantially in recent years there is still much to learn. In time, acetylation and subsequent deacetylation of proteins might be looked at in a similar fashion as phosphorylation and dephosphorylation in regulating cellular processes.

Histone deacetylases

Histone tails are normally positively charged due to amine groups present on their lysine and arginine amino acids. These positive charges help the histone tails to interact with and bind to the negatively charged phosphate groups on the DNA backbone. Chromatin acetylation correlates with transcriptional activity (euchromatin), whereas deacetylation correlates with gene silencing. HDACs are also involved in the reversible acetylation of non-histone proteins. Yeast cells contain a group of related HDACs that include Rpd3p, Hda1p, Hos1p, Hos2p and Hos3p [396,462]. The earliest example of the targeting of HDACs to a regulatory

sequence was that of the Rpd3p, which is a member of the class I HDACs (the mammalian orthologues of which include *HDAC1*), *HDAC2* and *HDAC3*. In yeast, Rpd3p belongs to a large multiprotein complex that includes Sin3p, Sap30p [537], Sds3p [33,98,266], Pho23p [283] and Ume1p [253], and several other uncharacterized factors [144]. Yeast Hda1p (which is a member of the class II HDACs) is related to *HDAC4*, *HDAC5* and *HDAC6* in mammals, and is recruited to its target promoters through the Tup1p repressor [515]. Similar to Rpd3p targeting, recruitment of the Hda1p complex results in local deacetylation in a region spanning about 2 nucleosomes that are adjacent and downstream of its recruitment site. However, unlike the broad substrate specificity of Rpd3p, the Hda1p complex only deacetylates Lys residues in histones H3 and H2B (but not the H4 or H2A) amino-terminal tails [515]. The acetyltransferase Esa1p, which is the catalytic component of the NuA4p (for nucleosome acetyltransferase of histone H4) multiprotein complex, acetylates H4 and H2A histones [8,453] and seems to be targeted to promoters of the ribosomal protein genes in yeast [381]. The functional roles of these various substrate specificities for HDACs and HATs are as yet unknown. Although acetylation microarray data for HATs have not been reported so far, the genome-wide expression analyses of deletion mutants (*GCN5* and *HDA1* as simple and double mutant) of these enzymes in *C. albicans* have provided some evidence for a division of labour among HATs and HDACs.

Crosstalk between histone acetylation/deacetylation and chromatin remodeling proteins

It has been shown that ATP-dependent chromatin remodeling factors cooperate with histone acetyltransferases (HATs) and histone deacetylases (HDACs) in gene regulation. The functional link between ATP-dependent remodeling and histone acetylation was first suggested from genetic studies in yeast [369]. Mutations in Gcn5p of the SAGA complex in combination with mutations in the SWI/SNF complex resulted in lethal phenotypes, indicating a concerted interaction between components of these two complexes. It is suggested that SWI/SNF-dependent remodeling occurs prior to Gcn5-dependent histone acetylation [248], and is required for acetylation to occur. This observation implies that histone acetyltransferase complex cannot penetrate the compact chromatin without remodeling. Alternatively, histone acetylation may occur followed by ATP-

dependent remodeling, thereby altering chromatin structure for general transcription factor binding [1], or providing a better surface for stabilization of ATP-dependent remodeling complex [179]. Isw2p is an ISWI family ATP-dependent remodeling complex in yeast. Although Isw2p and Sin3p-Rpd3p histone deacetylase complex have unique biochemical activities, genetic studies indicate that Isw2p and Sin3p-Rpd3p function synergistically to regulate gene repression [126]. Both nucleosome remodeling ATPase Mi-2 and histone deacetylases (*HDAC1/2* in mammals and *RPD3* in *X. laevis*) reside in the NURD (nucleosome remodeling and deacetylation) complex, implying that histone modification (deacetylation) and ATP-dependent remodeling are synergistic actions in chromatin remodelling [172,240]. The ATPase activity of Mi-2 was shown to be able to increase the efficiency of histone deacetylation by NURD complex [172].

cDNA microarray analysis of differential gene expression in *C. albicans*

The near completion of sequencing the *C. albicans* genome has made it possible to employ genomic technologies, such as microarray analysis, to aid in identifying key genes involved in such clinical problems as the acquisition of high-level resistance to azole antifungal agents. In addition, DNA microarrays provide a snapshot of an organism's genome in action by revealing the relative transcript levels of thousands of genes at a time [281]. Microarray analysis gives researchers the ability to identify genes involved in processes such as acquisition of azole resistance and to use the data in a way that may lead to clinical approaches to inactivate these genes and improve patient outcomes. Differences in gene expression underlie many of the phenotypic variations in *C. albicans*, yet approaches to characterize such differences on a genome-wide scale are well developed by using cDNA microarray techniques. Phenotypic diversity can often be traced to the differential expression of specific regulatory genes [56,153]. Recently, microarray experiments revealed large-scale differences in the genome-wide transcription response of related organisms to equivalent environmental conditions. For example, the transcription program underlying insect metamorphosis differs considerably between related species of the *Drosophila melanogaster* subgroup [384]. Similarly, both the meiotic and the mitotic cell cycle transcription program have diverged significantly between the budding and the fission yeasts [397]. The impact of such large-scale variations in gene expression on the phenotypes of the organisms is not yet understood.

Existing computational approaches for the comparative analysis of microarray gene expression data have focused primarily on evolutionarily distant model organisms, for which large sets of expression data are available [7,315]. Such studies demonstrated that conservation of co-expression can improve functional gene annotation [30,450]. Common expression programs are invoked by related perturbations, such as adult onset in the nematode *Caenorhabditis elegans*, and the fruit fly *D. melanogaster* [315].

Table 1.1 Some of example about cDNA microarray analysis of differential gene expression in *C. albicans*

Analysis/publication	Abstract of result
Drug resistance	
cDNA Array Analysis of the Differential Expression Change in Virulence-related Genes During the Development of Resistance in *Candida albicans* [520]	In this work, 16 virulence-related genes were identified, whose differential expressions were newly found to be associated with the resistant phenotype. Furthermore, the up-regulation of *EFG1, CPH2, TEC1, CDC24, SAP10, ALS9, SNF1, SPO72* and *BDF1*, and the down-regulation of *RAD32,* IPF3636 and *UBI4* resulted in stronger virulence and invasiveness in the resistant isolates compared with susceptible ones. These findings provide a link between the expression of virulence genes and development of resistance during *C. albicans* infection in bone marrow transplant (BMT) patients, where induces hyphal formation and expression change in multiple virulence factors.
Genome-wide expression profiling of the response to ciclopirox olamine in *Candida albicans* [272]	By cDNA microarray method, 49 genes were found to be responsive to ciclopirox olamine, including 36 up-regulated genes and 13 down-regulated genes. These included genes involved in small molecule transport (*HGT11*, *HXT5*, *ENA22*, *PHO84* and *CDR4*), iron uptake (*FRE30*, *FET34*, *FTR1*, *FTR2*, *SIT1*) and cell stress (*SOD1*, *SOD22*, *CDR1*, *DDR48*). Mutants disrupted for *CDR2* and both *CDR1* and *CDR2*, as well as a clinical isolate overexpressing *CDR1* and *CDR2*, showed no change in susceptibility to ciclopirox olamine compared with the respective parent.
A gain-of-function mutation in the transcription factor Upc2p causes up-regulation of ergosterol biosynthesis genes and increased fluconazole resistance in a clinical *Candida albicans* isolate.[114]	By comparing the gene expression profiles of the fluconazole-resistant isolate and of strains carrying wild-type and mutated *UPC2* alleles, they identified target genes that are controlled by Upc2p. They show for the first time that a gain-of-function mutation in *UPC2* leads to the increased expression of *ERG11* and imparts resistance to fluconazole in clinical isolates of *C. albicans*.
A genome-wide steroid response study of the major human fungal pathogen *Candida albicans* [21]	In the absence of steroid receptors and any known mechanism of gene regulation by steroid hormones in *Candida albicans.* They did a genomewide analysis of *C. albicans* cells treated with progesterone using cDNA microarrays to find the complete repertoire of steroid responsive genes. A total of 99 genes were found to be significantly regulated by progesterone, among them 60 were up-regulated and 39 were down-regulated. Several genes associated with hyphal induction and the establishment of pathogenesis was also found up-regulated. In silico search for various transcription factor (TF) binding sites in the promoter of the affected genes revealed that *EFG1, CPH1, NRG1, TUP1, MIG1* and AP-1 regulated genes are responsive to progesterone.
The transcription factor Mrr1p controls expression of the *MDR1* efflux pump and mediates multidrug resistance in *Candida albicans* [326]	By comparing the transcriptional profiles of drug-resistant *C. albicans* isolates and *mrr1*Δ mutants derived from them and of *C. albicans* strains carrying wild-type and mutated *MRR1* alleles, they defined the target genes that are controlled by Mrr1p. Many of the Mrr1p target genes encode oxidoreductases, whose up-regulation in fluconazole-resistant isolates may help to prevent cell damage resulting from the generation of toxic molecules in the presence of fluconazole and thereby contribute to drug resistance. The identification of *MRR1* as the central regulator of the *MDR1* efflux pump and the elucidation of the mutations that have occurred in fluconazole-resistant, clinical *C. albicans* isolates and result in constitutive activity of this trancription factor provide detailed insights into the molecular basis of multidrug resistance in this important human fungal pathogen.
Yeast-hypha transition	
The Flo8 transcription factor is essential for hyphal development and virulence in *Candida albicans* [54]	Genome-wide transcription profiling of *efg1/efg1* and *flo8/flo8* using a *C. albicans* DNA microarray suggests that Flo8 controls subsets of Efg1-regulated genes. Most of these genes are hypha specific, including *HGC1* and *IHD1*.
In vivo and *ex vivo* comparative transcriptional profiling of invasive and non-invasive *Candida albicans* isolates identifies genes associated with tissue invasion [466]	By analysing the different phases of intraperitoneal infection from attachment to tissue penetration in a time-course experiment and by comparing the profiles of an invasive with those of a non-invasive strain, they identified genes and transcriptional pattern which are associated with the invasion process. This includes genes involved in metabolism, stress, and nutrient uptake, as well as transcriptional programmes regulating morphology and environmental sensing.

A generalization of the singular value decomposition approach that is applicable for such a comparative study was applied to cell cycle datasets from *S. cerevisiae* and human [7]. Yet, the challenge of systematically comparing the gene expression program in related organisms is only starting to be addressed.

Polyamines

Although simple in structure, the polycationic polyamines spermidine and spermine and their diamine precursor putrescine are essential factors for growth in eukaryotic cells [146,313] (Figure 1.9). The total intracellular concentration of the polyamines is in the millimolar range; however, free polyamine concentrations are considerably lower, as they are mostly ionically bound to various anions in the cell including DNA, RNA, proteins and phospholipids. The original discovery of spermine dates back to Leuwenhoek in 1678, however the tools necessary to study the specific molecular functions in which the polyamines are intimately involved have only recently become available. Understanding the molecular functions of polyamines is complicated by the fact that most of the critical interactions in which the polyamines are involved in readily reversible ionic interactions. Among the roles that polyamines have in the support of cell growth are association with nucleic acids, maintenance of chromatin conformation, regulation of specific gene expression, ion-channel regulation, maintenance of membrane stability, provision of a precursor in the synthesis of eukaryotic translation initiation factor 5A (IF5A), and free-radical scavenging [137,363]. Several recent studies have been instrumental in furthering our understanding of the molecular functions of polyamines. Polyamines and polyamine metabolism could be used as targets for antiproliferative therapy. This fact is based on several observations suggest that cells synthesize more polyamines when induced to grow; and that the polyamine biosynthetic enzymes are co-ordinately regulated with growth controls. Polyamine metabolism is frequently dysregulated in cancers. Data indicating that the polyamine pathway is a downstream target for known oncogenes and that inhibition of polyamine synthesis disrupts the action of those genes have been published [63,349]. The fact that polyamines appear to be a site of intervention that is distal and common to a number of validated targets makes this target even more intriguing, and validates the active pursuit of agents that interfere with both polyamine metabolism and function as a strategy for antiproliferative intervention.

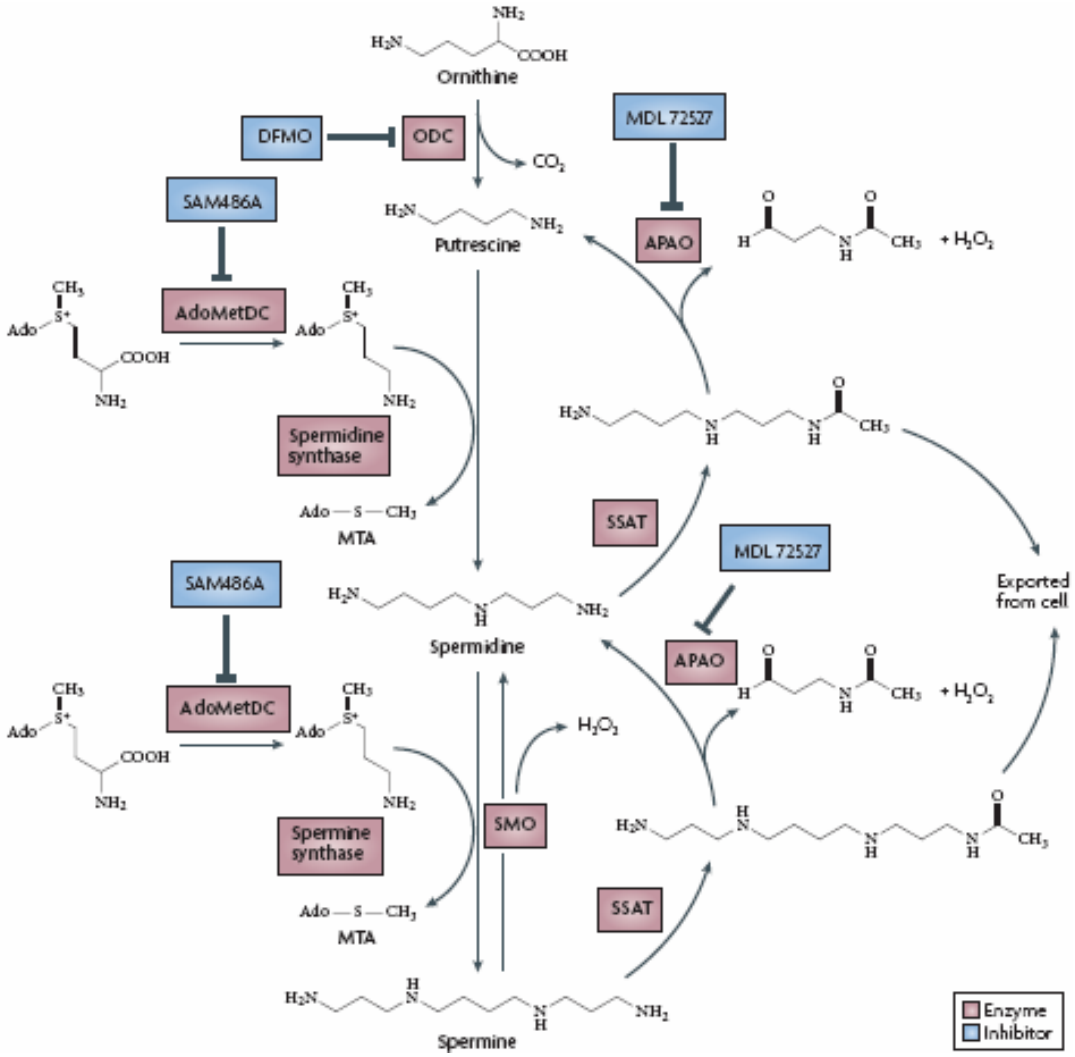

Figure 1.9 Pathway of polyamine synthesis in eukaryotic organisms **SAM**, S-adenosylmethionine; SAMdc and decarboxylated S-adenosylmethionine, difluoromethylornithine (**DFMO**), *N*, *N'*-bis(2,3-butadienyl)-1,4-butanediamine (**MDL-72,527**), *N*1-acetylpolyamine oxidase (**APAO**), spermidine/spermine *N*1-acetyltransferanse (**SSAT**), mammalian spermine oxidase (**SMO**), S-adenosylmethionine (**SAM486A**), S-Adenosylmethionine decarboxylase (**AdoMetDC**).

Although polyamines are abundant and ubiquitous components of all cells, relatively little is known about their function. In mammalian cells, there is a positive correlation between rapid growth and high levels of polyamines [14,319]. Furthermore, addition of growth factors to quiescent cells leads to a rapid induction of polyamine synthesis. The biosynthesis of the naturally occurring polyamines, spermidine and spermine, is tightly regulated by a variety of mechanisms [58,167,278,325,367,418]. Fine control of intracellular polyamine levels is necessary, because overproduction of these compounds can lead to cellular toxicity and oncogenic transformation [325]. In *C. albicans*, polyamine depletion is lethal and suboptimal concentrations inhibit the yeast-hypha transition [188].

Polyamine metabolism

The first rate-limiting step in polyamine biosynthesis is the production of putrescine by the pyridoxal, phosphate-dependent decarboxylase ornithine decarboxylase (ODC). The requirement for ODC activity (and putrescine production) in cell proliferation has been demonstrated in most eukaryots. *ODC* expression is tightly regulated at several steps from transcription to post-translational modification [365]. ODC is active as a homodimer with a half-life that is among the shortest of any known protein [490] (10-30 minutes in mammalian systems), and, like many short-lived proteins, ODC is destroyed by the 26S proteosome. However, unlike most proteins, ODC is not first ubiquitinated, instead a specialized regulatory protein, ODC antizyme [181], binds to the carboxylic end of the monomeric form of ODC and presents the ODC to the 26S proteasome for destruction [67,533]. *ODC* transcription is another important regulatory step in its expression and ODC was the first transcription target of the *MYC* oncogene to be reported [362], which demonstrates one link between two crucial growth-promoting pathways. It is important to note that spermine is primarily a polyamine found in eukaryotic cells, as prokaryotes do not have a spermine synthase homologue.

Ornithine decarboxylase (ODC)

Ornithine decarboxylase (ODC) catalyzes the conversion of ornithine into putrescine, the first step in the universal mechanism of polyamine biosynthesis (Figure 1.9), and is one of the most highly regulated enzymes of eukaryotic cells [69,101,206,219]. This control is essential, since polyamine excess is toxic and induces apoptosis [361,371]. The most important control mechanism of *ODC* in eukaryotic organisms operates at the post-translational level, regulating the amounts of the enzyme by a unique mechanism of proteasome-dependent degradation [180,186]. Originally described in higher eukaryotes, the mechanism has been further described in a few fungal species [87]. This is the first and rate-limiting reaction in the biosynthesis of polyamines in cells [365]. Even modest reductions in mammalian ODC activity can lead to marked resistance to tumor development [348,365]. ODC is turned over rapidly, and this, in part, is because of it being targeted to the 26S proteasome without ubiquitination by the protein antizyme, its key regulator [333,532]. Antizyme itself is negatively regulated by

antizyme inhibitor, a homolog of ODC with a higher affinity for antizyme that has lost the ability to decarboxylate ornithine [5,332,433,458]. In addition to its post-translational regulation, ODC is under transcriptional and translational layers of control. Intracellular levels of polyamines are regulated at various steps, including synthesis, degradation, uptake, and excretion, and cells have developed intricate mechanisms to ensure tight regulation of intracellular polyamine pools [492].

Function of polyamines in eukaryotic cells

The function of polyamines in mammalian cells may be much more complex, in view of their implicated roles in the regulation of diverse cellular activities such as transcriptional, translational, and posttranslational levels affecting proliferation, transformation, differentiation, and apoptosis. Indeed, by usinng inhibitors [351], have demonstrated an independent role for polyamines and eIF5A in supporting the proliferation of mouse mammary carcinoma cells. In addition, it has been shown [336], that either spermidine or spermine derivatives can support growth of polyamine depleted SV-3T3 cells without the need for metabolic conversion. Furthermore, cytostasis of mammalian cells induced by the ornithine decarboxylase (ODC) inhibitor DFMO or a *S*-adenosylmethionine decarboxylase inhibitor [530] has been reported to have two phases, an acute initial phase due to reduction of total cellular polyamines and a delayed second phase due to eventual deprivation of eIF5A. In fungi, polyamines are essential for distinct differentiation processes, such as spore germination, sporulation and dimorphism [395]. In *Ustilago maydis* [170] and *Yarrowia lipolytica* [221], polyamine concentrations higher than those necessary to ensure vegetative growth are required to sustain dimorphic transition. It has been reported that polyamines, mainly putrescine, might play an active role in the pathosystem maize -*U. maydis* [389].

Transcriptional regulation of the ODC-encoding gene in higher eukaryotes occurs under different conditions [68]. In fungi contrasting data exist. Evidence of transcriptional regulation of the homolog genes has been described in *Neurospora crassa* Spe1p [206] and *Mucor circinelloides* [35], whereas no transcriptional regulation of the corresponding genes was observed in *U. maydis* [170], *Candida albicans* [285] and *Coccidioides immitis* [169], during the dimorphic transition.

The possible significance of the transcriptional regulation by polyamines of the gene encoding *ODC* in fungi may depend on its interrelation with the post-

translational regulation of the enzyme. This offers a rapid response of the cell to the environmental conditions, but is energetically expensive, since it depends on the inactivation of *ODC* [67]. Transcriptional regulation would respond more slowly, but would save energy to the cell, controlling the amounts of transcript and enzyme. The simultaneous operation of both mechanisms would provide a more efficient mechanism for the control of the cellular functions that depend on the levels of polyamines. Jiménez-Bremont and Ruiz-Herrera in 2008 by analysis of the transcriptional regulation of *YLODC* gene from the dimorphic fungus *yarrowia lipolytica* reported that there exists a weak but reproducible transcriptional regulation of *YlODC* under the analyzed conditions. Their data suggest that gene transcription is down-regulated by putrescine, and stimulated by spermidine. It is suggested that the simultaneous operation of transcriptional and post-translational regulatory mechanisms endows the cell with a more efficient mechanism to control the physiological processes that depend on polyamines [220]. Accordingly, whether transcriptional regulation of the *ODC*-encoding genes is widespread, and how regulation occurs, remains unknown. In this work, we report a study of the transcriptional regulation of the gene encoding *ODC* in the dimorphic fungus *C. albicans* (Chapter IV).

Polyamine auxotrophs of *Candida albicans*

Strains of *C. albicans* have been constructed that are unable to synthesize ornithine and are thereby deficient in polyamine biosynthesis. This strain has been used to develop a protocol for observation of mutant phenotype cells under varius condition that blocked directly in polyamine synthesis. It has been demonstrated previously in our laboratory that low concentrations of putrescine (0.01 mM) are sufficient for near-normal growth of an *odc* mutant polyamine auxotroph of *C. albicans.* Homozygous null *Candida* mutant grew exclusively in the yeast form at low polyamine levels (0.01 mM putrescine) under all conditions tested. An increase in the polyamine concentration (10 mM putrescine) restored the capacity to switch from the yeast to the filamentous form. The strain with a deletion mutation also showed increased sensitivity to salts and Calcofluor white. This *Candida odc/odc* mutant was virulent in a mouse model. The results suggest a model in which polyamine levels exert a pleiotrophic effect on transcriptional activity, and that polyamine levels control a broad range of gene functions [188].

Transcriptional response of *Candida albicans* to temperature

Abstract

One of the main parameters involved in the yeast-to-hypha transition in *Candida albicans* is temperature and this transition is involved in pathogenicity. A complete switch between both forms -yeast and hypha- can be achieved by changing temperature from 28ºC to 37ºC in minimal medium; namely, Lee medium. Here we carried out a genome-wide analysis of the response of *C. albicans* to temperature after 15, 60 and 180 min exposure to 37ºC. Using a cDNA microarray method, a total of 781 genes were found to change at the consensus level of ≥ 1.5-fold, including 401 up-regulated genes and 380 down-regulated genes. 341, 403 and 251 genes changed after 15, 60, and 180 min of incubation at 37ºC, suggesting that the key time for controlling the temperature switch and the dimorphic change occurs very early and that mainly housekeeping hyphal genes are expressed after three hours.

Of those genes, 43 were common to the three times assayed (24 and 19 up- and down- respectively); 68 and 52 genes were common between 15 and 60 min. and 60 and 180 min., respectively, and variations in 222, 242 and 148 genes were found only at one time of induction (15, 60 and 180 min. respectively). Only 8 genes were common to 15 and 180 min. indicating a sequential gene expression and confirming the validity of our approach. Of the genes common for the three times assayed, 3 corresponded to genes coding for proteins of unknown function. The main overrepresented categories were cell wall genes with 9 genes (seven up-, *ALS1, ALS3*, *ECE1*, *HWP1, PHR1, RBT1*, *SUN41* and two down-, *PIR1* and *YWP1*), transporters CA3966, *ENA21, ENA2, GAP6* and *PHO84* up- and four down-, *MEP2, PMA1* and *OPT2* and *OPT3* (two oligopeptide transporters; genes induced transcriptionally) upon phagocytosis by macrophages; and fluconazole-induced, dehydrogenases (*ARD, PUT2* and *GDH3*), and oxidase/peroxidase-encoding genes (*AMO1, AOX2* and *GPX2*). We also detected the down-regulation of three glycolytic genes (*PDC11, PFK1* and *PFK2*) and the up- and down-regulation of two transcription factors: *GAT2* and *STP3* (the latter of which regulates *SAP2*). The main categories of common genes after 15 and 60 min of incubation at 37ºC belonged to the following categories: ribosomes (*ERF1*, *RPL25*, *RPL33, RPS10* and *RPS13*) and glycolysis (*ADH1*, *ADH2*, *ENO1*, *FBA1*, *PGI1*, *PGK1* and *TPI1*). Between 60 and 180 min, the main categories of common genes

corresponded to thiamine biosynthesis (*THI4*, *THI6* and *THI13*) and drug resistance proteins (*CDR1*, *CDR2* and *CDR4*). Representative categories of genes regulated at only one time were: (15min.) ergosterol biosynthesis (*ERG1*, *ERG3*, *ERG10*, *ERG11, ERG20* and *ERG251*), ferroxidases and ion transporters (*FET3*, *FET99*, *FTH1*, *FTR1* and *FTR2*), and histones (*HHF1*, *HHF22*, *HHT1, HHT21*, *HTA3* and *HTB1*); at 60 min: general amino acid permeases (*GAP1*, *GAP4* and *HIP1*), lipases (*LIP7*, *LIP8* and *LIP9*) and ribosomes (*RPL18*, *RPL24A*, *RPL38*, *RPS4A*, *RPS20* and *RPS22A*). Under our conditions, only a few transcription factors were detected, indicating that either shorter times of incubation should be assayed or that the limit of fold-change imposed should be changed.

Introduction

Candida albicans is a polymorphic organism able to grow in several yeast forms either in liquid media [431] or switching spontaneously in solid media to form colonies with different phenotypes [430]. *C. albicans* is also able to form chlamydospores that may allow survival under harsh environmental conditions, acting as resting forms, as has been described for other species, although this assumption and their putative role in infection remain to be confirmed [298,446]. Finally, *C. albicans* can undergo a dramatic change in morphogenesis due to environmental conditions when round yeast cells form pseudohyphal and true filaments, and both processes, which are controlled by a complex network of parallel pathways, play a role in virulence [34,451]. The well established DNA array technology allows efficient analysis of the genome-wide expression of multiple strains, mutants, and different growth conditions. Much work has been published about issues such as the yeast-hypha transition [342], cyclic AMP signalling [177], the regulation of morphogenesis and metabolism by APSES proteins [108], the role of the *FLO8* transcription factor in hyphal development [54], the convergent regulation of virulence by Cph1p, Cph2p and Efg1p [261], the Tac1 regulon [282], the response to steroids [21], etc. Despite such a large body of information, we still lack the transcriptomes of *C. albicans* obtained under standard and well defined sets of conditions, and neither has the kinetics of induction of the regulatory and structural genes yet been unravelled. In the present work, we analyzed the response of *C. albicans* in a minimal medium (Lee) dependent only on temperature at three induction times: 15, 60 and 180 min. We detected a peak in gene alteration

at 60 min., suggesting that most of the regulatory processes have been completed at this time and that at more prolonged times mainly housekeeping genes are expressed. In similar approaches, Nantel et al [342] found a regulated expression of 232 genes (up- and down-regulated) by comparison of the growth of *C. albicans* in several media, and Kadosh and Johnson [226] described 61 genes that were induced more than two-fold during the blastospore-to-filament transition. At all three incubation times assayed, we detected the regulation of most of the cell wall genes described by other authors [226,342] -*ECE1*, *HWP1, PHR1* and *RBT1*- although two exceptions were remarkable. We observed a putative change in the transcriptional regulator of the SAP family, not in the genes of that family, and we detected the coordinated expression of the genes involved in pathogenicity: the ALS and LIP families [201,410]. At least at one time, we also found 15 genes (out of 46) described specifically by Nantel et al [342] and we observed the regulation of three new families: BMT*,* the F_1F_0 ATPase complex, and THI. Several important sets of genes appeared misregulated at one of the times assayed; i.e. genes coding for histones, for general amino acid permeases (after 15 and 180 min. of incubation respectively), etc. Our results indicate that for modelling the yeast-hypha transition in *C. albicans* further experimental approaches with defined sets of parameters should be addressed.

Materials and methods

The strain of *C. albicans* used was CAI4, *Δura3*::*imm434*/*Δura3*::*imm434* [132]. It was maintained by periodic transfer to slants of yeast extract-dextrose (YEPD) medium (1% yeast extract, 1% glucose, 2% agar). Yeast growth was achieved in YNB (0.67% yeast nitrogen base (Difco), 0.5% ammonium sulphate, 0.2 mM uridine, 1% glucose) or Lee medium [270] containing 1.25% glucose as a carbon source and supplemented with 0.2 mM uridine, at 28ºC, (pH 6.8). The dimorphic transition was induced by changing the temperature to 37ºC in Lee medium. Cells were photographed using a Zeiss Axiophot phase-contrast photomicroscope equipped with a 35 mm camera using Ilford FP4 Plus film (125 ASA).

RNA isolation, cDNA preparation and microarray hybridization

C. albicans strain CAI4 was grown in MM or under our condition for the yeast-hypha transition. Cells were pre-grown in Lee medium up to the exponential phase

at 28ºC, washed, starved in water for 24 h at 4ºC, and then incubated in Lee medium at 37ºC. Samples (300 ml of cells, 10^7 cells/ml) were collected at 15, 60 and 180 min., harvested at room temperature, resuspended in a very small volume of the supernatant, and immediately frozen by releasing small drops of cell samples into liquid nitrogen. Cells were then stored at -80ºC until RNA extraction. Total RNA was isolated by breaking cells in a micro-dismembrator (Braun, Melsungen), followed by the trizol extraction method, as described in the Galar Fungail standard operating procedures for RNA extraction (*http://www.pasteur.fr/recherche/unites/Galar Fungail/*), keeping the cells frozen in liquid nitrogen at all times. RNA was checked for integrity by electrophoresis and quantified by spectrophotometric analysis at 260 nm.

About 15 μg of total RNA was resuspended in 20 μl of RNase-free H_2O along with 1 μl of RNasin ribonuclease inhibitor (Promega) and then used for labelled cDNA synthesis, following a protocol from Eurogentec, with minor modifications. Direct labelling reactions were carried out by reverse transcription using the SuperScript II RT™ enzyme (Invitrogen), the *Candida albicans* specific primer mix (Eurogentec), OligodT (12-18) (Sigma), the dNTP mix (Sigma) and Cyanine 3-dCTP/Cyanine 5-dCTP (Amersham Biosciences), DTT and 5× First-strand buffer. In short, total RNA isolated from *C. albicans* cells was used to obtain cDNA labelled with Cyanine 3-dCTP or Cyanine 5-dCTP by a reverse transcription reaction lasting 3 hours. Then, the reaction was stopped by the addition of EDTA, and the residual RNA was denatured by adding NaOH and incubating at 65ºC for 20 min. The labelled probe was then purified using the Qia-quick PCR purification kit (Qiagen). cDNA was checked for proper labelling by measuring the amount of probe and incorporated dye and calculating the frequency of incorporation (f.o.i). Only probes with more than 65 pmol of dye incorporated and an f.o.i greater than 20 labelled nucleotides per 1000 nucleotides were used for subsequent microarray hybridization. From each probe, the volume corresponding to 65 pmol of dye was concentrated to an approximate volume of 5 μl using Microcon-30 filters (Amicon). Concentrated Cy5/Cy3-labelled probes from treated and untreated cells were mixed. Then, 5 μl of denatured salmon sperm DNA (10 mg/ml) was added and after DNA denaturing DIG easy hybridization buffer (Roche) was incorporated to a final volume of 60 μl. The probe was hybridized onto the microarray underneath a coverslip (Sigma) and incubated overnight at 42ºC in a Corning chamber. Following this, the microarrays

were washed following the Eurogentec recommendations and dried by centrifugation at 1100 rpm at room temperature.

cDNA microarray data analysis

Transcriptional profiling was carried using cDNA microarrays containing 6039 *C. albicans* ORFs of strain SC5314 (~98% of total) in duplicates purchased from Eurogentec (Seraing, Belgium). The data analysed included 4 replicates, corresponding to 2 biological replicates (RNA from independent cultures), and a dye swap experiment for each one. Since the ORFs were spotted in duplicate onto each microarray, each gene was represented by eight datasets. The microarrays were scanned with a GenePix 4000B scanner, and intensity data were captured with GenePix Pro, version 4.0. Each microarray image was inspected to identify low-quality spots that were not included in the analysis. Normalization and statistical analyses were performed with GeneSpring software, version 5.0.3 (Silicon Genetics/Agilent Technologies, Redwood City, CA). Data were per-chip- and per-spot-normalized by intensity-dependent (Lowess) normalization applied to the print-tip region. Genes with differential expression were obtained by first filtering genes with a fold-change $\geq$ 1.5 in at least one hybridisation and then applying a "statistical group comparison" (Wilcoxon-Mann-Whitney test) and the multiple testing correction Benjamini and Hochberg False Discovery Rate (FDR) with a p-value $\leq$ 0.02. The first filter was introduced to control the stringency of the FDR correction, which is directly influenced by the number of genes analysed. Among the genes selected by the test, we considered genes as being significantly regulated only if they were regulated by at least a mean factor of 1.5. Cluster analyses and Venn diagrams were carried out with the GeneSpring program, using standard value settings. Gene designations were in accordance with the annotation of the Candida DB Web server (*http://genolist.pasteur.fr/CandidaDB/*) and the Candida Genome Database (*http://www.candidagenome.org/*). Total gene expression data are available in the Supplementary material.

Results

Morphology of the cultures

The yeast-hypha transition was induced from cells pre-grown in Lee medium up to the exponential phase at 28ºC, washed, starved in water for 24 h at 4ºC, and then

incubated in Lee medium at 37ºC. Under these conditions, the growth of most of the cells was arrested in the G1 phase of the cell cycle, and a reproducible and homogeneous hyphal formation was obtained, Figure 2.1.

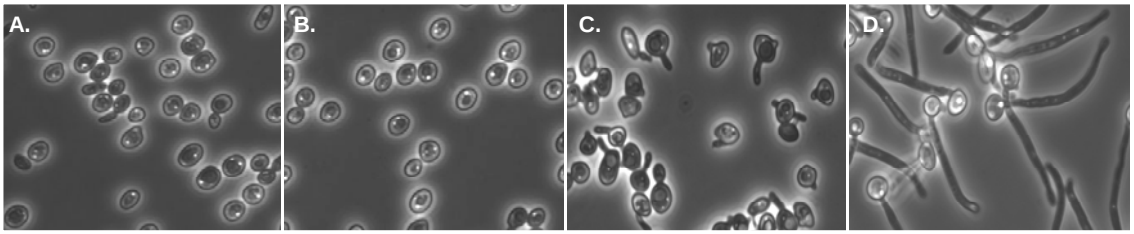

Figure 2.1 Morphology of *Candida albicans*. Yeast phenotype of the cells of the inoculum incubated in Lee medium up to the exponential phase at 28ºC, washed and starved in water for 24 h at 4ºC (**A**). Yeast-hypha transition after 15 (**B**), 60 (**C**) and 180 min (**D**)

Global gene expression profile

In previous studies, the transcriptomes of the *C. albicans* SC5314 and CAF2-1 strains were compared by induction of the yeast-hypha transition in YPD at 30ºC, YPD plus serum at 37ºC after 30 min., at 1 and 6 hours of induction, and at 25-37ºC and at 30-37ºC temperature shifts in the absence of serum [342], or by growing in YEPD at 1, 2, 3 and 5 hours [226]. As a means to identify the genes responding only to temperature, and in order to analyze the behavior of these, we performed gene expression profiling analysis at three shorter times of incubation at 37ºC -15, 60 and 180 min.- by comparison with yeast growing in the exponential phase at 28ºC. A total of 781 genes were found to change at the consensus level of ≥ 1.5 fold, including 401 up-regulated genes, and 380 down-regulated genes. The distribution of genes altered at the three time-points is represented in Figure 2.2, and the Venn diagram and the clustering of genes regulated by temperature are shown in Figure 2.3 (A and B).

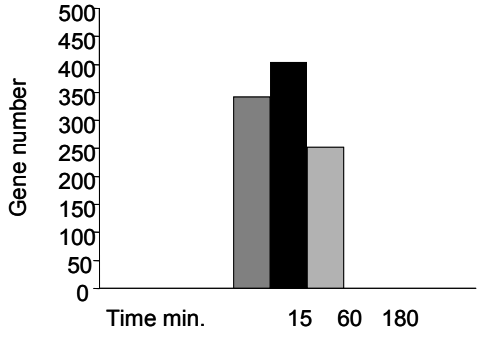

Figure 2.2 Total number of genes regulated by temperature at the ≥ 1.5-fold level

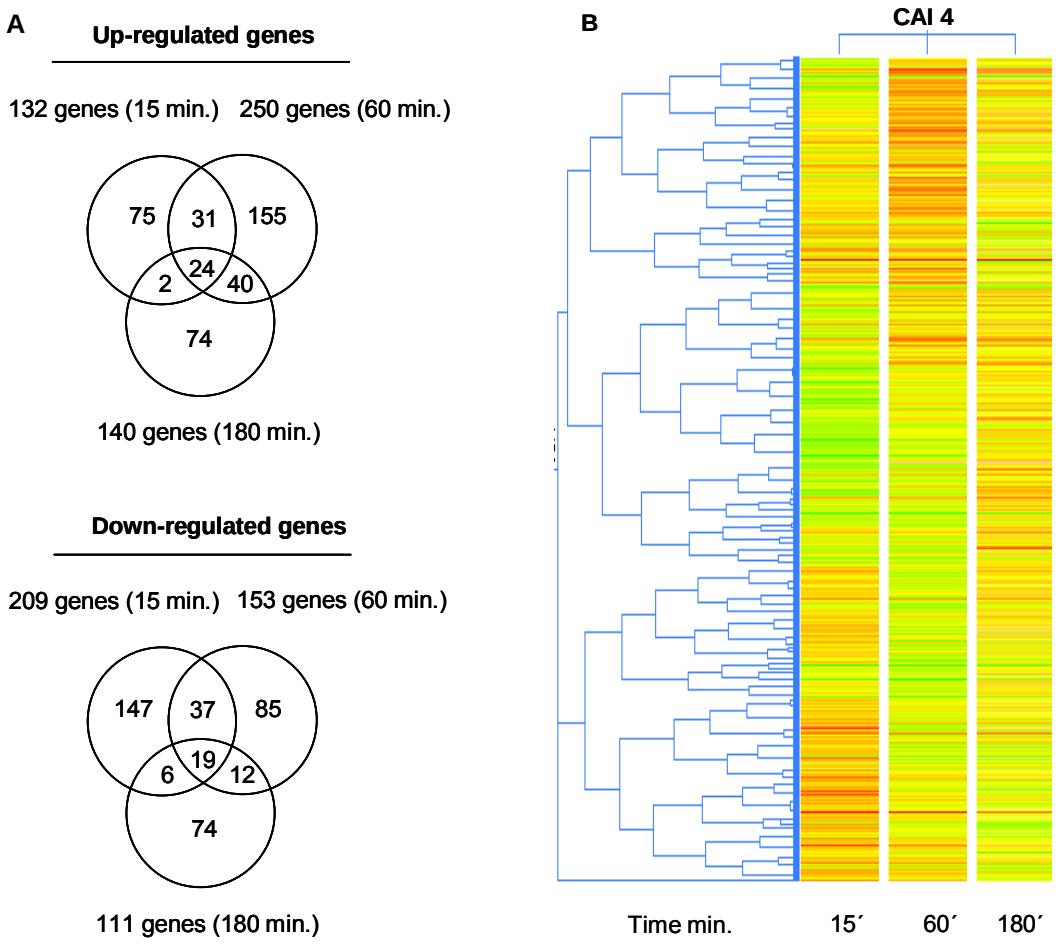

Figure 2.3 Genes regulated by temperature. (**A**) Venn diagram of up-regulated and down-regulated genes, including those common to all three times studied. (**B**) Clustering of genes regulated by temperature. Ratios of gene expression obtained by dividing the experimental samples by the reference samples are represented with a red-green colour scale.

The time-course of gene expression clearly indicates a peak of regulated genes after 60 min., suggesting that most of the signals and the regulatory genes involved in the morphogenetic switch exert their function rapidly. We observed a good statistical correlation in our results; thus, 43 genes were common to the three times assayed (24 and 19 up- and down- respectively), 68 genes at 15 and 60 min (31 up- and 37 down-) and 52 genes at 60 and 180 min. (40 up- and 12 down-). 222 genes (75 up- and 147 down-), 240 genes (155 up- and 85 down-), and 148 genes (74 up- and 74 down-) varied only at one induction time (15, 60 and 180 min. respectively). However, only 8 genes were common between 15 and 180 min. validating our experimental approach. The elevated number of regulated genes detected only at one time suggests a coordinate expression of genes and gene families and a rapid adaptation to temperature and to the morphogenetic switch. The 43 genes common to the three times are listed in Table 2.1.

Table 2.1 Genes whose transcription is induced more than 1.5 fold at 37ºC at the three times assayed, 15, 60 and 180 min.

Gene name	Accession no	Description (CandidaDB)	Fold regulation		
UP-regulated genes			**15 min**	**60 min**	**180 min**
Cell wall proteins					
ALS1	orf19.5741	Agglutinin-like protein	20.99	5.17	3.00
ALS3	orf19.1816	Agglutinin like protein	2.23	11.44	3.5
ECE1	orf19.3374	Cell Elongation Protein	22.72	26.57	14.00
HWP1	orf19.1321	Hyphal wall protein	6.43	16.97	11.60
PHR1	orf19.3829	GPI-anchored pH responsive glycosyl transferase	2.98	3.46	1.90
RBT1	orf19.1327	Putative cell wall protein with similarity to Hwp1p	1.62	4.10	2.85
SUN41	orf19.3642	Putative cell wall beta-glucosidase (by homology)	1.67	2.66	1.95
Dehydrogenases					
ARD	orf19.6322	D-arabinitol dehydrogenase	1.85	1.83	2.17
PUT2	orf19.3974	1-pyrroline-5-carboxylate dehydrogenase	2.24	1.84	2.22
Oxidases/peroxidises					
AOX2	orf19.4773	Alternative oxidase (by homology)	1.54	1.72	1.74
GPX2	orf19.85	Glutathione peroxidase (by homology)	3.60	3.63	3.80
Transporters					
ENA21	orf19.5170	P-type ATPase	2.93	2.15	1.96
ENA2	orf19.6070	P-type ATPase involved in Na^+ efflux	2.12	2.10	1.70
GAP6	orf19.6659	General amino acid permease	1.63	2.40	2.19
IPF9376	orf19.655	High-affinity inorganic phosphate/H^+ symporter	2.49	3.95	2.09
Transcription					
GAT2	orf19.4056	Putative DNA-binding transcription factor	1.73	2.39	1.82
Other functions					
ARP3	orf19.2289	Actin related protein (by homology)	2.25	2.90	2.18
DCK1	orf19.8435	Putative guanine nucleotide exchange factor	3.81	2.75	1.81
DEF1	orf19.7561	Protein required for filamentous growth	1.76	2.11	1.80
IPF11725	orf19.10979	A Gag-related protein; hyphal induced	2.82	2.94	3.01
PCK1	orf19.7514	Phosphoenolpyruvate carboxykinase	1.99	5.76	3.64
TMA19	orf19.3268	Ortholog of *S. cerevisiae* Tma19p (Ykl065cp)	1.74	1.92	1.73
Unknown function					
PET18	orf19.7330	Unknown	3.95	7.43	1.64
Down-regulated genes					
Cell wall proteins					
PIR1	orf19.220	Putative cell wall protein of the PIR family	0.30	0.24	0.35
YWP1	orf19.3618	Putative cell wall protein (by homology)	0.28	0.25	0.16
Dehydrogenases					
GDH3	orf19.4716	NADP-glutamate dehydrogenase	0.15	0.18	0.40
Oxidases/peroxidases					
AMO1	orf19.5784	Amine oxidase (by homology)	0.44	0.30	0.38
Transporters					
MEP2	orf19.5672	Ammonium permease	0.26	0.18	0.35
OPT2	orf19.3746	Oligopeptide transporter	0.34	0.40	0.23
OPT3	orf19.3749	Oligopeptide transporter	0.45	0.51	0.51
PMA1	orf19.5383	Plasma membrane H^+-transporting ATPase 1	0.40	0.50	0.56
Glycolysis					
PDC11	orf19.2877	Pyruvate decarboxylase (by homology)	0.33	0.19	0.60
PFK1	orf19.3967	6-phosphofructokinase alpha subunit	0.25	0.56	0.51
PFK2	orf19.6540	6-phosphofructokinase beta subunit	0.23	0.58	0.48
Transcription					
STP3	orf19.5917	Transcription factor	0.59	0.52	0.53
Other functions					
DUR1.2	orf19.780	Urea amidolyase (by homology)	0.60	0.46	0.52
FET99	orf19.11689	Cell surface ferroxidase (by homology)	0.23	0.33	0.46

FMA1	orf19.6837	Oxidoreductase	0.44	0.25	0.48
GCY1	orf19.6757	Aldo/keto reductase (by homology)	0.38	0.44	0.42
RNR22	orf19.1868	Ribonucleoside-diphosphate reductase	0.29	0.44	0.35
Unknown function					
IPF16598	orf19.7781	Unknown function	0.55	0.39	0.65
IPF20054	orf19.6117	Unknown function	0.40	0.60	0.55

The lowest fold-change detected was 0.15 for *GDH3* at 15 min. and the highest fold-change was 26.57 for *ECE1* at 60 min. These fold-changes match perfectly in the range of our assays (see Supplementary material Tables 2.1 and 2.2). Only 3 of the common genes corresponded to genes of unknown function. This percentage, roughly 7%, is one third of those found at global level (25%), as would be expected from our knowledge about the morphogenetic switch. Of the rest of the genes, 10 belonged to disperse functional categories; i.e., *ARP3*, actin-related protein; *RNR22*, ribonucleoside diphosphate reductase, etc., and 27 genes could be grouped in six functional categories. The main one corresponded to cell wall proteins and included the up-regulation of *HWP1,* a well known cell wall protein [443]; *ECE1,* a protein involved in cell elongation [27,32]; *PHR1*, a cell surface glycosidase [131], and *RBT1* [27,434], a putative cell wall protein with similarity to Hwp1p. We observed the down-regulation of *YWP1*, a cell wall and secreted protein [159], and of *PIR1*, a cell wall structural protein [309]. All these proteins have been described previously as being up- and down-regulated at similar levels of induction [226,342] and are regulated by Efg1p and Tup1p [108,226,434].

Also well represented were the following categories: transporters with 9 genes, including the two oligopeptide transporters, oxidases/peroxidses (4 genes), glycolysis (3 genes), and dehydrogenases (3 genes) (see below). Our results indicate an important bias toward the functional categories mentioned previously and show that the role of these genes in the yeast-hypha transition must be investigated more carefully.

Expression of transcription factors

Only one transcription factor was up-regulated at the three times of incubation; namely, *GAT2*, which has been described as a putative DNA-binding transcription factor with an unspecified role in morphogenesis that awaits further characterization. Three transcription factors -*CLN21*, *HAC1* and *UME6*- were up-regulated at two times: 15 and 60 min. *CLN21* is a hypha-specific G1 cyclin protein involved in the regulation of hyphal morphogenesis [428,543]; *HAC1* is a putative

transcription factor involved in unfolded protein response and the control of cell morphology [508], and *UME6* is a transcription factor required for wild-type hyphal extension [22,282]. Deletion of all three genes produces clear defects in hyphal formation and extension, and hence our results are in agreement with previous ones. However *CLN21* is also induced after overexpression of *EFH1* [108] and *HAC1* is down-regulated in an *efg1, efh1* mutant [108], suggesting that the regulatory circuits described to date are incomplete [34] and that our knowledge of them must be improved. The other up-regulated transcription factors (at 60 min. of incubation) were *SKO1,* a putative transcription factor involved in the cell wall damage response [379]; *TEC1,* a TEA/ATTS transcription factor involved in the regulation of hypha-specific genes [262], and *UME7,* a putative transcription factor with a zinc cluster DNA-binding motif [298]. At 180 min. of incubation we found *ZCF20*, a predicted zinc-finger protein of unknown function [298], to be regulated *UME7* and *ZCF20* have been characterized only by "*in silico*" analysis [298]. The expression of *TCC1,* a protein involved in the regulation of filamentous growth and virulence that interacts with Tup1p and acts in the transcriptional regulation of hypha-specific genes [229], and *CTA2*, a putative transcriptional activator down-regulated by Efg1p [108], was increased from 0.65 to 1.29 and from 0.50 to 5.50 (at 15 min. to 180 min. of incubation respectively). Both results again perfectly match the hypothesis of a coordinated developmental program. Of the 6 down-regulated transcription factors, only one appeared regulated at all three times; namely, *STP3*, which regulates *SAP2* [311] (see below). *STP4,* a putative transcription factor with a zinc-finger DNA-binding motif*,* was regulated at 60 min. [46]. At 180 min. of incubation at 37ºC we found: *HAP2*, a CCAAT-binding factor that regulates low-iron (chelation) induction of *FRP1* transcription [15]*; orf19.173,* with a zinc-finger DNA-binding motif [377] activated by *MNL1* (which regulates weak acid-induced stress responses); *MDM34,* with a zinc-finger DNA-binding motif [129]; *MNS4*, similar to *S. cerevisiae* Msn4p [354] (all three putative transcription factors), and *NRG1*, a well known transcriptional repressor that regulates hyphal genes [38,229,331].

Time course of gene induction in the main categories detected

In general, our results are in good agreement with those described by several authors [226,334,342,427], with only one remarkable exception; we were unable to detect the induction of the genes of the SAP family. This is probably due to the

conditions we used to induce the morphogenetic switch. We only performed a change in temperature, and we were working with a minimal medium (Lee), while other authors changed temperature and either added serum or grew the culture in YPD medium. Our results are supported by the fact that the only transcription factor that was regulated at all three times was *STP3*, which in turn regulates *SAP2* [311]: the enzyme of the family predominantly expressed under proteinase-inducing conditions *in vitro* [211,339] and also the most important Sap enzyme in virulence [103]. To analyze the other regulated gene categories, we clustered together the main representative groups whose expression level changed at least at one time of incubation, by functional categories and all of them exhibited "kinetic" behavior.

Cell wall genes. Figure 2.4A shows the clustering of the cell wall genes affected. The ALS family includes 8 genes and of these, six changed their expression. Four increased their expression: *ALS1* and *ALS3* at all three times; *ALS9* at two times (15 and 60 min.), and *ALS5* only at 60 min. Our results revealed higher levels of expression for the *ALS1* and *ALS3* genes (up to 20 and 11 times respectively) and lower ones for the *ALS9* and *ALS5* genes (up to 2 and 1.5 times respectively), and they are in complete agreement with those described by other authors, who demonstrated the role of *ALS3* in adhesion [541] and analysed *ALS* gene expression in budding and filamentous cultures of *C. albicans* by RT-PCR [164]. However, two of the *ALS* genes strongly decreased their expression; namely, *ALS2* and *ALS4* (Figure 2.4A).

Glucanases, glucosidases and mannosyltransferases. *SUN41*, a putative cell wall glucanase (the orthologue in *S. cerevisiae* is *SUN4*, [487]), was up-regulated at the three times of induction. Deletion of both alleles of the gene results in defects in hyphal formation [130] and a down-regulation of the gene during protoplast regeneration [62]. *BGL2*, which catalyzes the splitting (removing a disaccharide) and linkage of β-1,3-glucan molecules, was up-regulated at 60 and 180 min. of incubation (microarray analysis revealed that *BGL2* was induced during cell wall regeneration, its maximum value being reached after 2 hours [62]). *BGL22* also showed increased induction kinetics, a value of 1.55 times being reached at 180 min. of incubation at 37ºC. A third putative glucanase, *UTR2* [6], again regulated during protoplast regeneration [62], was also up-regulated.

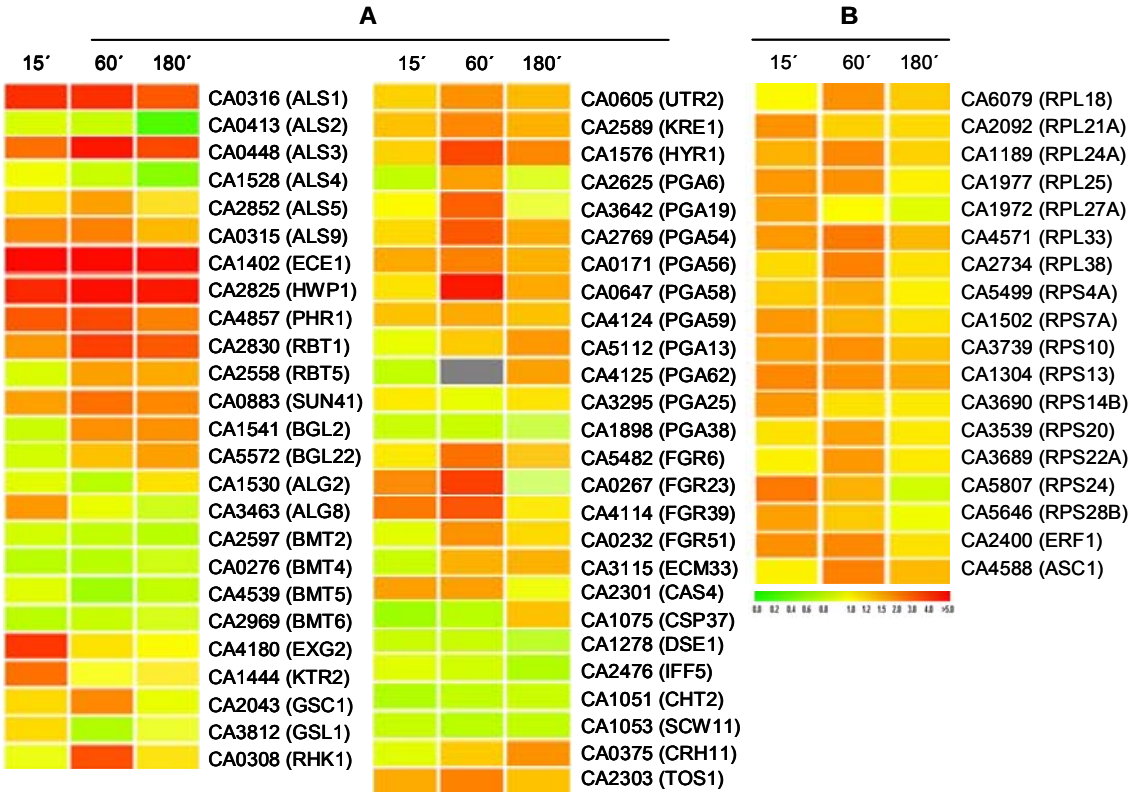

Figure 2.4 Transcriptional regulation by temperature. Cell wall, (**A**); and ribosomal, (**B**), genes Representative genes were grouped by gene families

More cell wall genes changed their expression: *ALG2* and *ALG8* (two putative glucosyltransferases involved in cell wall mannan biosynthesis), and *GSL1* and *GSL2*, subunits of beta-1,3-glucan synthases [322], which showed a compensatory effect (see Figure 2.4). *EXG2,* an exo-1,3-beta-glucosidase induced during cell wall regeneration [62]; *KTR2*, a fungal-specific mannosyltransferase; *RHK1,* a putative hitherto uncharacterized alpha-1,3-mannosyltransferase; *CAS4*, a protein the of RAM cell wall integrity signalling network required for hyphal growth [439], *TOS1*, a protein described as being similar to the alpha agglutinin anchor subunit [73], and *DSC1* and *SCW11*, which are predicted cell wall proteins whose transcription is decreased in mutants lacking *ACE2* [232]. Of interest was the down-regulation of several putative beta-mannosyltransferases: *BMT2, BMT4, BMT5* and *BMT6* [321]. Finally, *CHT2,* a chitinase GPI anchor induced in yeast-form cells [115,177], was down-regulated at all three times of incubation.

GPI-anchored proteins

A large group of genes coding for putative GPI-anchored proteins showed a single peak of induction at 60 min: *KRE1* (also found to be highly regulated during *C.*

albicans protoplast regeneration [62]); *HYR1* (induced in hypha at 15 and 60 min). [229], and *RBT5, PGA6*, *19*, *54*, *56*, *58* and *59* [104] (*PGA13* and *PGA62* were also up-regulated, but at 180 min.). We also observed the regulation of a set of genes coding for proteins lacking an orthologue in *S. cerevisiae* -transposon mutation affects filamentous growth- *FGR 6*, *23*, *39* and *51* [477] (see Figure 2.4A); *ECM33* (also modulated by adenylyl cyclase, [177]), and *CRH11*, a predicted glycosyl hydrolase [104]. Very few cell wall genes were down-regulated: *CSP37* (at 15 and 60 min.), *PGA25* (at 60 min.), and *PGA38* and *IFF5* (at 180 min.).

Genes involved in protein synthesis and ribosomal proteins

A second important category included the up-regulation of 18 genes coding for ribosomal proteins and involved in protein synthesis. Five of those genes were up-regulated at 15 and 60 min.; 6 genes at 15 min., and the other 7 genes at 60 min. (Figure 2.4B). No genes of this category were found up-regulated at 180 min. or down-regulated at any time. Almost no data about the behavior of this group of genes under standard conditions of the yeast-hypha transition (serum or temperature) have been reported, and only *RPS24* has been reported to be down-regulated by cyclic AMP signalling [177,208,229,342]. Many of them have been described as up-regulated by comparison of different mutants with a wild-type strain: the *Cacwt1* mutant [324] and the *Caefg1* and *Caefh1* mutants [108]. However, most of them are down-regulated upon *C. albicans* macrophage internalization [286].

Central carbon metabolism

The regulation of 40 genes (35 down-regulated after 15 min of incubation) involved in the glycolytic pathway, the TCA and glyoxylate cycles and gluconeogenesis (see Figure 2.5A, B and C) was detected.

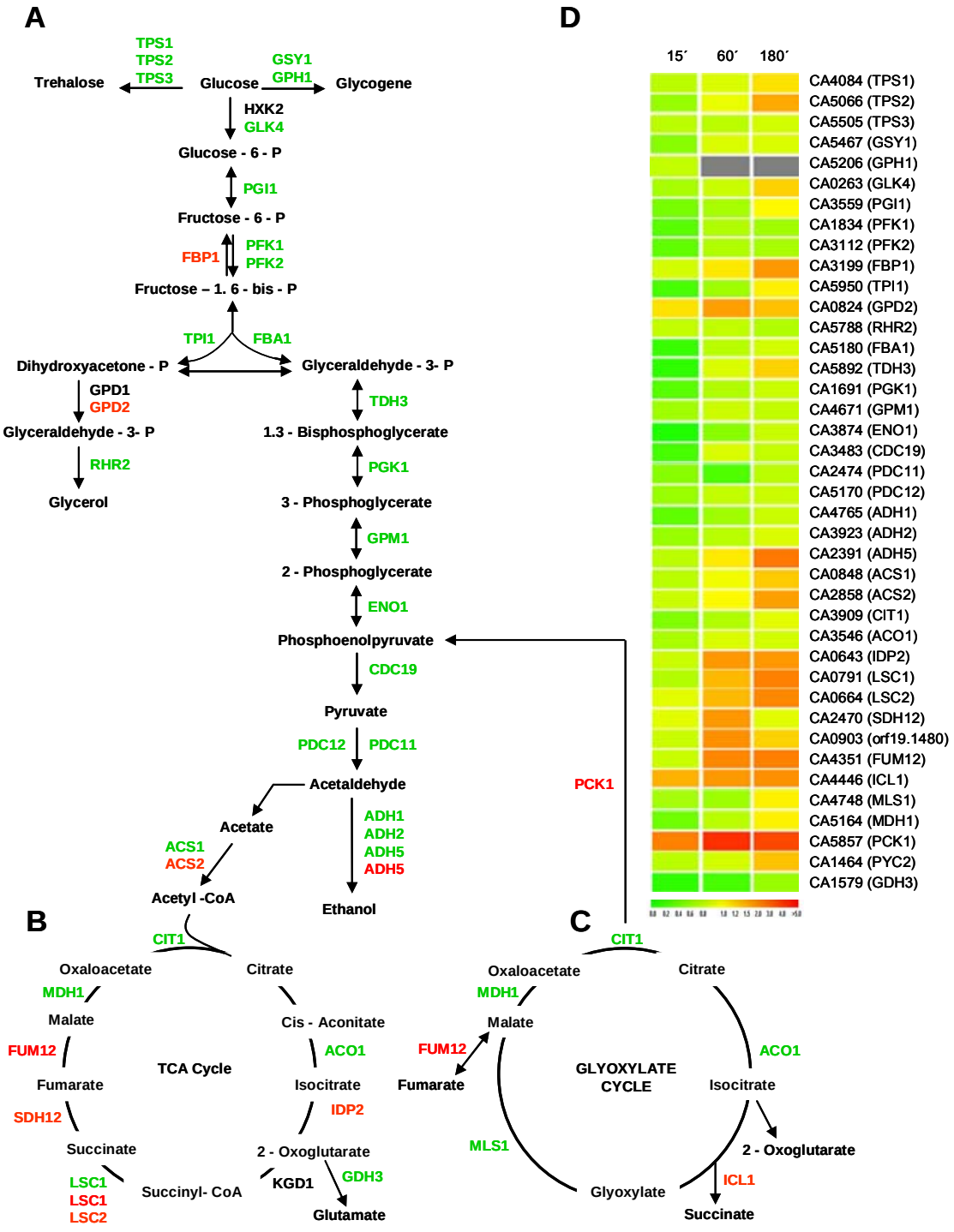

Figure 2.5 Transcriptional regulation by temperature of genes involved in glycolysis and gluconeogenesis (**A**) and TCA (**B**) and glyoxylate (**C**) cycles. Green and red colours represent down- and up-regulation respectively.

The expression of all of them increased with time (Figure 2.5A), suggesting an adaptation to the hyphal mode of growth. In general our results are in agreement with those described by many authors [92,108,177,208,226,342,427], and they raise the question of the putative effect of mutations on the pathways [108]. Two

genes involved in gluconeogenesis were up-regulated *GPD2* and *PCK1.*

Transporters and oligopeptide transporters

Nine genes were regulated at the three times of incubation (four genes down- and five up-, Table 2.1) and 25 more at one or two times (Table 2.2).

Table 2.2 Regulated genes involved in transport

Gene name	Accession no	Description (CandidaDB)	Fold regulation		
			15 min	60 min	180 min
ALP1	orf19.2337	Amino-acid permease	1.09	1.73	0.99
CTR1	orf19.3646	Copper transport protein	1.07	1.09	1.76
DAL4	orf19.313	Probable uracile or allantoin permease	0.84	0.66	0.82
DUR3	orf19.781	Putative urea transporter	0.70	0.55	0.71
FEN2	orf19.12981	Predicted membrane transporter	1.11	0.69	0.42
FET34	orf19.4215	Iron transport multicopper oxidase	1.10	2.16	1.15
FTH1	orf19.4802	Iron transporter	0.59	0.99	1.06
FTR2	orf19.7231	High affinity iron permease	0.59	0.80	0.90
GAP1	orf19.4304	General amino acid permease	1.14	2.90	1.24
GAP4	orf19.4456	General amino acid permease	1.28	1.72	1.40
GNP1	orf19.8784	Asparagine and glutamine permease	0.95	1.92	1.19
HAK1	orf19.6249	Putative potassium transporter	0.72	0.71	0.62
HGT1	orf19.4527	High-affinity glucose transporter	1.01	1.37	2.60
HGT7	orf19.2023	Sugar transporter	0.57	0.74	0.94
HGT8	orf19.2021	Sugar transporter	0.47	0.83	1.03
HIP1	orf19.3195	General amino-acid permease	1.42	1.94	1.15
IPF14040	orf19.2397	Probable transporter	1.90	1.41	0.94
IPF1992	orf19.7336	Predicted membrane transporter	1.55	0.86	0.74
MAL31	orf19.3981	Maltose permease	1.14	1.87	1.22
PHM7	orf19.2170	Membrane transporter	0.81	2.13	1.33
PHO88	orf19.7327	Involved in phosphate transport	1.52	1.31	1.20
PTR2	orf19.6937	Oligopeptide transporter	0.60	1.23	1.07
SMF12	orf19.2270	Manganese transporter	0.93	1.88	1.36
ZRT1	orf19.3112	High-affinity zinc transport protein	1.04	0.96	2.08
ZRT2	orf19.1585	Zinc transport protein	0.30	0.72	2.15

A significant group of genes down-regulated at 15 min. corresponded to iron transporters (*FTH1*, *FTR1* and *FTR2*) [259]. Surprisingly, a regulation in the opposite way has been described for the two FTR genes [376]. The expression of *FTR1* is induced under iron-limited conditions and repressed when the iron supply is sufficient, the opposite being observed for *FTR2*. *FTR1* has also been reported as a virulence factor by the same authors [376] (Figure 2.6A).

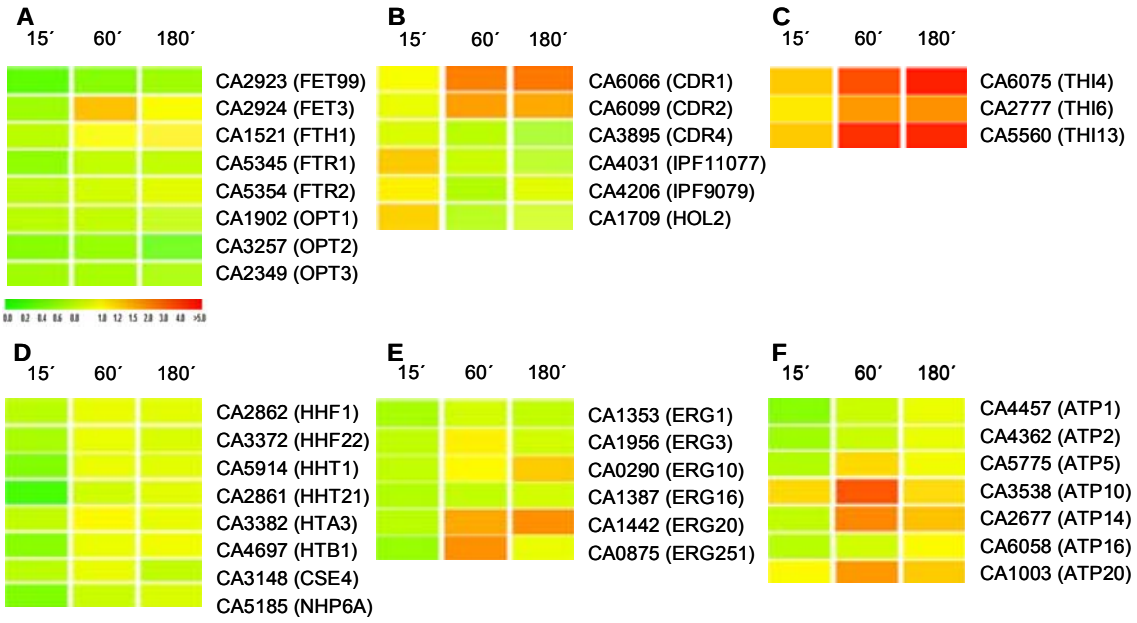

Figure 2.6 Transcriptional regulation by temperature of genes involved in transporters, (**A**); drug resistance, (**B**); thiamine biosynthesis, (**C**); histones, (**D**); ergosterol biosynthesis, (**E**); and F_1F_0 ATPase complex (**F**). Representative genes were grouped by gene families.

The genes up-regulated at 60 min. include several amino acid permeases (*ALP1, GAP1*, *GAP4, GNP1* and *HIP1)*. Two oligopeptide transporters -*OPT2* and *OPT3*- were down-regulated at the three times of incubation. Another one, *OPT1*, was also down-regulated at 15 and 60 min. of incubation [383] (Figure 2.6A). Another oligopeptide transporter, *PTR2*, with a discrepancy in the annotation -described as *PTR21* (CA4707) or *PTR2* (CA2690, CA2691)- was also detected.

Drug resistance

Three of the five putative genes belonging to the multidrug transporter of ATP-binding cassette (ABC) superfamily were regulated at 60 and 180 min. of incubation. Two of them -*CDR1* and *CDR2* [105]- were up-regulated and the other one, *CDR4*, with a less defined function, was down-regulated. Cdr1p and Cdr2p have an established role in conferring azole resistance [372] and both proteins also act as phospholipid translocases, which transport phospholipids in an in-to-out direction [432]. According to our data, it is tempting to speculate that the up-regulation of drug transporters may favour the invasion of tissues by adapting cell membranes to a different composition. We also observed the down-regulation of CA1709, CA4031 and CA4206, three predicted membrane transporters, and members of the drug: proton antiporter (12 spanner, DHA1 family) of the major facilitator superfamily (MFS) [143] (Figure 2.6B).

Oxidases/peroxidases

Two genes, *AOX2* and *GPX2*, were up-regulated and one gene, *AMO1*, was down-regulated at the three times of incubation. Other regulated genes were *AOX1*, *PRX1* and two putative d-amino acid oxidases: *IFG3* and *IFG4*. We detected two multicopper oxidases, *FET3* and *FET99,* also required for growth under low-iron conditions [424].

Hydrolases/Dehydrogenases

We mainly detected the up-regulation of *IMH3* (Inosine monophosphate (IMP) dehydrogenase [24]), *SUR2* (a protein described as a ceramide hydroxylase); *ALD5* (a protein described as an aldehyde dehydrogenase [177]); *IFD6* and *CSH1* (members of the aldo-keto reductase family, similar to aryl alcohol dehydrogenases [255]), and *GDH2* (a putative NAD-specific glutamate dehydrogenase, fungal-specific; (no human or murine homolog), and the down-regulation of *GND1,* a putative 6-phosphogluconate dehydrogenase.

Thiamine biosynthesis

After 60 and 180 min. of incubation, we detected the up-regulation of three genes involved in thiamine biosynthesis -*THI4*, *THI6*, and *THI13*- two of them (*THI4* and *THI6*) being fungal-specific (Figure 2.6C). *THI4*, identified in *S. cerevisiae* as the gene involved in the synthesis of HET-P, is a single gene but its transcript is one of the most abundant under thiamine-depleted conditions [328]. Also, *THI6* is a single-copy gene and *thi6* null strains have an absolute requirement for thiamine [357].

Sets of genes regulated only at one incubation time

After 15 and 60 minutes of incubation at 37ºC, several sets of genes were either up- or down-regulated. The main groups of up-regulated genes (at 15 min.) included two RNA helicases; one of the DEAD box family; *DBP2*, and one ATP-dependent RNA-helicase; namely, *DBP3*. The genes up-regulated at 60 min included lipases -*LIP7*, *LIP8* and *LIP9*- while *LIP10* was found to be down-regulated. Among the down-regulated genes after 15 min. of incubation, the following should be noted: histones (Figure 2.6D), with six genes (of the eleven genes coding for histones described in *C. albicans*: *HHF1*, *HHF22*, *HHT1*, *HHT21*, *HTA3* and *HTB1*), including H2A, H2B, H3 and H4. We failed to detect the

regulation of genes coding for histone H1. We also detected the down-regulation of *CSE4*, a centromere-associated protein; it is essential and is similar to CENP-A (centromeric histone H3 variant [383], and of *NHP6A,* a protein described as non-histone chromatin component [281]. A second important category corresponded to genes involved in ergosterol biosynthesis (Figure 2.6E), with another six genes (*ERG1*, *ERG3*, *ERG10*, *ERG11, ERG20* and *ERG251*). Both results suggest a rapid remodelling of chromatin, probably for it to be adapted to a new transcriptional profile, together with a change in membrane composition during hypha formation. The genes of the F_1F_0 ATPase complex *ATP1*, *ATP2*, *ATP5* and *ATP16* were down-regulated at 15 min. of incubation at 37ºC, a normal value being attained after 180 min. of incubation. However, another set of genes of the same complex -*ATP10*, *ATP14* and *ATP20*- displayed an induction peak at 60 min. of incubation, [467] (Figure 2.6F). No specific sets of genes were found at 180 min of incubation, suggesting that at this time *C. albicans* has reached a general balance in its main regulatory metabolic pathways.

Discussion

In *Candida albicans,* a total of 781 genes out of the 6039 present in the microarray changed due to a variation of temperature -28ºC to 37ºC, in a minimal medium (Lee)- at one of the three incubation times assayed (15, 60 and 180 min.) at the arbitrary, although normally used, limit of ≥ 1.5 fold. These 781 genes represent a percentage of 13%. 198 genes, representing 25% of the genes that varied, were classified as being of unknown function, indicating that we need to improve our knowledge of the *Candida* genome, even as regards the morphogenetic process, which is one of the aspects most widely studied in this organism. Only two transcription factors were regulated at the three times assayed, and others were induced at two times (3), or at one time (15). Assuming a similar number of transcription factors to those described for *S. cerevisiae*, 482 (*http://www.yeastgenome.org/*), for *C. albicans*, our results show that this category is underrepresented at 2% (17 out 783) instead of the expected 8% (482 out of 6033). Two hypothesis can be advanced to account for this observation. The first is that transcription factors act faster in the first 15 minutes of incubation and that after 15 min. most of them have already performed their function. The second one is that the arbitrary limit of ≥ 1.5-fold variation is too high and that transcription factors act

with more subtle variations. We are in favour of the second hypothesis, because analysis of the behavior of several transcription factors in a random way indicated that many of them consistently changed outside the imposed limit, i.e. *CRZ1* (1.18, 1.16, 1.13); *CZF1* (1.22, 1.11, 1.13); *EFH1* (1.41, 1.34, 1.02); *ROX1* (1.31, 1.22, 0.80); *ASH1* (0.80, 0.79, 0.82) or *MIG1* (0.89, 0.78, 0.69) (values at the three times assayed: 15, 60 and 180 min. respectively). We found at least 51 genes corresponding to well-characterized cell wall proteins. Two of the highly induced genes, *ALS1* and *ALS3*, and two repressed genes -*ALS2* and *ALS4*- are all classified in the same subfamily [200]. Functional analyses with RT-PCR and/or by comparison of the behavior of several mutants have been carried out [164,540,541]. A weaker induction was observed in one member of each of the other subfamilies -*ALS5* and *ALS9*- respectively [200]. In our strain, *ALS1*, *ALS5* and *ALS9* are in one chromosome [542]. Whether an independent induction or a joint induction occurs must be clarified. Although the genes encoding these proteins share a cross-hybridizing tandem repeat domain, our data suggest that each protein has an different adhesive function, and they are in agreement with those described by Hoyer's group, who show that strains lacking *ALS3* or *ALS1* do not exhibit a defect in germ tube formation when grown in RPMI 1640 medium, while the *als1*/*als1* mutant formed significantly fewer germ tubes in Lee medium [540]. Our data and those reported previously [164,540,541] point to a different type of regulation for each ALS protein in *C. albicans* and suggest a role of the *ALS* genes in cell wall remodelling *per se* during the yeast-hypha transition, independently of virulence or any other factors, and this is also in agreement with the strong induction of *ALS1* during *C. albicans* protoplast regeneration [62].

Upon comparing our results concerning PGA genes with those described by Sentandreu's group [62] on the regeneration/formation of cell wall after *C. albicans* protoplasting, we also observed the up-regulation of *PGA6, 13, 54, 56, 59* and *62* (although slightly earlier) and the down-regulation of *PGA38*. Our only discrepancy concerns *SUN41;* we found this gene to be up-regulated at the three times of induction, while a down-regulation was described in that work [62].

Another interesting fact is that unlike the up-regulation found in most of the cell wall genes, we observed the down-regulation of 4 putative beta-mannosyltransferases, *BMT2, BMT4, BMT5* and *BMT6*, out of the 9 described for *C. albicans* [321]. This implies that β-mannosylation is also involved in cell wall remodelling in the yeast-

hypha transition. It may be predicted that this group of genes, together with those involved in O-mannosylation [274], will be essential for understanding why *C. albicans* is virulent. Several authors have described changes in genes involved in the glycolysis and citric acid and glyoxylate cycles under many different conditions; for example, during phenotypic switching [258], the yeast-hypha transition [342], mutants in APSES proteins [108], and after macrophage internalization [286] or antifungal treatment [281], etc. Our results in general agree with previous ones, but some differences should be noted. Thus, we detected more regulated genes involved in all three pathways than other authors, 38, and most of them were down-regulated at 15 min. of incubation at 37ºC (Figure 2.4). After 180 min. many of the genes had reached a normal level, except 10 that appeared slightly overexpressed. We found a down-regulation of all the genes involved in the citric acid cycle that we were able to detect, while in the APSES mutants these genes always were up-regulated. However, genes involved in glycolysis and in reserve carbohydrates were down-regulated in the APSES mutants, as in our experimental conditions [108]. This discrepancy could be due to the different times of incubation or to a direct effect of the *efg1* and *efh1* APSES mutations, which would directly regulate the genes involved in the citric acid cycle. A switch from glycolysis to gluconeogenesis in phagocytosed *C .albicans* cells has been described [286]. It has also been shown that key enzymes such as aconitase (*ACO1*), citrate synthase (*CIT1*), isocitrate lyase (*ICL1*), malate synthase (*MLS1*) and malate dehydrogenase (*MDH1*) are strongly induced in phagocytosed populations of both *C. albicans* and *S. cerevisiae* [134,286,287]. Our results, however, point in the opposite direction. Thus, we observed a down-regulation of those genes (except *ICL1*), confirming that the metabolic changes are indeed involved in phagocytosis. Genes involved in carbohydrate metabolism do not seem to be an important group in the response to *C. albicans* treatment with antifungal agents [281].

We detected an up-regulation of the components of the translation apparatus: 18 genes (up to the ~50 genes well characterized). The regulation of this group of genes has not been reported in previous works describing the yeast-hypha transition [226,342,427], although up-regulation was found by comparison of a wild-type strain with the *efg1*, and *efh1* [108] and *cwt1* [324] *C. albicans* mutants. Interestingly, and unlike our results, phagocytosed cells rapidly down-regulate the genes encoding the translation machinery and those coding for a large number of

transport proteins, including multiple oligopeptide transporters [286]. We detected a decrease in three oligopeptide transporters (*OPT1*, *OPT2* and *OPT3*) (see Figure 2.6A). Both results are again in agreement with those described in the previous paragraph and support the notion that phagocytosis involves a strong metabolic change.

Many genes related to transport (Table 2.2), oxidases/peorxidases and hydrolases/ dehydrogenases were regulated. Several of them are related to iron transport or to encoding general amino acid permeases. Owing to the large amount of disperse data [15,24,177,226,255,259,342,376] it is difficult to analyze their role, but their prevalence under all kinds of experimental conditions suggests that this group of genes deserves further, more careful analysis in order to establish their specific functions in dimorphism and virulence. The expression of *CDR1* and *CDR2*, which encode multidrug transporters of the ABC family, is up-regulated in the presence of estrogen [21,91,287] and antifungal agents [105,281] and *CDR2* is also up-regulated in a *C. albicans* double mutant: *efg1, efh1* [108]. Our results suggest that the increase in expression of both genes is due only to temperature, and that this (temperature) can act as a kind of pre-adaptation of the *Candida* machinery to survival in the human host. Our findings also agree with the observed down-regulation of *CDR4* in the *efg1* mutant [108]. Finally, four groups of genes must be mentioned: two of them have been described previously in several works, although not within our experimental context: histone-, and ergosterol-encoding genes. Histone genes are up-regulated in the *C. albicans efg1* and *efg1, efh1* mutants and after exposure to antifungal agents [281]. Ergosterol genes are also mainly up-regulated after exposure to antifungal agents [231,281]. No previous data have been reported for the other two sets of genes described in this Chapter; namely, those coding for thiamine and for the F_1F_0 ATPase complex. From our results it may be concluded that the morphogenetic switch involves a strong remodelling of the yeast cell wall and membrane and a change in metabolism. These processes are independent of virulence, although both can be interconnected through an adaptation of *C. albicans* to the host. The data described with both protoplasts and with different sets of mutants are in agreement with our own [62,108,324] and with those obtained from the transcriptional response of *C. albicans* upon internalization by macrophages [286]. The only apparent discrepancy -the different regulation of histone genes- can be explained by a rapid remodelling of chromatin to allow, in

conjunction with the corresponding transcription factors, the expression of specific hyphal genes, as has been demonstrated by the regulation of *efg1* [463], the main transcription factor involved in morphogenesis, by chromatin-remodelling factors.

Phenotypic, virulence, and transcriptome analysis of the *Candida albicans gcn5, hda1* double mutant: Comparison with the *gcn5* and *hda1* simple mutants

Abstract

The fungal pathogen *C. albicans* switches from a yeast-like to a filamentous mode of growth in response to a variety of environmental conditions. Histone acetylases and deacetylases play important roles in eukaryotic gene regulation. Here we deleted the *C. albicans GCN5* gene in a homozygous *hda1* mutant genetic background. We generated a *gcn5, hda1* double mutant strain and examined its behavior. The behavior of the double *gcn5, hda1* mutant resembled that of the simple *gcn5* mutant with respect to growth rate, hyphal outgrowth, chlamydospore formation and sensitivity to Calcofluor white, SDS, Congo red, Hygromycin, Amphotericin B, Fluconazole and Caffeine. Whole-genome microarray analysis of the *gcn5, hda1* double mutant revealed an altered expression of 180 (2.98%) and 664 (10.99%) genes during the yeast mode of growth and during the yeast-hypha transition, respectively. Thirty-nine genes were common to the three times of the yeast-hypha transition assayed (15, 60 and 180 min); 35 genes were common between 15 and 60 min; 33 at 60 and 180 min and 31 at 15 and 180 min. Of the common up-regulated genes for the three times assayed, 5 genes corresponded to putative transcription factors (*BDF1, GAL4, NRG1, TYE7* and *ZCF39*); 4 to cell wall genes (*CRH11, PIR1, RBT2* and *RHD3*), and 4 to transporter genes (*ENA22, GAP5, PMC1* and *STL1*). Some hypha-specific genes including *ALS1, ALS3, ECE1* and *HWP1*, fatty-acid metabolism-related genes (*CYB5, FAD2* and *SUR2*) and vitamin biosynthesis-related genes (*BIO2* and *BIO3*) were down-regulated during all three times of induction. The expression of other cell wall genes (*ALS4, GLC3, GSL21, PGA6* and *SUN41*) was down-regulated at 15 and 60 min of induction. We detected the up-regulation of genes encoding for transcription factors *CTA24* (15 and 60 min), *ZCF1* (60 and 180 min), *MOT1* and *ROX1* (15 and 180 min), *MDM34* and *TOA2* (15 min), *RIM101* (60 min), *GCN4, SSN6, STP4, TBP1* and *ZMS1* (180 min), and down-regulation of *HAC1* (15 min), *TSM1* and *UME6* (60 min) and *SPT20* (60 and 180 min). Our results include a global transcriptome analysis of the cellular response to the double deletion of the *GCN5* and *HDA1* genes and afford a basis for a better understanding of the role of this histone acetyltransferase and histone deacetylase in *C. albicans*. Finally we show that the *gcn5* simple mutant is

avirulent in a mouse model.

Introduction

The histone acetyltransferase (HAT) Gcn5p plays a pivotal role in chromatin structure and in the regulation of gene expression acting as a catalytic component of multiprotein complexes [64]. In order to facilitate transcription in an activated chromatin environment, the HATs (*GCN5*) transcription machinery is required for chromatin remodelling and transcriptional activation [374]. Additionally, histone deacetylases (HDACs) remove the acetyl groups of lysine residues of histone tails, leading to chromatin compaction and transcriptional repression [308]. The discovery that a well known transcriptional co-activator, Gcn5p, also has intrinsic HAT activity provided the first direct link between histone acetylation and gene activation [45]. Since then, many studies have confirmed and extended the initial findings in different eukaryotic systems [323,514]. In yeast, Gcn5p is the catalytic component of at least two multi-protein complexes: SAGA and ADA [117,162]. Consistent with a role for Gcn5p in the chromatin remodelling of promoters, the recruitment of SAGA by a transcriptional activator leads to histone acetylation and concomitant activation of transcription *in vitro* [479]. Moreover, the mutation of sequences encoding residues critical for Gcn5p HAT activity *in vitro* is sufficient to significantly affect the function of the protein in transcriptional activation *in vivo* [249] and to generate localized alterations in the chromatin structure of target gene promoters [166,250]. Gcn5p and Hda1p are highly conserved among eukaryotes and can acetylate and deacetylate H3, H4 and H2B *in vitro*. Gcn5p and Hda1p might act as a 'chromatin conditioner' in generally establishing and maintaining active chromatin in eukaryotes and in the dynamic maintenance of chromatin organization, respectively. The genome of the yeast *Saccharomyces cerevisiae* also encodes other HATs, such as Esa1p, Sas2p, Sas3p, Hpa2p, Hat2p and Hat1p [13,42,237,346,382] and HDACs such as Hos1p, Hos2p, Hos3p, Rpd3p and Hda1p. In *C. albicans,* the *gcn5* mutant grows poorly in YEPD rich medium and fails to undergo hypha formation (switching-defective) [106], and the *hda1* mutant displays a normal phenotype under both growth conditions [307]. The double mutant displays a phenotype similar to that of the *gcn5* mutant. The molecular basis for these growth phenotypes appears to be due to problems with the activation of many genes. In *S. cerevisiae* the *gcn5* mutant strain fails to activate

the *HIS4* gene in response to low nitrogen [289], and also shows reduced activation of HO and some additional genes [248,369]. The somewhat limited phenotypic consequences of the deletion of the transcription-related HATs and HDACs described above could be explained in terms of the existence of functional redundancy. Cellular functions such as transcriptional activation might still proceed almost normally when one HAT complex is non-functional. Similar redundancies could underlie the limited consequence of deleting genes encoding HDACs [396]. Here we suggest that the deletion of *GCN5* in double and simple mutants confers typical phenotypes such as slow growth adaptation, defects in yeast-hypha switching, chlamydospore formation, and antifungal sensitivity. In support of the existence of functional overlaps between double and *gcn5* mutant strains, we suggest that the acetyltransferase activities of Gcn5p would elicit severe consequences for cell phenotypes. These consequences, however, can also be counteracted by mutating the genes encoding the HDACs: *HDA1* and *HOS2* [512].

Materials and methods

Strains and plasmids manipulation

The *Escherichia coli* strain used here was grown at 37ºC in Luria-Bertani medium supplemented with 100 μg/mL of ampicillin for plasmid selection [399].

The *C. albicans* strains used in this study are listed in Table 3.1. Strains were maintained by periodic transfer to slants of YEPD medium [1% Yeast extract (Difco), 2% Bactopeptone (Difco), 1% Glucose (Difco)]. Yeast growth was obtained in YNB medium at 28ºC supplemented with 0.2 mM uridine. Solid medium was obtained by adding agar (2%). Our solid medium for inducing the yeast-hypha transition was Lee medium, in which glucose was replaced by *N*-acetylglucosamine (1.25%), Spider medium [280], synthetic low-ammonium dextrose medium (SLAHD) [48,60,270] and Embedded medium [43].

Table 3.1 Strains used in this study

Strain	Genotype	Parental strain	Reference
SC5314	Clinical isolate		[149]
CAI4	*ura3::imm434/ura3::imm434*	CAF2-1	[132]
CAMR	*ura3::imm434/URA3*	CAI4	(Rodriguez and Dominguez, 2005)
NDH4	*ura3::imm434/ura3::imm434 hda1::hisG/hda1::hisG*	CAI4	(Martin and Dominguez, 2000)
SGM1	*ura3::imm434/ura3::imm434 hda1::hisG/hda1::hisG gcn5 hisGURA3hisG/GCN5*	NDH4	This work
SGM2	*ura3::imm434/ura3::imm434 hda1::hisG/hda1::hisG gcn5::hisG/GCN5*	SGM1	This work
SGM3	*ura3::imm434/ura3::imm434 hda1::hisG/hda1::hisG gcn5::hisGURA3hisG/gcn5*	SGM2	This work
SGM4	*ura3::imm434/ura3::imm434 hda1::hisG/hda1::hisG gcn5::hisG/gcn5::hisG*	SGM3	This Work
RAS1	*ura3::imm434/URA3 gcn5::hisG/gcn5::hisG*	RDG4	This work
HAJ1	*ura3::imm434/URA3 gcn5::hisG/gcn5::hisG hda1::hisG/hda1::hisG*		
RDG4	*ura3::imm434/ura3::imm434 gcn5::hisG/gcn5::hisG*	CAI4	(Degano and Dominguez, 2000)

The dimorphic transition was induced in Lee medium by changing the temperature to 37ºC, by adding 4% bovine calf serum (GIBCO/BRL), or by changing the carbon source. Uracil prototrophic (Ura^+) transformants were further subjected to 5-fluoroorotic acid (5-Foa; Sigma) selection for excision of the *URA3* marker [132]. Strains for generation time assays were grown on rich medium (YEPD) at an $O.D_{600nm}$ of 0.1, and growth was monitored over a period of 34 h by measuring the optical density of the cultures. To induce hyphal development on solid media, *C. albicans* cells were grown overnight at 28ºC in YEPD liquid medium and washed twice with water before being plated. Photomicrographs of colonies and invasive growth were taken with a DMRXA microscope (Leica, Germany). To observe chlamydospore formation, a CAI4 strain -CAI4, an isogenic series of *C. albicans* strain derived from SC5314- and the double mutant were streaked out lightly on cornmeal agar (Difco)-0.33% Tween 80, coverslipped, and incubated at 25ºC for 14 days.

DNA manipulation

Total DNA from *C. albicans* was prepared as described previously for filamentous fungi [399,400]. Restriction enzyme digestions and DNA ligations were performed according to the recommendations of the manufacturers. Isolation of plasmid DNA from *E. coli* was performed by standard procedures [399]. *C. albicans* cells were transformed using the spheroplast protocol [391]. Oligonucleotides were supplied by Roche (*www.roche-as.es*). A list of the primers used can be found in Table 3.2. A southern analysis was performed using a radioactive procedure. DNA fragments used as probes were labelled by the Rediprime™ II Random Prime labelling system from Amersham-Biosciences with (α-^{32}P) dCTP (Boehringer Mannheim) and were used according to the manufacturer's instructions.

Table 3.2 Primers used in this study

Primer	Sequence 5´-3	Primer	Sequence 5´-3´
RD56	TAT CCA TTG TCC AGG TGG TT	**URA1**	GGA TAC TAT CAA ACA AGA GG
RD57	ACT GAA GTA GAC TTC CCA CT	**AHM IP2**	TTA CAA TCA AAG GTG GTC C
RD64	TTC AGT TGA CGA GAT AGC AC	**AHM IP3**	GGT ACA GTT CCT CAC ATC
RD54	ATT GAA AGA CTG CAG TTT TGT	**GCN5-Inner**	CTT TCT CAC AAC TGC CAT TGA
RD55	CTT ATT CTC GAG CAA TGC TT	**5´ RACE-Inner**	GCG GGA TCC GAA CAC TGC GTT TGC TGG CTT TGA TG
URA2	AAT GCT GGT TGG AAT GCT TA		
AHM1	CCG AAC ATC AAC CAA TCG T	**5´ RACE-Outer**	GCT GAT GGC ATG AAT GAA CACTG
GCN5-Outer	ACG GTT GTT AAA TGG ACG GTA		

Chromosomal disruption of the *GCN5* gene

Deletion of *GCN5* in the *hda1* background strain was achieved by the "Ura-blaster" procedure [132]. Plasmid pCJR3 was used to generate the double mutant strain. This plasmid was constructed by Degano at our laboratory. Strain NDH4 was transformed by the protoplast method. Resistance to 5-fluoroorotic acid led to spontaneous loss of the hisG-*URA3* cassette. Deletion of the gene and loss of the *URA3* gene was confirmed by Southern blot analysis.

RLM-RACE

The *C. albicans* transcript was mapped using the FirstChoice RLM-RACE kit (Ambion). RNA was isolated from CAI4 at exponential time by breaking the cells in a micro-dismembrator (Braun, Melsungen), followed by the trizol extraction method as described in Galar Fungal standard operating procedures for RNA extraction. Total RNA was treated with Calf Intestinal alkaline Phosphatase (CIP) to remove

the 5´-phosphate from non-full-length uncapped RNA. The 5´ cap was removed from full-length mRNA by treatment with Tobacco Acid Pyrophosphatase (TAP), leaving a 5´-monophosphate to which a 5´ RACE adapter (0.3 μg/ml) oligonucleotide (5´GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA 3´) was ligated using T4 RNA ligase. Random-primed reverse transcription and nested PCR were used to amplify the 5´ transcript. The outer PCR reaction used the 5´ RACE outer primers, whereas the inner PCR reaction used the 5´ RACE inner primers (Table 3.2). The 5´ RACE inner PCR products were cloned into pGEM-T (Promega) and sequenced.

NaCl, Calcofluor white, Caffeine, SDS, Fluconazole, Itraconazole, Amphotericin B and Hygromycin sensitivities

The methods used to test the *C. albicans* strains were similar for all the effectors. Cultures were grown in 50 ml of YEPD medium with 1% glucose until the exponential phase and diluted to an $O.D_{600nm}$ of 0.4. Five microlitres of pure and 1/10 serial dilutions of each cell culture were spotted onto YEPD plates containing NaCl (0.5 to 1 M), Calcofluor white (0.05 to 1 mg/ml), Caffeine (15 mM), SDS (0.005% to 0.05%), Fluconazole (4 μg/ml), Amphotericin B (50 to 100 μg/ml), Hygromycin (100 to 300 μg/ml) and Congo red (0.1 to 0.5 mg/ml). Differences in growth were recorded after incubation of the plates at 28ºC for 72 h.

DNA sequencing and sequence analysis

Both strands of the PCR product described above were sequenced using the DNA Sequencing Service of the University of Salamanca with an Applied Biosystems ABI PRISM 3100 Genetic Analyzer.

Zymolyase sensitivity phenotypic test

Cultures of the CAI4, *gcn5, hda1* and both simple mutant strains were grown in YEPD medium up to the exponential phase. Cells were washed twice in water and resuspended in 10 mM Tris-HCl (pH 7.5) and 0.3% β-mercaptoethanol. 2×10^7 cells were resuspended in the same buffer containing 20T Zymolyase at a concentration of 0.01 mg/ml. The optical density at 600_{nm} was measured at the start of the incubation and every 20 min thereafter. The decrease in optical density reflected the portion of cells that had lysed.

Alcian blue binding assay

The Alcian blue binding assay was carried out using the method of Herrero et al [189]. A series of solutions containing different amounts of Alcian blue were prepared in 0.02 N HCl and the optical density at 600_{nm} of each solution was determined. A standard curve was plotted of the O.D values *versus* the amounts of Alcian blue. To quantify Alcian blue binding to the cell surface, an aliquot of 1000 μl of exponential-phase yeast cells ($O.D_{600nm}$, 5) was centrifuged, and the cells were washed twice with 1 ml of 0.02 N HCl and resuspended in 1 ml 0.02 N HCl containing 100 μg Alcian blue. The cell suspension was allowed to stand for 10 min at room temperature and then centrifuged for 3 min to pellet the cells. Then, the $O.D_{600nm}$ of the supernatant was measured. The amount of dye bound to the cells was calculated by subtracting the amount of dye in the supernatant from 100 μg.

RNA isolation, cDNA preparation and microarray hybridization

C. albicans CAI4 and SGM4 strains were grown in YNB [0.67% Yeast Nitrogen Base without amino acids (YNB w/o aa, Difco), 1% Glucose (Difco)] and Lee for yeast and hypha growth conditions, respectively. RNA isolation, cDNA preparation and microarray hybridization were carried out as in the previous chapter II.

Results

Determination of transcription initiation sites using RNA ligase-mediated Rapid Amplification of 5' cDNA Ends (5' RLM-RACE) of *GCN5* gene

To identify transcription initiation sites, RLM-RACE was performed. The results are shown in Figure 3.1. We amplified one band of ~500 bp, which was cloned in pGEM-T. After sequencing 5 different clones, three putative transcription start sites (three identical and two different) were identified. These products may have originated from different transcription start sites. The three positions 22, 32 and 39 bp were taken as the transcription start site (Figure 3.1). In any case, the *GCN5* transcript is relatively short only a few bp upstream from the ATG codon. It should be noted that very few data about the length of transcripts are available for *C. albicans.*

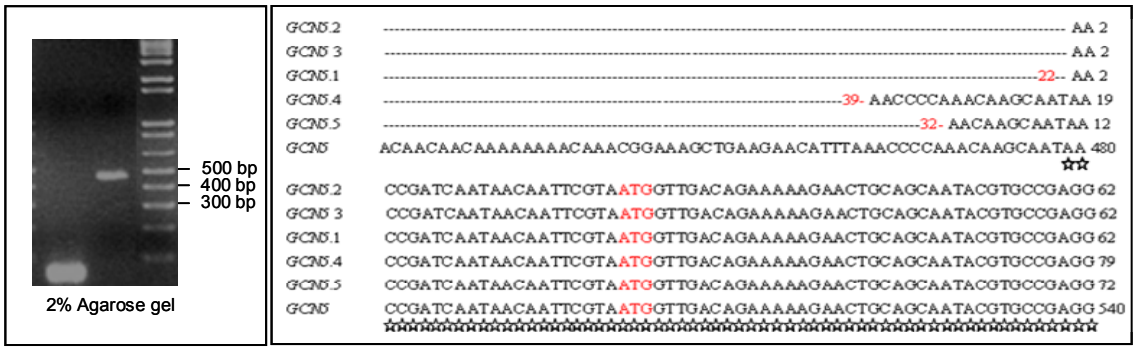

Figure 3.1 Mapping of *GCN5* transcription initiation sites by RLM-RACE. Predicted transcription start site of the *GCN5* gene

Disruption of *GCN5* in an *hda1* mutant background strain

We deleted the ORF of the *GCN5* gene with the "Ura-blaster" method [132] using protoplasts [391] in a *hda1* background strain. Deletion was verified by using two pairs of primers for PCR analysis (data not shown) and by Southern blot analysis (Figure 3.2). With the double mutant we performed a whole set of experiments and below we describe the results obtained.

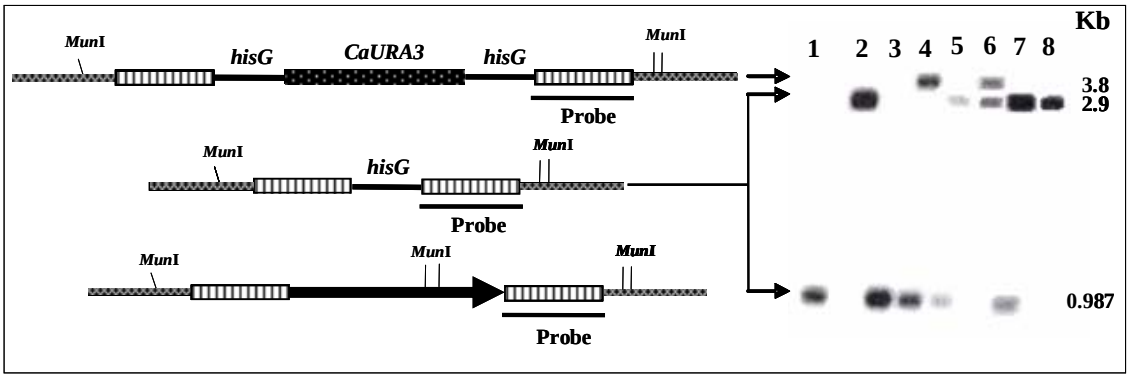

Figure 3.2 Southern blot analysis of the SGM4 double mutant *gcn5, hda1* using the *Rsa*I/*Eco*RI fragment of the *GCN5* gene as probe. Genomic DNA samples were digested with *Mun*I. Lane **1**, CAI4 *(ura3/ura3 GCN5/GCN5).*
Lane **2**, RDG4 *(ura3/ura3 gcn5/gcn5::hisG).* Lane **3**, NDH4 *(ura3/ura3 hda1/hda1::hisG)*
Lane **4**, SGM1 *(ura3::imm434/ura3::imm434hda1::hisG/hda1::hisG gcn5::hisGURA3hisG /GCN5)*
Lane **5**, SGM2 *(ura3::imm434/ura3::imm434hda1::hisG/hda1::hisGgcn5::hisG/GCN5)*
Lane **6**, SGM3 *(ura3/ura3 gcn5::hisG-URA3-hisG/gcn5::hisG)*
Lane **7**, SGM4-3 *(ura3::imm434/ura3::imm434 hda1::hisG/hda1::hisG gcn5::hisG/gcn5::hisG)*
Lane **8**, SGM4-11 *(ura3::imm434/ura3::imm434 hda1::hisG/hda1::hisGgcn5::hisG/gcn5::hisG).*

The *gcn5, hda1* double mutant has a growth defect

To analyze the growth rate of the double mutant we performed a comparison of growth between all four strains: CAI4 (*GCN5, HDA1*), NDH4 (*hda1*), RDG4 (*gcn5*) and SGM4 (*gcn5, hda1*) (Figure 3.3). The beginning of the exponential growth phase in the double mutant, as in the case of the *gcn5* simple mutant, was delayed

by up to 22 h. At our laboratory Martin [307] failed to detect any significant difference in growth rate, cell size or morphology between the *hda1* mutant and the CAI4 strain. Furthermore, the homozygous mutant of *gcn5* [106] shows a delay in the entry into the logarithmic phase (Figure 3.3).

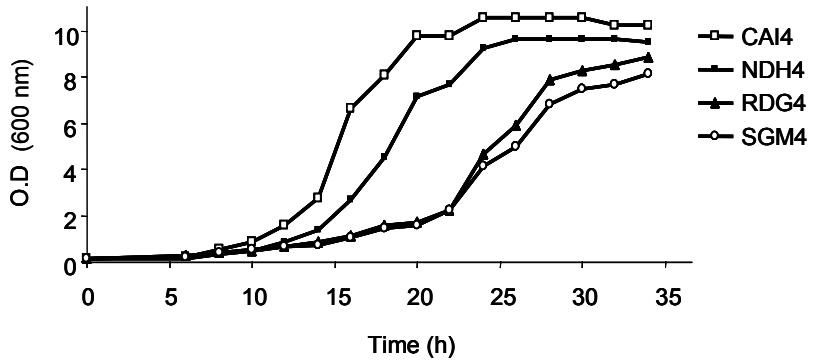

Figure 3.3 Growth curves of CAI4, NDH4 (*hda1*), RDG4 (*gcn5*) and SGM4 (*gcn5, hda1*). Strains were pre-grown on rich medium containing 1% glucose and inoculated at an O.D at 600_{nm} (0.1) YEPD. Growth was monitored every two hours over a period of 34 h by measuring the optical density of the cultures.

These results suggest that *GCN5* would be involved in vegetative growth in *C. albicans* and that *HDA1* would be unable to compensate this effect. Morphologically, the double mutant, like the *gcn5* simple mutant, is very different to the CAI4 strain, as shown in Figure 3.4. The culture of the *gcn5, hda1* double mutant in YEPD liquid medium was composed of a mixture of spherical cells and short chains of elongated, aberrantly shaped cells attached to one another (Figure 3.4B), suggesting that Gcn5p activates or suppresses the transcription of genes that directly or indirectly control cell morphology.

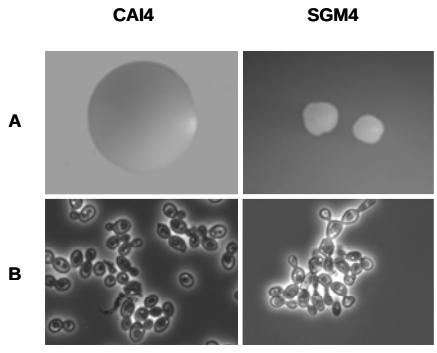

Figure 3.4 (**A**) Colony growth and (**B**) morphological characteristics of *C. albicans* homozygous strain CAI4 and double mutant *gcn5, hda1* in YEPD medium.

Behavior of the double mutant *gcn5, hda1* in solid media

For the observation of filamentous growth on solid media, cells of the simple and

double mutants as well as the CAI4 strain were plated at 50 ufc on plates. Solid media normally induce filamentous growth after 3 days. The CAI4 strain displayed normal filamentous growth, with long chains of hypha growing outwards from the colony (Figure 3.5). In contrast, the *gcn5, hda1* mutant was defective for filamentous growth in all media assayed. The colonies had completely smooth edges and all cells examined were in the budding yeast form (data not shown). These results indicated that both the *GCN5* and *HDA1* genes were necessary for efficient filamentation on solid media. We also observed that, like both simple mutants, the double mutant was unable to form hypha in Embedded medium (Figure 3.5).

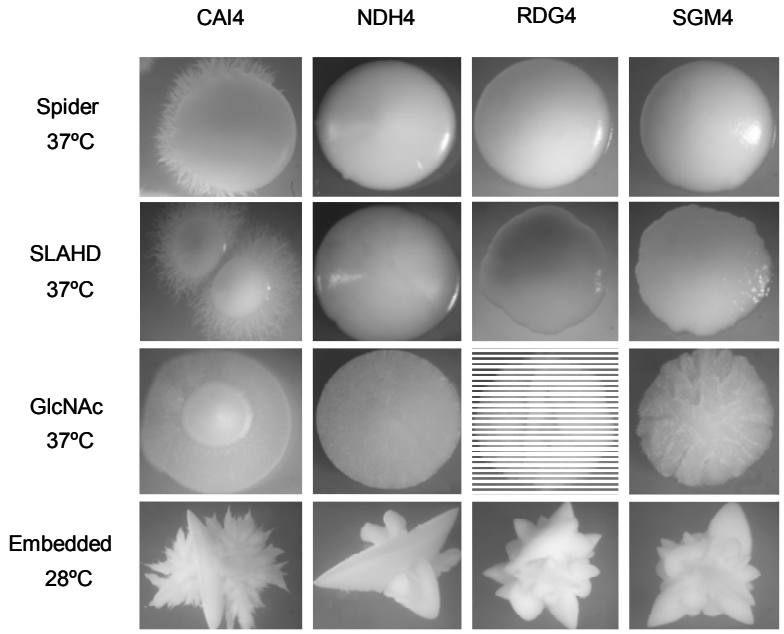

Figure 3.5 Phenotypes of the CAI4, NDH4 (*hda1*), RDG4 (*gcn5*) and SGM4 (*gcn5, had1*) on solid media. Plates were incubated for 5 days at 37ºC.

Behavior of the *gcn5, hda*1 mutant in liquid media

The behavior of the double mutant strain was next investigated by assessing hyphal formation in Lee (pH 6.8, 37ºC) or in serum inducing media. Figure 3.6 shows that the double *gcn5, hda1* mutant failed to form germ tubes in any of the liquid media assayed.

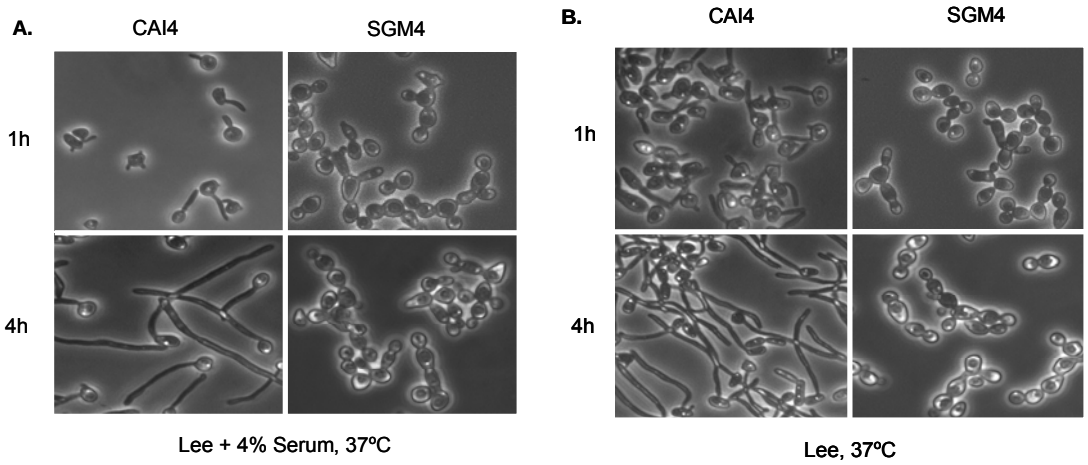

Figure 3.6 Cellular morphology of CAI4 and SGM4 (*gcn5, hda1*) strains during the yeast-hypha transition in Lee + 4% serum (**A**) and Lee medium (**B**) at 37ºC.

Chlamydospore formation

We attempted to determine whether the deletion of both genes might be involved in the formation of chlamydospores. The results are shown in Figure 3.7. The *gcn5, hda1* double mutant, like the *gcn5* simple mutant, was completely deficient in chlamydospore formation. In contrast, *hda1* [307] and the CAI4 strain were able to form chlamydospores under the same conditions. These results confirm that Gcn5p is required for the activation of the expression of chlamydospore formation-related genes and again demonstrate that there is no compensatory effect on Hda1p.

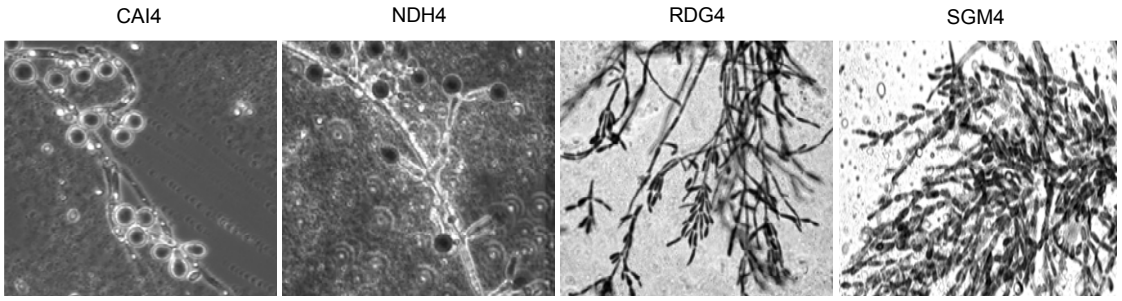

Figure 3.7 Chlamydospore formation in *C. albicans* CAI4, NDH4 (*hda1*), RDG4 (*gcn5*) and SGM4 (*gcn5, hda1)* mutant.. The strains were lightly streaked out on chlamydospore induction medium (cornmeal agar (Difco)–0.33% Tween 80 and incubated at 25ºC for 14 days.

Sensitivity to CFW, Congo red, NaCl and SDS

Using a screening method designed to identify genes involved in cell surface assembly [293], we found that the *gcn5* and *gcn5, hda1* double mutant showed a moderate sensitivity to Calcofluor white and NaCl, and hypersensitivity to Congo red and SDS; the results are shown in Figure 3.8. These findings prompted us to

carry out a further six phenotypic tests (Hygromycin, Amphotericin B, Fluconazole, Caffeine and Zymolyase sensitivity) in order to bether characterize and define the *gcn5, hda1* simple and double mutants. The *gcn5* and the *gcn5, hda1* double mutant were hypersensitive to Hygromycin, Amphotericin B, Fluconazole and Caffeine (Figure 3.8). The growth of the *hda1* mutant and CAI4 was not affected at the same concentrations.

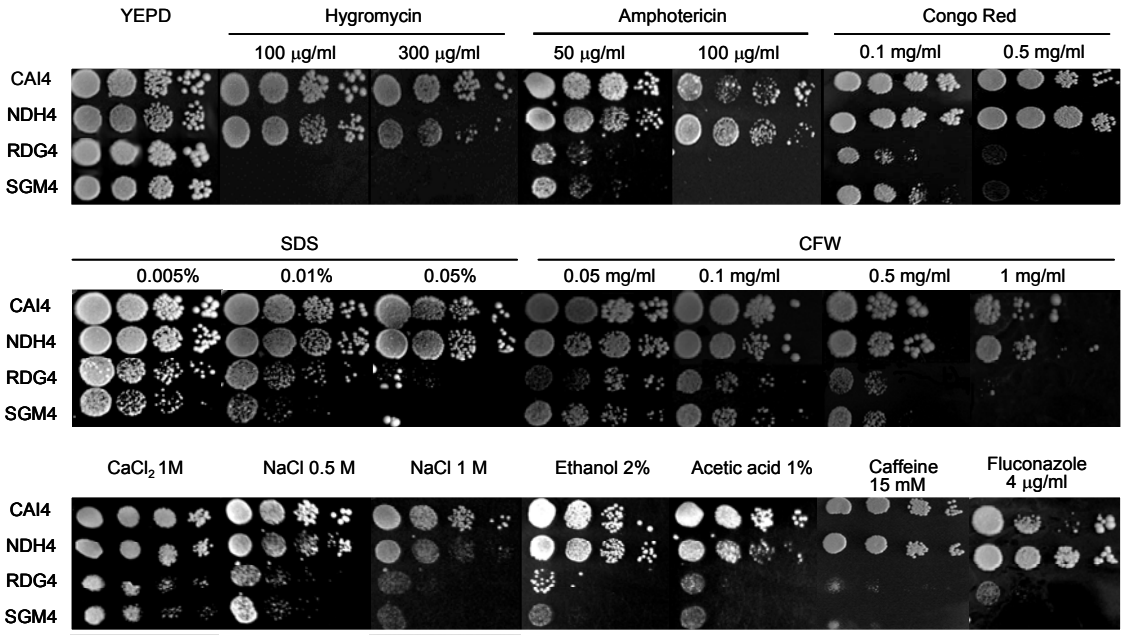

Figure 3.8 Sensitivity assays of CAI4, NDH4 (*hda1*), RDG4 (*gcn5*) and SGM4 (*gcn5, hda1*) to different agents. All strains were grown in liquid YEPD. The optical density at 600_{nm} of the cultures was adjusted to 0.4 with the same medium, and 5 μl aliquots from the cultures and from 10-fold serial dilutions was spotted onto YEPD plates supplemented with different agents. Plates were incubated at 28ºC for 4 days.

The sensitivity of yeast cells to Zymolyase has been used to detect changes in cell wall composition and arrangement [375]. Our results indicated that there were no significant differences between the CAI4 and any of the mutant strains as regards sensitivity to Zymolyase (Figure 3.9).

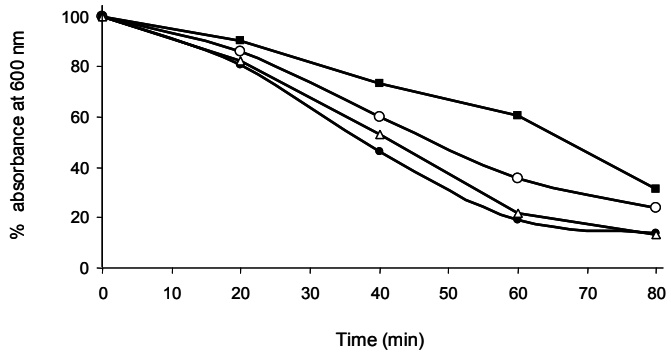

Figure 3.9 Resistance to a cell wall-degrading enzymatic complex of *C. albicans* CAI4 (○) NDH4 (■), RDG4 (●) and SGM4 (Δ) were grown up to the exponential phase in YEPD at 28ºC. Aliquots (2×10^7 cells) were resuspended in 10 mM Tris-HCl, pH 7.5, and treated with 10 μg of Zymolyase per ml. The decrease in optical density (O.D) (percentage of resistant cells) was monitored as described in Methods.

Alcian blue binding assay

We then determined the level of Alcian blue binding of CAI4 and the three mutant strains of *C. albicans,* using the method previously described [189]. All three mutants showed a significant increase in Alcian blue binding, suggesting a strong acidification of the cell wall composition (Figure 3.10). In this case, an additive effect was detected. Thus, the *gcn5, hda1* double mutant increased binding by 85%, which roughly corresponded to the addition of the increase in Alcian blue binding of the simple mutants: *hda1*, 20%, and *gcn5*, 55%.

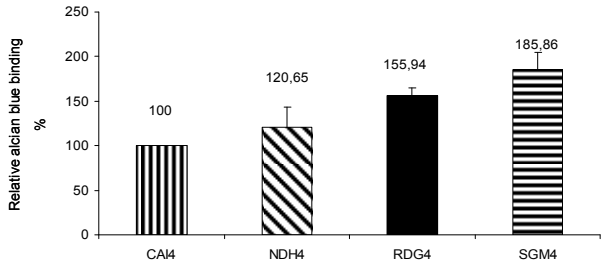

Figure 3.10 **Alcian blue binding assays**. Relative dye binding was calculated as the percentage of dye bound compared with results obtained for the parental strain CAI4

The *GCN5* gene is required for *C. albicans* virulence

Our observation that the *C. albicans GCN5* gene was required for hyphal emergence and/or elongation *in vitro,* led us to examine whether *GCN5* might also be required for *C. albicans* virulence. Mice were injected intra-peritoneally with a reconstructed *URA3 gcn5* and the double mutant strain and survival was monitored over a period of 30 days. Two days after infection, all the mice injected with the CAMR strain showed signs of systemic disease, including weight loss (data not show) and rapid mortality. Twenty percent of the mice injected with *gcn5, hda1* double mutant cells survived. No difference in mortility was found between mice infected with the *hda1* [307] and the CAMR strains. In contrast, 80% of the mice injected with 5×10^8 cells of the *gcn5* null mutant survived for the observational period of 30 days (Figure 3.11).

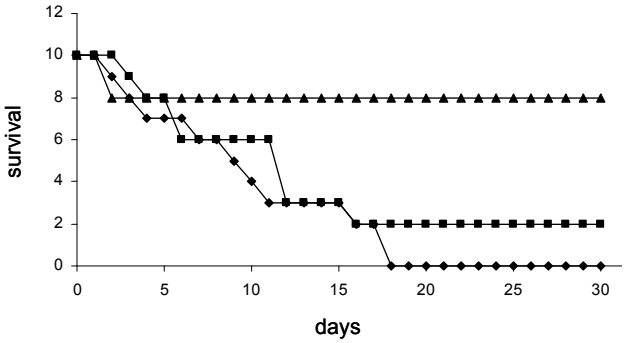

Figure 3.11 Virulence assays. Survival curves for mice (n = 10 for each *C. albicans* strain at each inoculation dose) infected with 5×10^8 cells of *C. albicans.*♦ **CAMR** (CAI4-*URA*$^+$), ▲ **RAS1** (*gcn5/gcn5-URA*$^+$), ■ **HAJI1** (*gcn5, hda1-URA*$^+$).

Genome-wide analysis

Transcript profiling was used to further examine the role of the Gcn5p and Hda1p proteins in *C. albicans.* The transcriptome of the *gcn5, hda1* double mutant was compared with the CAI4 strain during the yeast mode of growth and during the yeast-hypha transition. Previously, at our laboratory we had performed experiments in which the transcriptomes of the *gcn5* and *hda1* simple mutants were analyzed. Genes that displayed reproductive and statistically significant changes in expression were identified under both yeast growth conditions and during the yeast-hypha switch. A list of differentially expressed genes in the double mutant is provided as Supplementary data (Supplementary Tables 3.1 and 3.2). To explore the functions of the *gcn5, hda1* double mutant under conditions that promoted growth in the yeast form, we compared the transcriptomes of CAI4 and the double mutant strain in YNB medium at 28ºC. In the *gcn5, hda1* double mutant we detected an altered expression of 180 genes by ≥ 1.5-fold: 84 genes being up-regulated and 96 being down-regulated.

Only eight genes were affected by the deletion of the *HDA1* gene (all genes were down-regulated) (Figure 3.12). Three genes -*ALS2, ALS4* and *BGL22*- were commonly affected in both the double and simple mutants. The simultaneous deletion of *GCN5* and *HDA1* showed a transcriptional pattern that mainly overlapped with that of the *gcn5* single mutant (47 genes) (Figure 3.12).

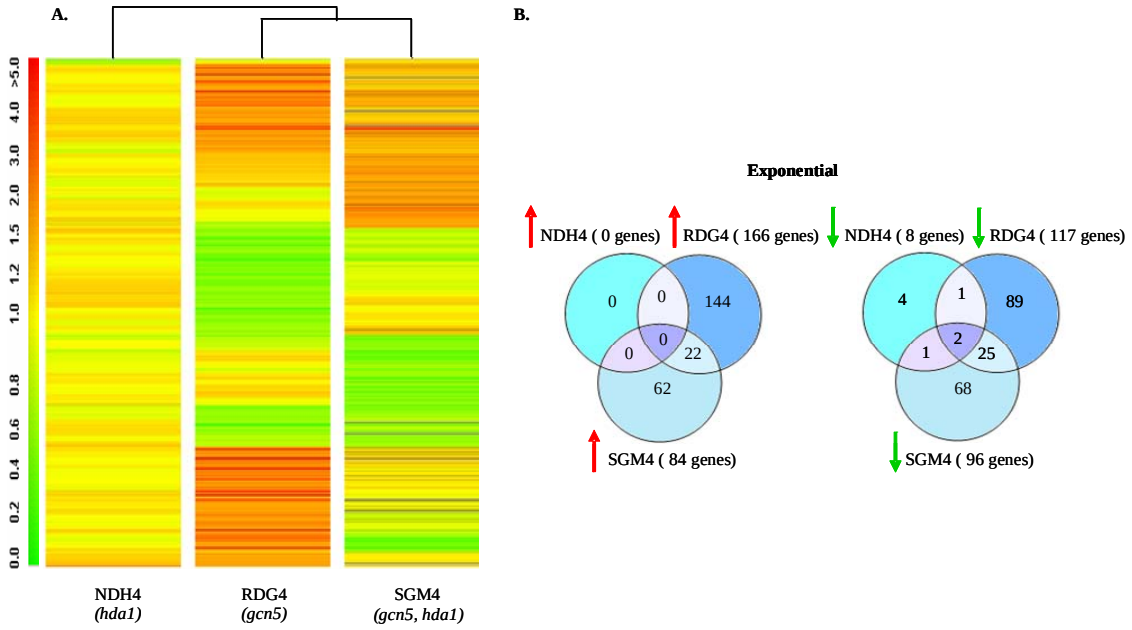

Figure 3.12 **A.** Transcript profiles of the *C. albicans gcn5* and *hda1* single and double mutants during the yeast growth form (**A**). Venn diagrams of genes regulated by Gcn5p and Hda1p in the single and double mutant strains. The CAI4 strain was used as a reference for the *gcn5* mutant (RDG4), the *hda1* mutant (NDH4) and the *gcn5, hda1* double mutant (SGM4), which were all grown under identical conditions: YNB/28ºC (non-inducing) (**B**).

However, a subset of 130 genes was affected in the double mutant that was not altered in either of the simple mutants. These results indicate that under standard growth conditions both genes jointly regulate an important set of genes.

Categorization of the regulated genes according to their functional categories of the MIPS database (*http://mips.gsf.de/genre/proj/yeast/searchCatalogFirstAction*. do? db_CYGD) revealed that double deletion of the *GCN5* and *HDA1* genes had a striking effect on carbon metabolism. The transcript levels of almost all the glycolytic genes (e.g., *FBA1, PFK1* and *PFK2*) and of the genes involved in the metabolism of the reserve sugars trehalose and glycogen (e.g., *TPS2* and *GSY1*) were reduced in the double mutant by a factor of 1.5 (Table 3.2). In addition, the transcript levels of the *ACO1* (aconitate hydratase), *LSC2* (succinate-CoA ligase, beta subunit) and *TAL1* (transaldolase) were increased (Supplementary Table 3.1).

Table 3.3 Differentially expressed genes in the *gcn5, hda1* mutant during the yeast mode of growth

Gene name	Accession no	Description	Fold regulation
Up-regulated genes			**EXP**
Cell wall			
AAF1	orf19.7436	Adhesion and aggregation mediating surface antigen	1.68
CSP37	orf19.2531	Plasma membrane hyphal cell wall protein	1.66
PGA13	orf19.6420	Putative GPI-anchored protein	1.57
PGA56	orf19.1105.2	Regulator of sorbose utilization; putative GPI-anchor	1.73
Transcription			
RPD32	orf19.6801	Histone deacetylase B	1.59
ZCF32	orf19.5940	Putative transcriptional regulator	1.80
Down-regulated genes			
Glycolysis and gluconeogenesis			
ADH1	orf19.3997	Alcohol dehydrogenase I	0.21
ADH5	orf19.2608	Probable alcohol dehydrogenase	0.63
CDC19	orf19.3575	Pyruvate kinase	0.35
FBA1	orf19.4618	Fructose-bisphosphate aldolase	0.30
GPM2	orf19.1067	Phosphoglycerate mutase	0.48
HXK2	orf19.8176	Hexokinase II	0.39
PDC11	orf19.2877	Pyruvate decarboxylase	0.35
PFK1	orf19.3967	6-phosphofructokinase, Alpha subunit	0.31
PFK2	orf19.6540	6-phosphofructokinase, Beta subunit	0.23
PGI1	orf19.3888	Glucose-6-phosphate isomerase	0.63
PGK1	orf19.3651	Phosphoglycerate kinase	0.47
PYC2	orf19.789	Pyruvate carboxylase 2	0.54
TDH3	orf19.6814	Glyceraldehyde-3-phosphate dehydrogenase	0.35
TPI1	orf19.6745	Triose phosphate isomerase	0.46
Amino acid metabolism			
ARO8	orf19.2098	Aromatic amino acid aminotransferase I	0.61
BAT22	orf19.6994	Branched chain amino acid aminotransferase	0.65
ECM17	orf19.4099	Putative sulfite reductase	0.62
HOM3	orf19.1235	Aspartokinase	0.68
LYS12	orf19.2525	Homo-isocitrate dehydrogenase	0.65
MET6	orf19.10083	Methyltetrahydropteroyltriglutamate	0.60
MIS11	orf19.2364	Mitochondrial C1-tetrahydrofolate synthase	0.55
SAM2	orf19.657	S-adenosylmethionine synthetase 2	0.47
Drug resistance			
CDR1	orf19.6000	Multidrug resistance protein	0.65
CDR4	orf19.5079	Multidrug resistance protein	0.66
Ferroxidase and ion transport			
FET99	orf19.4212	Cell surface ferroxidase	0.60
FET34	orf19.4215	Multicopy oxidase	0.66
FTR1	orf19.7219	High affinity iron permease	0.62
Lipid, Fatty-acid and isoprenoid metabolism			
FAS2	orf19.5951	Fatty-acyl-coa synthase, Alpha chain	0.50
ERG1	orf19.406	Squalene epoxidase	0.44
SHM2	orf19.5750	Serine hydroxymethyltransferase precursor	0.66
SUR2	orf19.5818	Hydroxylation of C-4 of the sphingoid moiety of ceramide	0.36
Metabolism of energy reserves (glycogen. Trehalose)			
GSY1	orf19.3278	UDP glucose-starch glucosyltransferase	0.55
TPS2	orf19.3038	Threalose-6-phosphate phosphatise	0.54
Ribosome biogenesis			
RPA1	orf19.2992	60S ribosomal protein	0.66
RPL10A	orf19.3465	L10A ribosomal protein	0.61
RPL10E	orf19.7015	Ribosomal protein L10. Cytosolic	0.58
RPL81	orf19.6002	60S ribosomal protein l7a.e.B	0.51
RPL82	orf19.2311	60S ribosomal protein l7a.e.B	0.67
RPS12	orf19.6785	Acidic ribosomal protein S12	0.63
RPS15	orf19.5927	40S ribosomal protein S15	0.49
RPS19A	orf19.5996.1	Ribosomal protein S19.e	0.54
RPS22A	orf19.6265	Ribosomal protein s15a.e.c10	0.66

RPS7A	orf19.9267	Ribosomal protein	0.67
RPS8A	orf19.6873	Ribosomal protein	0.56
YST1	orf19.6975	Ribosomal protein	0.57
Transcription factor			
EFG1	orf19.8243	Enhanced filamentous growth factor	0.68
TYE7	orf19.4941	Basic helix-loop-helix transcription factor	0.69
UPC2	orf19.391	Similar to *S. cerevisiae* Upc2p	0.63
Transport			
ATP5	orf19.5419	F1F0-ATPase complex	0.66
HXT62	orf19.2023	Sugar transporter	0.29

Description as Candida Genome date bases

In our experiments, we observed the down-regulation of *EFG1*. It has been reported that the transcript levels of almost all glycolytic genes and of genes involved in the metabolism of the reserve sugars trehalose and glycogen are reduced in an *efg1* mutant [108]. Our result also suggested that there is a indirect control on the expression of glycolytic genes in double mutant cells. We found 3 genes (*CSP37*, *PGA13* and *PGA56*), encoding for cell wall and cell surface proteins, to be up-regulated in the double mutant. *CSP37* is a membrane-associated protein and the deletion of both alleles of the gene showed no apparent defect in cell viability, growth, or cell wall assembly but displayed attenuated virulence in systemic infections [416]. The other two genes -*PGA13* and *PGA5*- correspond to mucins that are induced during cell wall regeneration [62]. Another interesting group of down-regulated genes were genes encoding for ribosomal proteins. The expression of 12 genes involved in ribosomal proteins was down-regulated in the double mutant cells. It has been reported that the expression of 63 genes out of 117 (53%) is down-regulated during the yeast mode of growth in the *gcn5* simple mutant, raising the possibility that Gcn5 is a major regulator of genes involved in ribosomal proteins.

We observed that two (*CDR1* and *CDR4*) of the five putative genes belonging to the multidrug transporter of the ATP-binding cassette (ABC) super family were down-regulated. The over-expression of *CDR1* in an azole-resistant strain has been reported [522]. We detected the down-regulation of two multicopper oxidases, *FET34* and *FET99,* required for growth under low iron conditions [424]. The deletion of *GCN5* and *HDA1* is associated with decreased expression of genes involved in amino acid metabolism and lipid, fatty-acid and isoprenoid metabolism (*ERG1, FAS2, SHM2* and *SUR2*) during the yeast mode of growth. Finally, we detected the down-regulation of *EFG1, TYE7* and *UCP2*. *UCP2* is a transcriptional regulator of ergosterol biosynthetic genes and sterol uptake [427].

Genome-wide analysis during morphogenesis

Next, we focused on the identification of genes whose expression was altered in the double mutant strain during the yeast-hypha transition. We generated the transcriptional profiles for the *gcn5, hda1* double mutant in Lee medium for 15, 60 and 180 min at 37ºC using the CAI4 strain as reference (Figure 3.13).

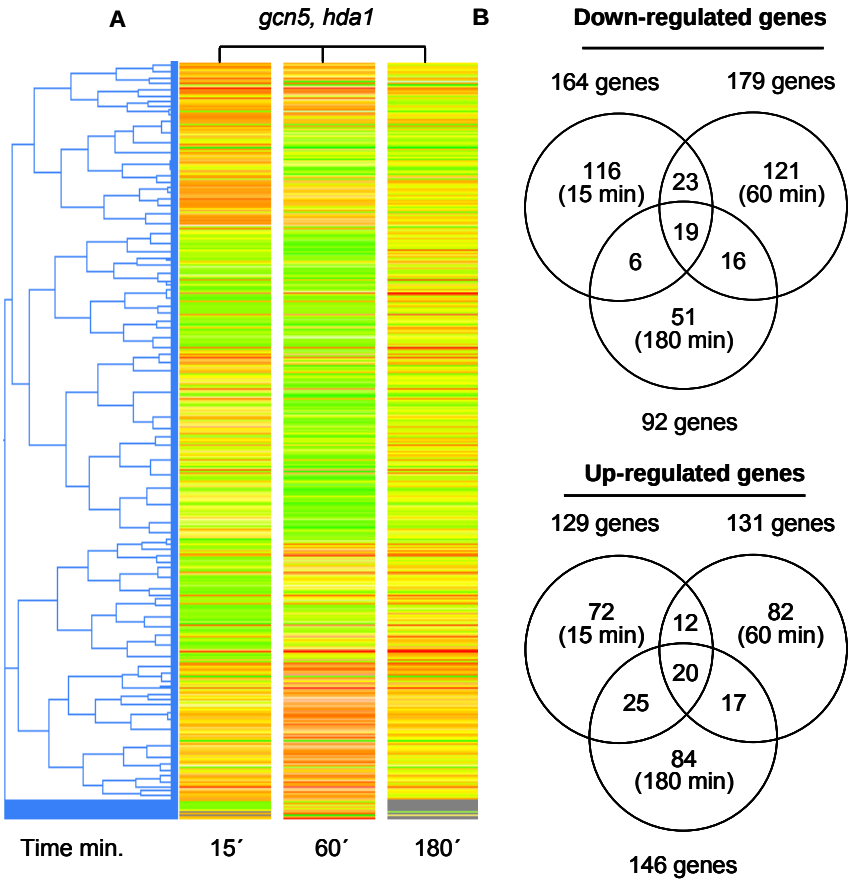

Figure 3.13 Transcript profiles of *C. albicans* double mutant during the yeast-hypha transition (15, 60 and 180 min) (**A**). Venn diagrams of genes regulated during different times of the hypha transition. The CAI4 strain was used as a reference for the double mutant (*gcn5, hda1*), which was grown in Lee/37ºC (inducing) (**B**).

A total of 664 genes were found to change at the consensus level of ≥ 1.5 fold, including 312 up-regulated and 352 down-regulated genes. The highest fold-change detected was for CA0410, at 60 min, and the lowest fold-change for *ECE1*, at 180 min. The distribution of genes altered at the three points is represented in the Venn diagram, and the clustering of genes regulated by temperature is represented in Figure 3.13 (A and B).

Table 3.4 Differentially expressed genes during the three times of incubation in the double mutant strain

Gene name	Accession no	Description (CandidaDB)	Fold regulation		
Up-regulated genes			**15min**	**60min**	**180min**
Cell wall					
CRH11	orf19.2706	putative GPI-anchor; localizes to cell wall	1.77	1.97	3.80
KRE1	orf19.4377	Predicted GPI-anchor	1.64	1.67	3.31
PIR1	orf19.220	Putative cell wall protein of the PIR family	3.85	3.28	2.55
RHD3	orf19.5305	Putative GPI-anchored protein	2.38	1.78	7.19
Oxidoreductase					
FRE10	orf19.1415	Major cell-surface ferric reductase	2.37	3.46	4.18
Transporter					
ENA22	orf19.6070	P-type ATPase involved in Na^+ efflux	2.63	1.90	3.66
FTR1	orf19.7219	High affinity iron permease	3.59	2.08	6.83
GAP5	orf19.4456	General amino acid permease	2.12	1.59	2.53
HGT10	orf19.13176	Sugar transporter	1.79	1.70	1.62
PMC1	orf19.1727	Ca^{2+} transporting P-type ATPase	1.61	2.03	2.06
SHA3	orf19.3669	Involved in glucose transport	2.85	2.00	1.71
Transcription					
BDF1	orf19.978	Putative ranscription factor	1.70	1.53	1.66
GAL4	orf19.5338	Transcriptional activator	1.63	1.82	2.53
NRG1	orf19.7150	Transcriptional repressor Nrg1p/Nrg2p	1.95	1.75	2.52
TYE7	orf19.4941	Basic helix-loop-helix transcription factor	2.05	1.70	2.97
ZCF39	orf19.7583	Putative transcription factor	1.57	1.55	2.05
Other function					
GAL7	orf19.3675	UDP-glucose-hexose-1-phosphate	2.67	1.57	2.57
Unknown function					
IPF15217	orf19.3779	WD-repeat protein	1.53	1.65	1.60
REP1	orf19.7521	Unknown function	2.13	1.98	3.81
IPF4861	orf19.6274	Unknown function	3.56	2.99	2.58
Down-regulated genes					
Cell wall					
ALS1	orf19.5741	Agglutinin-like protein	0.40	0.52	0.45
ALS3	orf19.2355	Agglutinin like protein	0.22	0.52	0.13
ECE1	orf19.3374	Cell Elongation Protein	0.10	0.15	0.04
HWP1	orf19.1321	Hyphal wall protein	0.15	0.45	0.13
MNT1	orf19.1665	Mannosyltransferase	0.32	0.45	0.53
Transporter					
FCY24	orf19.7331	Purine-cytosine transport protein	0.64	0.57	0.54
HNM3	orf19.2587	Choline permease	0.53	0.37	0.52
IPF14040	orf19.2397	probable transporter	0.20	0.38	0.22
Vitamin biosynthesis					
BIO2	orf19.2593	Biotin synthetase	0.59	0.33	0.42
BIO3	orf19.2591	DAPA aminotransferase	0.60	0.50	0.47
Other function					
ASC1	orf19.6906	A 40S ribosomal subunit	0.55	040	0.55
CYB5	orf19.7049	Cytochrome b5	0.52	0.63	0.50
ICL1	orf19.6844	Isocitrate lyase	0.57	0.60	0.64
FAD2	orf19.7765	Delta-12 fatty acid desaturase	0.60	0.34	0.57
PFK2	orf19.6540	6-phosphofructokinase	0.44	0.41	0.47
POR1	orf19.1042	Mitochondrial outer membrane	0.57	0.32	0.48
RPL12	orf19.1635	Ribosomal protein	0.59	0.56	0.45
SUR2	orf19.5818	Hydroxylation of C-4 sphingoid	0.56	0.54	0.55
Unknown Function					
PET18	orf19.7330	Uncharacterized protein	0.56	0.65	0.45

Description as Candida Genome date bases

We found that 39 genes were common to the three times assayed (20 and 19 up- and down- respectively); 35 genes at 15 and 60 min (12 up- and 23 down-), and 33 genes at 60 and 180 min (17 up- and 16 down-). 188 genes (72 up- and 116 down-), 203 genes (82 up- and 121 down-) and 135 genes (84 up- and 51 down-) varied only at one time of induction (15, 60 and 180 min respectively). The 39 common genes at the three times are listed in Table 3.4. Only 3 of the common genes corresponded to genes of unknown function. Of the rest of the genes, 13 belonged to disperse functional categories- i.e. *CYB5,* cytochrome b5; *SUR2,* hydroxylation of C-4 of the sphingoid moiety of ceramide, etc., and 26 genes could be grouped into four functional categories.

The main category corresponds to cell wall proteins and includes the up-regulation of *RHD3*, a putative GPI-anchored protein that localizes in the cell wall and whose transcription is decreased upon yeast-hyphal switch [61,104,342], *PIR1*, a structural protein of the cell wall [309]; *KRE1* a predicted GPI-anchor, with a role in 1,6-beta-D-glucan biosynthesis [36,46], and *CHR11*, a putative GPI-anchor, that localizes to the cell wall [6,61]. We observed down-regulation of *ECE1*, a protein involved in cell elongation [27,32]; of *HWP1*, a well known cell wall protein [443]; two genes belonging to the *ALS* family, *ALS1* and *ALS3*, and the alpha-1,2-mannosyl transferase, *MNT1*. All these down-regulated genes have been described previously as being up-regulated during the hypha transition [39,342].

Other genes regulated during all three times of incubation were grouped in the following categories: transporters (9 genes), transcription factors (5 genes) and vitamin biosynthesis (2 genes). As mentioned above, the double mutant strain failed to form hypha under inducing condition and the down-regulation of hypha-specific genes is consistent with our results.

Expression of transcription factors

Our microarray analysis indicated that five genes encoding for transcription factors *BDF1* [28,302], *GAL4* [306], *NRG1* [330,331], *TYE7* [177,231,281,342], and *ZCF39* were up-regulated at three times (Table 3.4). Sixteen genes encoding transcriptional regulators were found to be regulated at one and two times. Twelve of those genes were up-regulated, and four were down-regulated (Supplementary Tables 3.3 and 3.4). Fifteen genes encoding transcriptional regulators (13 up- and 2 down-regulated) have been reported to be regulated by *GCN5* at one and two

times. In the *hda1* simple mutant, the up-regulation of *TYE7, UME6, ZCF20* and the down-regulation of *HAC1* at 15 min of incubation have been reported. Seven of the twenty-one regulated transcriptional factors in the double mutant were overlapped with *gcn5* simple mutant. The rest of the transcriptional regulators may be involved in eliciting changes in gene expression that affect the reprogramming of *C. albicans* physiology in response to the deletion of *GCN5* and *HDA1*.

Time-course of gene induction of the main significant categories

The genes belonging to diverse functional categories whose expression level changed at least at one time of incubation were clustered together. These diverse functional categories include genes that are both up- and down-regulated, indicating a differential regulation of these functions in the double mutant. Several genes can not be readily categorized into any functional group and represent a wide range of cellular metabolic processes (Supplementary Tables 3.3 and 3.4). The microarray results demonstrate that the lack of Gcn5p and Hda1p affects a large number of cell functions and most likely serves to globally reprogram *C. albicans* physiology to allow the double mutant to adapt to this environment.

Cell wall genes:

Four genes detected by our microarray analysis, *ALS1* and *ALS3* (at three times), *ALS2* (15 min) and *ALS4* (15 and 60 min) encode an agglutinin-like protein and were found to be down-regulated in the double mutant (Figure 3.14). It has been reported down-regulation of *ALS4* (15 min), *ALS3* (15 and 180 min) and *ALS1, ALS2* at the three times of incubation in the *gcn5* simple mutant.
In the *hda1* simple mutant the down-regulation of *ALS1* (15 min) and *ALS3* (180 min) has been reported. The expression of *ALS* genes can affect the adhesion [541] of *C. albicans*, and could be related to the increase in *C. albicans* virulence under host invasion. This finding and is consistent with the phenotypic results, which indicated that the double mutant failed to form true hypha in either liquid or solid media. Decrease in expression *ALS1* and *ALS3* (Figure 3.14) in the both simple and double mutant during the yeast-hypha transition suggested that these genes separately regulated by Gcn5p and Hda1p.

Glucanases, glucosidases and mannosyltransferases:

Our microarray analysis indicated that twelve genes encoding glucanases, glucosidases and mannosyltransferases were regulated at one, two and three times (Supplementary Tables 3.3, 3.4 and Figure 3.14). Two of these genes were up-regulated (CA5339 and *GSL22*), and ten were down-regulated (e.g *MNT1*, *GSL21*, *GLC3*, *SUN41, MNT3, PMT2* and *PMT4*). The change in expression of these genes may be involved in eliciting changes in cell wall composition.

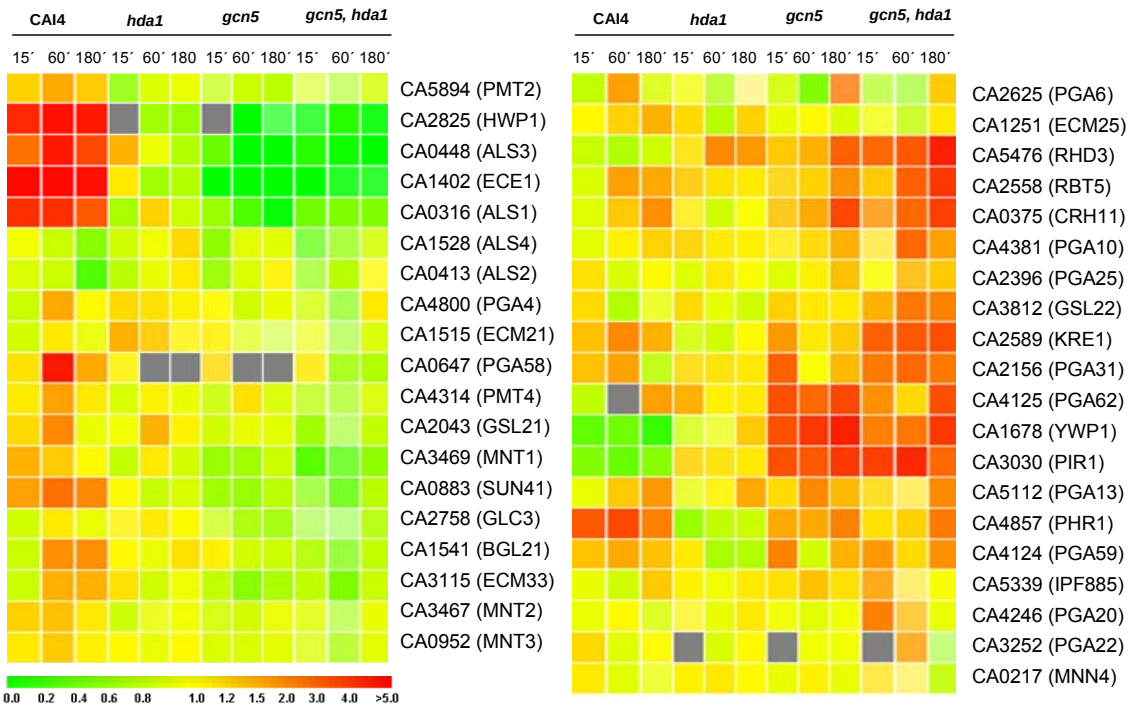

Figure 3.14 Transcriptional regulation of genes involved in the cell wall in the CAI4 strain, both simple and double mutants Representative genes responsible for specific cellular functions are grouped and their regulation during 15, 60 and 180 min of yeast-hypha transition in the double mutant and strain relative to the CAI4 reference-type strain is listed.

The up-regulation of *MNN4* (15 min), *BMT4* (180 min) and *SUN42* (60 and 180 min) and down-regulation of *MNT1* and *SUN41* at 15 and 60 min of incubation has also been reported in the *gcn5* simple mutant. In the *hda1* simple mutant, the down-regulation of *PMT1, PMT2* (15 min) and *BGL22* (60 min) has been described. These results suggest that, in addition to common regulated genes between both the simple and double mutant cells under the yeast-hypha transition (Figure 3.14), some genes were regulated jointly by *GCN5* and *HDA1*.

GPI-anchored proteins

Our results indicate that the transcript levels of sixteen genes belonging to a large class of functionally diverse proteins (GPI-anchored proteins) were regulated in the double mutant at least at one time during the hypha transition. Of these, *PGA20* (15 min), *PGA22* and *PGA25* (60 min), *PGA13* and *PHR1* (180 min), *PGA10* and *RBT5* (60 and 180 min) and *PGA31, PGA59, PGA62* (15 and 180 min) were up-regulated and *ECM21, ECM25, ECM33, PGA4* and *PGA58* (60 min), *PGA6* (15 and 60 min) were down-regulated (Figure 3.14). GPI-anchored proteins may be enzymes, surface antigens, adhesion molecules, or surface receptors [86,200,455]. Ten genes -*PGA6*, *PGA7*, *RBT1*, *HYR3*, *PGA13*, *PGA10*, *PGA26*, *RBT5, PGA62*, *PHR1* and five genes - *PGA48* and *PGA62, PHR1* (15 min) and *RHD3, PGA59* (60 and 180 min) encoding known or putative GPI-anchored proteins have been described to be regulated in the *gcn5* and *hda1* simple mutant respectively. Our results indicate a role of histone acetyltransferase (*GCN5*) and histone deacetylase (*HDA1*), jointly or separately, in the regulation of genes encoding GPI-anchored proteins during the hypha transition.

Genes involved in protein synthesis and ribosomal proteins

A second important category includes the down-regulation of 19 genes coding for ribosomal proteins and involved in protein synthesis. Twelve of these genes were down-regulated at 15 min; 3 at 60 min, 2 at 180 min, one at 15 and 180 min and another one at three times of incubation (Figure 3.15). Only three genes (*MRPL36, MRPL17,* and *RSA2*) of this category were found to be up-regulated after 15 and 60 min in the double mutant strain (Supplementary Table 3.3). No data about the behavior of this group of genes under standard conditions of the yeast-hypha transition (serum or temperature) have been reported, and only 11 are down-regulated in the *gcn5* simple mutant at one and two times of incubation. Many of them have been described as down-regulated by comparison of the *hda1* simple mutant with a CAI4 strain during one and two times of hypha transition (Figure 3.15).

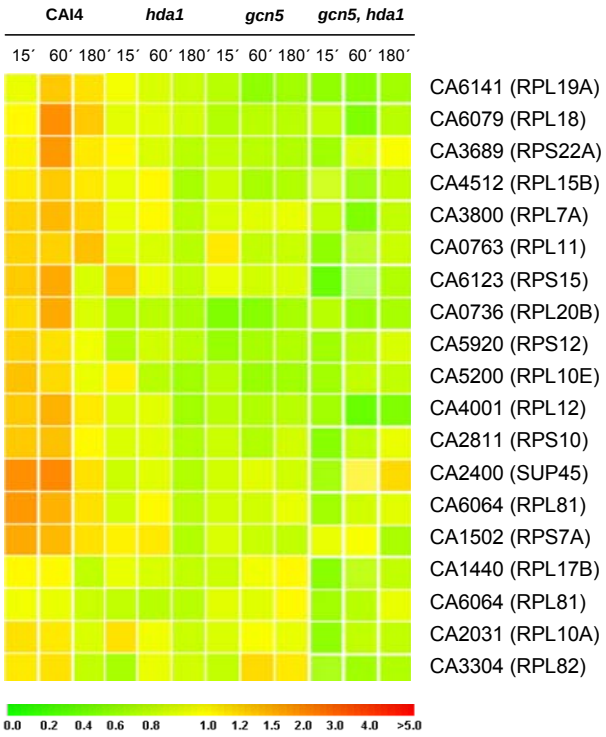

Figure 3.15. Transcriptional regulations of genes coding for ribosomal proteins in the CAI4 strain, both simple and double mutants during the yeast-hypha transiton.

Central carbon metabolism

The regulation of several genes (18 down-regulated after one, two and three times of incubation) involved in the glycolytic pathway, TCA cycles and gluconeogenesis was detected (Figure 3.16). The expression of most of them increased with time, suggesting an adaptation to the hyphal mode of growth. It has been reported that 20 genes involved in glycolysis and gluconeogenesis are regulated (15 down-regulated) in the *gcn5* simple mutant and that 20 genes involved in glycolysis, gluconeogenesis and the TCA cycle are up-regulated in the *hda1* simple mutant at one, two and three times of incubation (Figure 3.16). The down-regulation of most genes involved in central carbon metabolism in the double mutant and the *gcn5* simple mutant is in agreement with dominant of *gcn5* phenotype in the double mutant strain.

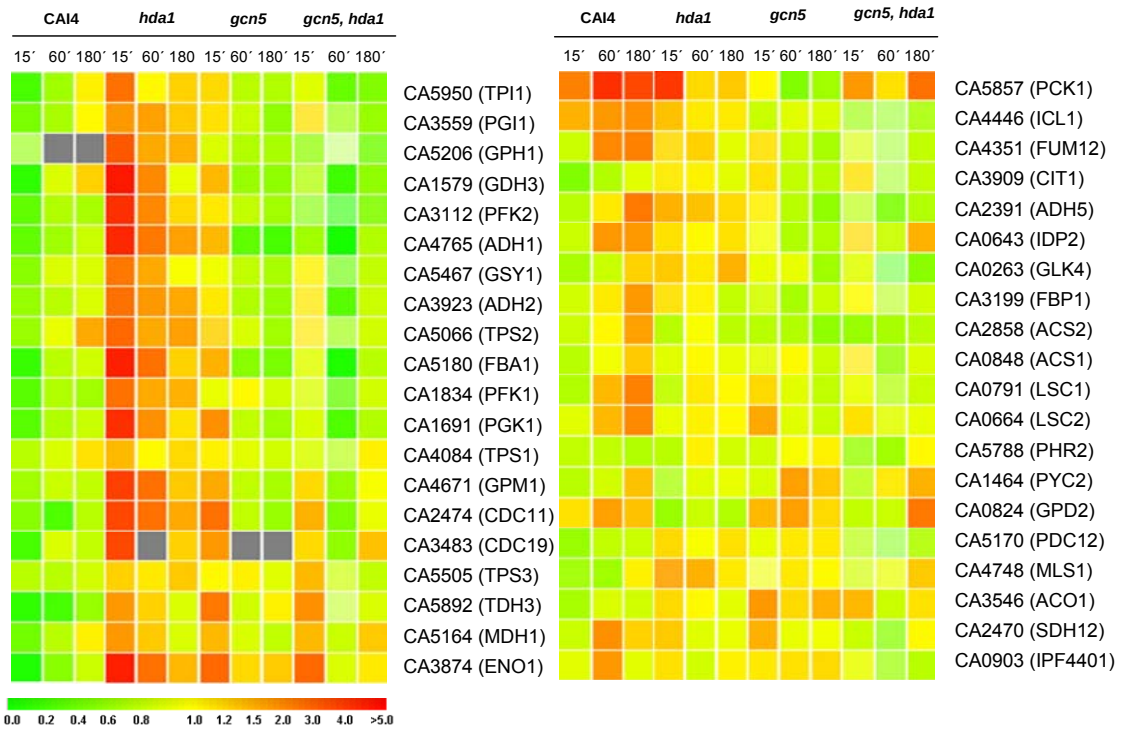

Figure 3.16 Transcriptional regulation of genes involved in the glycolytic pathway in the CAI4 strain, both simple and double mutants during the yeast-hypha transition.

Transporters

Nine genes were regulated at the three times of incubation (three genes down- and six up-, Table 3.4) and 20 genes at one or two times (Table 3.5). At 15 min, a number of ion transporters including *ENA21, PHO84, PMA1* and *RHO88* were down-regulated. At 180 min, some genes encoding for amino acid-transporter permeases, including *AGP1, AGP3, DIP51* and *GAP6,* were up-regulated. Martin [307] detected up-regulation of genes involved in glucose transport *HGT11, HGT12* (15 min), *SNF31* (180 min) and *HGT7* (15 and 60 min) in the *hda1* mutant. Degano [106] reported up-regulation of 4 genes encoding for amino acid-transporter permeases at 180 min of incubation in the *gcn5* simple mutant. The up-regulation of genes involved in the amino acid-transporter permeases in both the double and *gcn5* simple mutant is again in agreement with the dominant of phenotype of *gcn5* in the double mutant strain.

Table 3.5 Regulated genes involved in transport

Gene name	Accession no	Description	Fold regulation		
Up-regulated genes					
AGP1	orf19.8784	Asparagine and glutamine permease	0.48	0.58	2.89
AGP3	orf19.3795	Amino acid-permease	1.37	0.94	1.80
CAN2	orf19.111	Amino acid permease	0.58	0.72	0.95
CAN5	orf19.3641	Amino acid permease	1.02	0.57	0.95
CTR1	orf19.3646	Copper transport protein	0.80	0.82	0.50
DIP51	orf19.2942	Dicarboxylic amino acid permease	1.44	1.15	1.62
DUR32	orf19.5017	Urea transport protein	0.88	0.86	0.64
ENA21	orf19.5170	P-type ATPase	0.61	1.23	1.23
FTR2	orf19.7231	High affinity iron permease	1.34	2.15	2.57
GAP6	orf19.6659	General amino acid permease	1.12	0.97	2.20
HGT8	orf19.2021	Putative glucose transporter	1.56	0.93	2.92
HGT6	orf19.2020	Sugar transporter	1.12	0.80	1.76
IPF1992	orf19.7336	Putative MFS transporter	1.19	1.69	1.35
IPF9079	orf19.4550	Membrane transporter	1.23	2.27	1.21
JEN2	orf19.12767	Carboxylic acid transporter protein	1.85	1.81	1.43
PHO84	orf19.1172	Inorganic phosphate transport protein	0.57	1.08	0.75
PHO88	orf19.7327	Involved in phosphate transport	0.51	0.69	0.81
PMA1	orf19.5383	Plasma membrane H^+-transporting ATPase	0.64	0.70	0,91
TPO4	orf19.473	Putative sperimidine transporter	0.73	0.80	0.63
ZRT1	orf19.3112	High-affinity zinc transport protein	0.94	1.16	0.60

Description as Candida Genome date bases

Vitamin biosynthesis

We detected the down-regulation of two genes involved in biotin biosynthesis -*BIO2*, *BIO3*- at three times, and *BIO4* at 60 min of incubation in the double mutant cells. We identified the down-regulation of two genes involved in thiamine biosynthesis -*THI13* (60 min) and *THI4*- at 15 and 180 min of induction. The down-regulation of *BIO2* at 180 min and of two genes involved in thiamine biosynthesis (*THI4* and *THI13*) at one and two times of incubation in the *gcn5* simple mutant has been described. This result again is in agreement with the dominance of the *gcn5* phenotype in the double mutant strain.

Set of genes regulated only at one incubation time

The up-regulation of two components of the F_1F_0 ATPase complex (*ATP2* and *ATP16*), subunits of cytochrome b and cytochrome c (*COB, COX3A* and *COX3B*), subunits of the NADH ubiquinone oxidoreductase (*NAD1, NAD4, NAD5* and *NAD6*), three genes encoding 26S proteasome (*RPN1, RPN9* and *RPT4*) and two components of superoxide dismutase (*SOD1* and *SOD2*) were detected at 15 min of incubation. Of those genes grouped in the secretory pathway (*ERV25, ERV46, SEC24* and *SEC31*) and methionine biosynthesis (*CYS3, MET2, MET3, MET10*, *MET14, MET15* and *SAM2*) were down-regulated at 15 min of induction (Figure 3.17). Some of these genes (*MET3* and *MET14*) overlap with the *gcn5* simple

mutant at 15 min of incubation. No genes of this category were found up-regulated or down-regulated at any time in the *hda1* simple mutant.

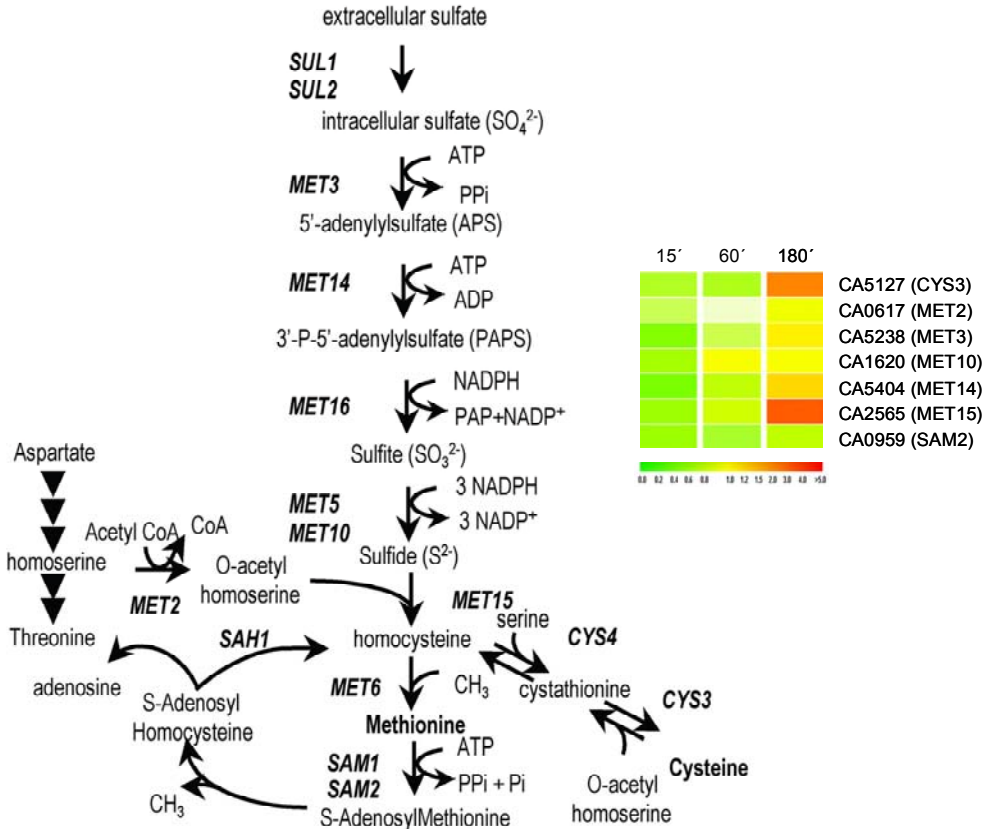

Figure 3.17 Differential expressions of the genes involved in sulphur amino acid biosynthesis in the *gcn5, hda1* double mutant strain.

At 60 min of incubation, the down-regulation of genes involved in ergoestrol biosynthesis (Figure 3.18) and respiration (*COX12, CYT12, NUC2* and *QCR8*) was observed. At 60 and 180 min, we detected regulation two and four genes encoding histones *HHF1, HHF22, HHT1, HHT21* and *HTB1*. However three of these genes, *HHF21, HHF22* and *HHT1,* were up-regulated in the *hda1* simple mutant [307]. While five genes were down-regulated in the *gcn5* simple mutant [106].

These results suggest a remodelling of chromatin, probably for it to be adapted to a new transcriptional profile. No specific set of down-regulated genes was found at 180 min of incubation, suggesting that at this time the double mutant cells had reached a general balance in their main regulatory metabolic pathways.

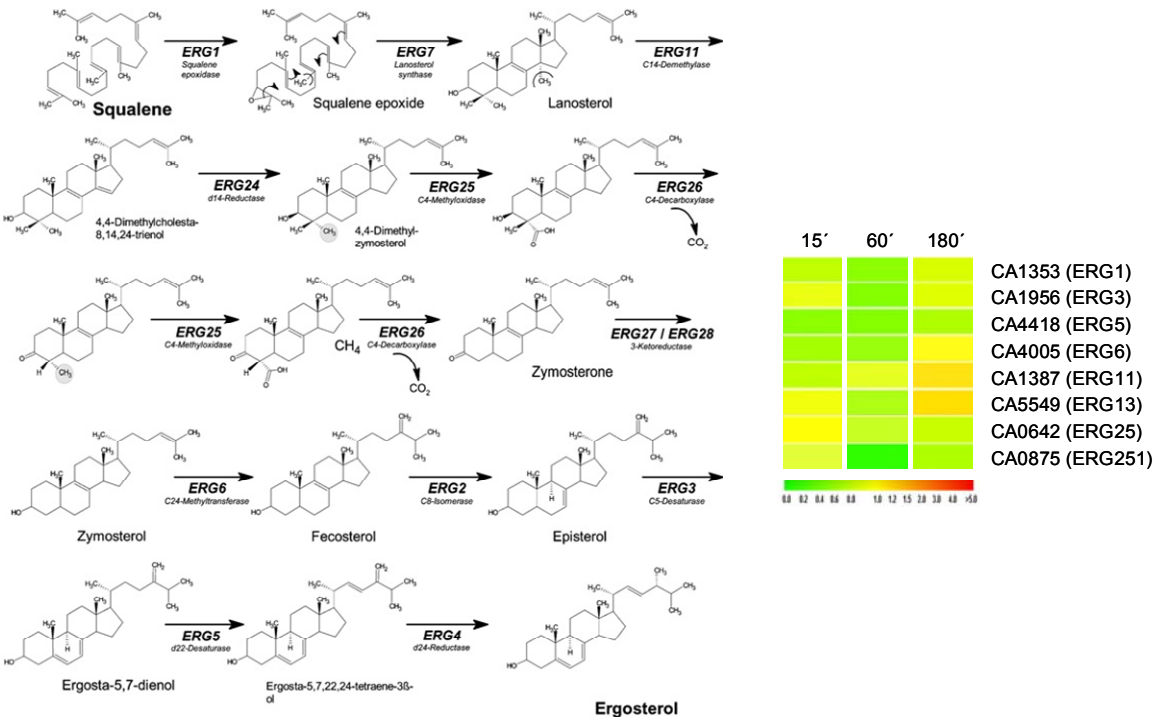

Figure 3.18 Sphingolipid biosynthesis pathway in the yeast *S. cerevisiae* according to [486]. Differential expression of the genes involved in sphingolipid biosynthesis. Expression ratios of the four paired comparisons (log scale) are represented during the yeast-hypha transition (15, 60 and 180 min).

Discussion

Candida albicans is able to establish mucosal and invasive disease by means of different virulence factors of unknown regulation. The focus of our work was on understanding the possible compensatory effects of deletion in the same strain of a histone acetyltransferase (*GCN5*) and histone deacetylase (*HDA1*) by analyzing the whole transcriptional profile during the yeast mode of growth and during the yeast-hypha transition. It has been reported that HDAC inhibitors cause a 90% reduction in the adherence of *C. albicans* to human cultured pneumocytes and significantly inhibit serum-induced germination [426]. The functions of *GCN5* and *HDA1* on the known filamentation pathways in *C. albicans* are as yet unknown. In our laboratory the *GCN5* and *HDA1* genes, have been previosly deleted in a CAI4 strain. In this work, we deleted the *GCN5* gene in an *hda1* mutant background strain. Deletions of *GCN5* appeared to block hyphal formation under a variety of standard inducing conditions; for example in the presence of serum or GlcNAc or during growth on Spider medium [106]. The deletion of *HDA1* does not block hypha formation in all liquid media, but does block the formation of true hypha in some solid media [307]. In the present work we show that deletion of the *GCN5* and

HDA1 such as the *gcn5* simple mutant strain is associated with a detectable deficiency in the rate of colony formation during growth on rich-medium plates at 28ºC. Furthermore, there was a significant difference between the growth rate of the double mutant and CAI4 during exponential growth in rich liquid medium at 28ºC. Morphologically, like the *gcn5* simple mutant, the *gcn5, hda1* double mutant is very different from the CAI4 strain. The main phenotype of the double mutant is its inability to switch from the yeast to the filamentous form in liquid media containing serum or at 37ºC and in all the solid media assayed. Our data suggest that the lack of Gcn5p down-regulates alternative morphogenetic pathways, thereby preventing filamentation under these conditions. However, Gcn5p is required for maintenance of the yeast growth form because the *gcn5* mutant growth like small pseudohypha. Our results show that in the *gcn5, hda1* double mutant, as well as in the *gcn5* simple mutant, chlamydospore formation is completely abolished. Chlamydospores can be induced to form under nutrient-poor oxygen-limited conditions at low temperatures [52]. Chlamydospores have been found in the lung of AIDS patient [80,353], and thus may be relevant to infection. The ability to form chlamydospores is almost universal among the *C. albicans* clinical isolates [2], thus suggesting that chlamydospores would have a functional role in *C. albicans* biology. A recent study has shown that in *C. albicans* genes such *CYP56, ISW2, MDS3, RIM13, RIM101, SCH9* and *SUV3* are required for efficient chlamydospore formation [317,353], we have found that *GCN5* is another gene required for efficient chlamydospore formation.

In the double mutant cells, a total of 664 genes, out of 6039 present in the microarray, changed due to deletion of *GCN5* and *HDA1*, in a minimal medium (Lee) at one of the three incubation times assayed (15, 60 and 180 min). These represent a percentage of 10.99%. 180 genes, while only 2.98% of the genes changed during the yeast form of growth. Only five transcription factors were up-regulated at the three times assayed and others were regulated at two times (five), or at one time (nine). The regulation of some well characterized transcription factors e.g., *NRG1* [330] and *UME6* [22] is in agreement with the phenotype of the double mutant cells during hypha development.

We found, regulation at least 40 genes corresponding to well cell wall proteins. Four of the repressed genes, *ALS1* [205], *ALS2* [204], *ALS3* [203] and *ALS4* [204] are all classified in the same subfamily [200]. We detected the down-regulation of

ALS2 and *ALS4* during the yeast form growth in both the simple and double mutants. The down-regulation of this group of genes is in agreement with the significant increase in the binding of the positively charged dye Alcian blue, suggesting an increase in cell wall phosphate contents in the double mutant cells. The down-regulation of *ALS2* and *ALS4* during the yeast form of growth and the increase in the binding of the positively charged dye Alcian blue has been reported in both simple mutants. We also observed the down-regulation of *PGA4, PGA6, PGA58* and the up-regulation of *PGA10, PGA13, PGA20, PGA22, PGA25, PGA31, PGA59,* and *PGA62* at one and two times of incubation, suggesting a cell wall remodelling during the hypha transition. We detected the down-regulation of *SUN41* (0.60, 0.49, 0.78) during the hypha transition. The inactivation of *SUN41* affords defects in cell separation and hyphal elongation [130] and elicits a higher sensitivity towards the cell wall-disturbing agent Congo red [191]. In agreement with the down-regulation of *SUN41*, our mutant also showed higher sensitivity towards the cell wall-disturbing agent Congo red. Another group of down-regulated genes included *ECM21, ECM25* and *ECM33* and in *C. albicans,* these are involved in cell wall biogenesis and architecture [281]. *ECM33* is a GPI-anchored protein and is important for proper cell wall ultrastructural organization and also for the correct assembly of the mannoprotein outer layer of the cell wall. We found that the double mutant was sensitive to CWF, NaCl, Caffeine and SDS. This result confirms that the double mutant cells undergo a change in cell wall composition. We observed the down-regulation a number of genes involved in lipid, fatty acid, and sterol metabolism, such as *CYB5, ERG1, ERG3, FAS1* and *FAS2* [281]. The decrease in the expression of a number of genes involved in lipid, fatty acid, and sterol metabolism is in agreement with the sensitive phenotype of the double mutant to Fluconazole.

The expression of genes involved in glycolytic pathway and carbohydrate metabolism was decreased in the double mutant in both the yeast and hyphal forms. The same genes were not always identified during yeast mode of growth and during the yeast-hypha transition (Table 3.3 and Supplementary Table 3.4), suggesting a specific regulation that deserves further investigation. It has been demonstrated that the addition of glycolytic inhibitors blocks filamentation in *C. albicans* in germ-tube-inducing medium [260], indicating that the glycolytic pathway is required for filamentous growth. The genes involved in glycolysis and in reserve

carbohydrates were down-regulated in the yeast mode of growth and during the yeast hypha transition, as in the APSES mutants [108]. A large number of genes involved in glycolysis and gluconeogenesis has been reported to be down-regulated in the *gcn5* deletion strain, especially at one and two times of incubation, and to be up-regulated in the *hda1* simple mutant, suggesting that a common pathway may be regulated differently in both single mutants. Our results demonstrated that the double mutant strain underwent a metabolic change, including genes involved in carbohydrate metabolism during the yeast mode of growth and the yeast-hypha transition. It has been reported that genes involved in carbohydrate metabolisms do not appear as an important group in response to the treatment of *C. albicans* with antifungal agents [281]. Barrelle et al [23] have shown that the progression of systemic disease caused by *C. albicans* is dependent on glycolysis. Strains that are deleted for some glycolysis specific genes have a dramatically reduced virulence in animal models [23]. In addition, it has been reported that *PFK2* (also specific for glycolysis) is highly expressed during systemic infection. It is therefore possible that the dramatic reduction in virulence seen in the *gcn5* deletion mutant could be caused, at least partially, by a down-regulation of glycolysis.

We observed the down-regulation of components of the translation apparatus: 19 genes during one, two and three times of incubation. The down-regulation of this group of genes has been reported in previous work describing the yeast-hypha transition of both the *gcn5* and *hda1* simple mutant strains. We described an up-regulation of components of the translation apparatus in yeast-hypha transition of the CAI4 strain due to a change of temperature in chapter II. We also detected the down-regulation of most of these genes during the yeast mode of growth. In another work carried out at our laboratory, it has been reported that in the *gcn5* simple mutant 63 out of 117 down-regulated genes during the yeast mode of growth coded for components of the translation apparatus. These results suggested that Gcn5p would mainly be involved in the regulation of the components of the translation apparatus.

Finally, many genes related to transport (Table 3.5), methionine synthesis and vitamin synthesis were regulated in the double mutant strain. Several of them are related to iron transport or encode general amino acid permeases. No previous data have been reported for the regulation of these groups of genes during the

hypha transition.

Overall, the transcript levels of a significant number of genes were differentially expressed in the *gcn5* or the *hda1* strains, suggesting that there are several pathways that may be regulated in a different way by both proteins.

Global transcriptome of *Candida albicans odc* null mutant during the yeast mode of growth and during the yeast-hypha transition

Abstract

By comparison of the transcriptional profiles of a *C. albicans odc/odc* mutant during the yeast mode of growth in the exponential phase and during the yeast-hypha transition at low (0.01 mM) and high (10 mM) polyamine levels, we found that about one-third (31.14%) of the genes were differentially regulated. A total of 137 and 1981 genes changed during the yeast mode of growth and during the yeast-hypha transition, respectively. 502 (25.39%) genes commonly change at the three times of the yeast-hypha transition.

The main categories of up-regulated genes during the yeast mode of growth were cell wall, histone assembly and transport. The main down-regulated genes corresponded to those coding for cell wall proteins, drug resistance, glycolytic pathway, glucose transport and transcription factors.

The genes differentially expressed at the three times of yeast hypha transition are (i) genes coding for amino acid permeases (*AGP1, CAN2, CAN5, HNM3,* IPF4580 and *MAL31*), (ii) genes coding for (a) cell-wall formation (down-regulated: *AAF1, ALS3, BGL22, CHS5, DPM1, ECE1, ECM17, ECM33, FGR6, GLC3, HYR1, HWP1, IPP1, PGA26, PGA36, PGA48, PGA53, PGA54, PGA59, RBT1, SUN41;* and up-regulated: *BMS1, ECM1, MNN11, MNN4, PGA31, PHR2*, *PIR1*), cell polarity and filament formation (*ACT1, ARF3, ARP3, BET3, CDC12, CDC23, CHC1, COF1, GDI1, IPF1022, MHP1, RDI1, SEC24, TEM1* and *TPM2);* (b) ergosterol biosynthesis (*DAP1, ERG1, ERG24 and ERG251*); (c) glycolysis *(CDC19, ENO1, FAB1, FBA1, GPM1, HXK2, PDC11, PDI1, PFK1, PFK2* and *PGK1);* (d) lysine biosynthesis *(LYS1, LYS12, LYS7* and *LYS9*); (e) respiration and electron transport *(ATP1, COX13, COX3A, COX3B, COX4, CUP5,* IPF6566*, NAD6, STF2, VMA1* and *VAM6),* (iii) transcription factors (down-regulated: *GCF1,* IPF29*,* IPF9826*, TIP120, TYE7 and ZCF4;* and up-regulated: *ACE2, BCR1, BRE1, CAP1, CTA8, DAL81, GCN4, INO2,* IPF6857*, NRG1, RMS1, SFU1, TAF19, TBP1, WOR2, ZCF3* and *ZPR1*), (iv) DNA-directed RNA polymerases (*RPB5, RPB8, RPC25, RPC40, RPC53, RPC82* and *PAF1*), (v) mitochondrial ribosomal proteins (*IPF3361, IPF16564, MRP1, MRP17, MRP20, MRP7, MRPL17, MRPL40, MRPS28* and *YML6*), (vi) nucleolar proteins (*ENP1, ENP2, NOP1, NOP14, NOP15, NOP2, NUP133* and *NUP84*), (vii) cytoplasmic ribosomal proteins (*RPF1, RPL23, RPN4,*

RPS27A, RRP42, RRP6, RRP8, RRP9, RRS1 and *IMP3*), (viii) RNA helicases (*DBP2, DBP3, DBP8, DBP9, CHR1, DRS1, HAS1, PF15646, MTR4, RRP3* and *SUV3*) and (ix) translation initiation factors (*CDC95, GCD11, GCD7, NIP1, SUA5, SUI1, SUI3* and *TIF1*). About 32% of the differentially regulated genes included transcripts encoding products not yet classified for their functional attributes. The results suggest that polyamine levels exert a pleiotrophic effect on transcriptional activity.

Introduction

Candida albicans is an opportunistic fungus that generally exists in the oral cavity, skin, vagina and intestinal organs [340]. *C. albicans* is dimorphic and undergoes transition from the yeast form to the hyphal form depending on the growth conditions [155,451]. It grows in yeast form on membranes and skin surfaces, but in the hyphal form, it occurs at deep-seated mycoses [151]. The hyphal transition is induced by activation of the MAP kinase and cAMP pathways, which are controlled by the RAS protein [16,17,218,403]. Polyamines are synthesised from ornithine by ornithine decarboxylase (ODC) and are inducers of the enzyme adenylate cyclase [285,476]. Polyamines are essential for cell growth, and their metabolism is extensively regulated [507]. The *C. albicans* ODC gene (*ODC1*), showing high homology with other eukaryotic ODCs at both the amino acid and nucleic acid levels, is required for the synthesis of polyamines [188,316,350]. *ODC1* catalyses the conversion of ornithine into putrescine, which is the first step in the universal mechanism of polyamine biosynthesis, and it is one of the most highly regulated enzymes of eukaryotic cells [69,101,206,219]. This control is essential, because excess polyamine is toxic and induces apoptosis [361,371]. The most important control mechanism of *ODC1* in eukaryotic organisms operates at the post-translational level, regulating the amounts of the enzyme by a unique mechanism of proteasome-dependent degradation [180,186]. Because of the narrow concentration range of polyamines needed for execution of their multiple roles, *ODC1* expression is tightly controlled. Even modest reductions in mammalian *ODC1* activity can lead to marked resistance to tumour development [348,365]. Intracellular levels of polyamines are regulated at various steps, including synthesis, degradation, uptake and excretion, and cells have developed intricate mechanisms to ensure a tight regulation of intracellular polyamine pools [68,492].

In fungi, polyamines are essential for distinct differentiation processes, such as spore germination, sporulation and dimorphism [395]. The possible significance of the transcriptional regulation of the gene encoding *ODC1* by polyamines in fungi may depend on its interrelation with the post-translational regulation of the enzyme. The simultaneous operation of transcriptional and post-translational regulatory mechanisms has been reported to endow the cell with a more efficient mechanism to control the physiological processes that depend on polyamines [220]. To study polyamine regulation in *C. albicans*, a *odc/odc* mutant, which behaved as a polyamine auxotroph and grew exclusively in the yeast form at low polyamine levels (0.01 mM putrescine) under all conditions tested, has been generated [188]. An increase in the polyamine concentration (to 10 mM putrescine) restored the capacity to switch from the yeast form to the filamentous form, proving that polyamine concentrations are essential for the switchover [188]. Deletion of *C. albicans* ODC gene provide an excellent genetic system to study the effects of polyamines on gene expression during the yeast mode of growth and during the yeast-hypha transition, thereby facilitating the exploration of the possible mode of their regulation.

In this work, we report a study of the transcriptional profile of a *C. albicans odc/odc* mutant during the exponential phase and during the yeast-hypha transition at low (0.01 mM) and high (10 mM) levels of putrescine.

The information thus generated has enabled us to develop our knowledge about polyamine-mediated coordination between different levels of regulation, which may eventually explain the role of polyamines in yeast-form growth and hyphal transition in *C. albicans*.

Materials and methods

Yeast strain, media and growth conditions

The strain of *C. albicans* used was ABH4 (*ura3/ura3 odc1*::*hisG/odc1*::*hisG*) [188]. The strain was grown in a medium containing 1.25% glucose as a carbon source and supplemented with 0.2 mM uridine. Solid medium was obtained by adding agar (2%). The dimorphic transition was induced by changing the temperature to 37ºC. Putrescine was added at low and high concentrations (0.01 mM and 10 mM, respectively).

RNA isolation, microarray design and preparation

For yeast-form growth of the *C. albicans odc* mutant strain, ABH4 was grown in Lee medium cultures supplemented with 0.01 mM and 10 mM of putrescine till the mid-log phase (OD_{600nm}, 1.5). The dimorphic transition was induced by changing the temperature to 37ºC. Cell were pre-grown at 28ºC in Lee medium without polyamine until the exponential phase, washed, starved in water for 24 h at 4ºC and incubated in Lee medium (pH 6.8, 37ºC) supplemented with either 0.01 mM or 10 mM of putrescine. Cells were harvested at room temperature, resuspended in a very small volume of the supernatant and immediately frozen by releasing small drops of cell samples into liquid nitrogen. Cells were then stored at −80ºC until RNA extraction was carried out. Total RNA isolation, microarray design and cDNA microarray data analysis were carried out as described in Chapter II.

Results

Global transcriptomal response of the *odc* mutant

The *C. albicans odc/odc* mutant grown at low polyamine levels (0.01 mM of putrescine) failed to form filaments in response to media with serum or any other known inducers of filamentous growth. In addition, null strain was hypersensitive to Calcofluor and salts, indicating that polyamine levels control a broad range of gene functions [188]. A comparison of the transcript profiles between the low (0.01 mM putrescine) and high (10 mM putrescine) polyamine levels was first ascertained using cDNA microarrays. The data analysis was based on statistical confidence ($p < 0.02$) and at least 1.5-fold expression change. Initially, a comparison of *odc* mutants growing in Lee medium at 28ºC with low (0.01 mM) and high (10 mM) levels of putrescine in the exponential growth phase (OD_{600nm}, 1.5) was carried out. The deletion of *ODC* produced ≥1.5-fold changes in the expression of 137 genes, with 52 up-regulated and 85 down-regulated genes during the yeast mode of growth. When transcriptomes of cells growing in Lee medium at 37ºC during yeast-hyphal transition (15, 60 and 180 min) were compared, 1981 genes had modified expression levels: 1056 genes were up-regulated and 925 were down-regulated. As we have found in the other *C. albicans* mutants the number of misregulated genes in the mutant was greater during the yeast hypha transition than during the yeast-mode of growth (Figure 4.1).

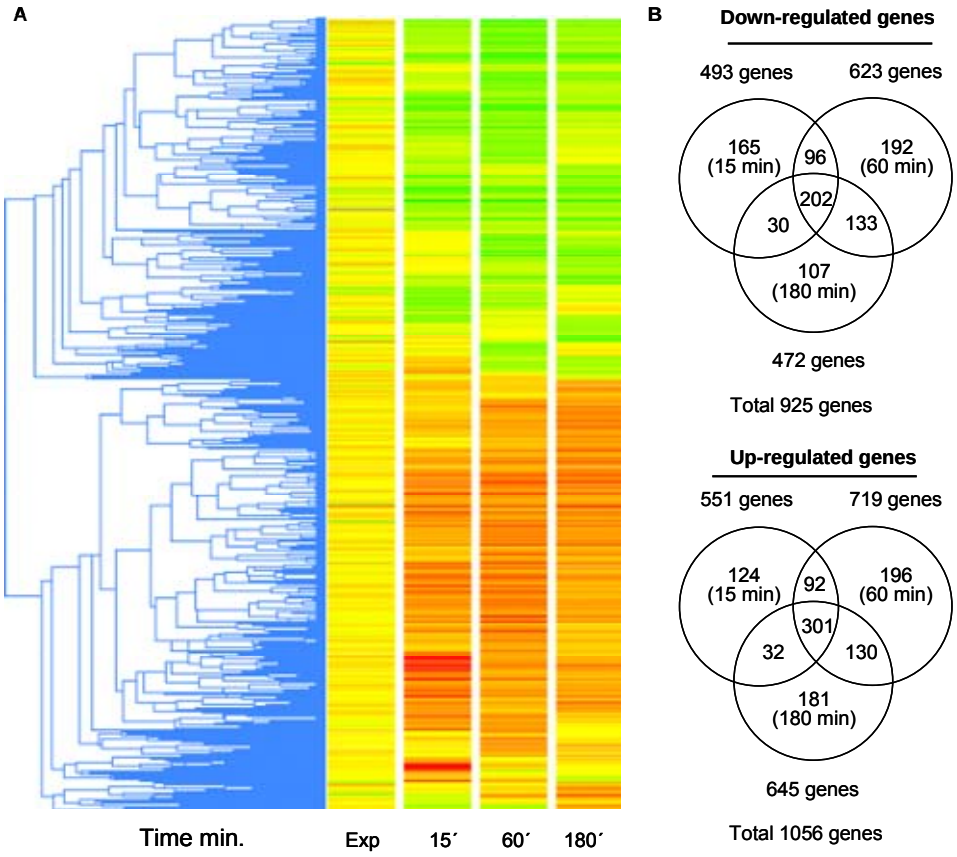

Figure 4.1 (**A**) **Transcriptional Genes Tree and statistical comparisons of polyamines-responsive genes in a *C. albicans odc/odc* mutant strain grown in Lee medium supplemented with 0.01 mM of putrescine *vs* 10 mM of putrescine**. The transcriptional profiles of genes whose transcript levels responded significantly ($\rho < 0.02$) shift to the 0.01 mM of putrescine (*odc* mutant strain) were clustered using a gene tree program of Genespring Gx 7.1.1). Panel Exp is a Gene Tree map of the transcriptional changes in gene expression in the yeast mode of growth shift to 0.01 mM of putrescine relative to the 10 mM of putrescine (control cell). Green indicates down-regulated expression and red indicates up-regulated expression. Panels 15, 60 and 180 min show the response of the same genes shown in panel EXP but during the yeast-hypha transition (15, 60 and 180 min). *C. albicans* Gene Tree transcriptional pattern indicate genes that showed a differential response ($\rho < 0.02$) during the yeast-hypha transition in the Lee medium (0.01 mM of putrescine *versus* 10 mM of putrescine) (**B**) Venn diagram for genes whose expression was significantly altered during the yeast-hypha transition.

Transcriptome of *odc* mutant strain in exponentially growing cells

The analysis of the microarray data of cells in the exponential growth phase showed 137 affected genes (Supplementary Tables 4.1 and 4.2). Of the total number of altered genes, 38 (27.73%) were genes with unknown functions. Among the rest of the genes, *RBT5*, encoding a glycosylphosphatidylinositol (GPI) cell-wall protein that is regulated by Rfg1p, Rim101p, Tbf1p, Sfu1p, Hog1p and Tup1p [29,37,196,227,259,441], showed the highest change (3.51 fold), whereas the lowest expression level in this experiment was observed for *HGT7*, which encodes a putative protein involved in glucose transport, (−6.17 fold) [124]. Genes expressed differentially between the low (0.01 mM) and the high (10 mM) levels of putrescine are listed in Supplementary Tables 4.1 and 4.2. Endogenous increase in polyamines has been reported to result in significant changes in gene-expression

patterns [442]. Due to the large number of genes found to be up- or down-regulated, this analysis was focussed on main the functional categories detected (e.g cell wall formation, drug resistance, hexose transporters and histone assembly) (Table 4.1).

Table 4.1 Distribution of differentially expressed genes during the yeast mode of growth into functional categories genes that are at least 1.5-fold up- or down-regulated in the *C. albicans odc/odc*. Growth was carried out in Lee medium supplemented with 0.01 mM and 10mM of puterescine.

Gene name	Accession no	Description (CandidaDB)	Fold regulation
UP-regulated genes			
Cell wall			
PGA10	orf19.5674	Plasma membrane protein involved in heme-iron utilization	1.75
PGA45	orf19.2451	Cell wall protein; putative GPI-anchor	1.56
PGA7	orf19.5635	Mycelial surface antigen precursor	2.19
PRA1	orf19.3111	PH-regulated antigen	1.58
RBT5	orf19.5636	Repressed by *TUP1* protein 5	3.51
Histone assembly			
HHF21	orf19.1059	Histone H_4	1.98
HHF22	orf19.1854	Histone H_4 (by homology)	1.92
HTA1	orf19.6924	Histone H_2A	1.74
HTB1	orf19.6925	Histone H_2B	1.86
Transcription factor			
GCN4	orf19.1358	Transcriptional activator	1.54
Transporter			
FRE5	orf19.5634	Ferric reductase transmembrane component	1.55
HGT14	orf19.2633	Putative glucose transporter	1.85
IPF1992	orf19.7336	Putative MFS transporter	1.56
JEN1	orf19.7447	Carboxylic acid transporter protein	1.90
Other Function			
ACH1	orf19.10681	Acetyl-coenzyme-A hydrolase	1.54
CDC54	orf19.11245	Transcription is regulated by Nrg1p and Mig1p	1.75
CSA1	orf19.7114	Mycelial surface antigen	1.87
CSA2	orf19.3117	Down-regulated by Rim101p at ph 8	2.65
FEN11	orf19.6343	Fatty acid elongase required for sphingolipid formation	1.63
HMO1	orf19.6645	High-mobility protein 1	2.34
IFQ4	orf19.1464	Putative beta-mannosyltransferase	1.66
INO1	orf19.7585	Myo-inositol-1-phosphate synthase	1.66
IPF4164	orf19.5455	Similar to *Saccharomyces cerevisiae* Vtc1p	1.61
LSP1	orf19.3149	Caspofungin repressed; biofilm induced	1.78
MRS4	orf19.2178	RNA splicing protein	1.75
NHP6A	orf19.4623.3	Nonhistone chromosomal protein related to *HMG1*	2.07
OPS4	orf19.4934	Opaque-phase specific protein OP4, precursor	1.67
SPB8	orf19.7290	Suppressor of *PAB1* (by homology)	1.65
UBI4	orf19.6771	Polyubiquitin	1.54
Unknown function			
23 genes			
Down-regulated genes			
Amino acid permease			
AGP1	orf19.8784	Asparagine and glutamine permease	0.52
GAP5	orf19.4456	General amino acid permease (by homology)	0.61
Cell wall			
ALS1	orf19.5741	Agglutinin-like protein	0.54
BMT4	orf19.13055	Putative beta-mannosyltransferase	0.62
HYR1	orf19.12440	Hyphally regulated protein	0.62
IPF3964	orf19.675	Similar to cell wall proteins; induced in core stress response	0.58
PGA26	orf19.2475	Putative GPI-anchored protein of unknown function	0.64
PGA31	orf19.5302	Cell wall protein; putative GPI anchor	0.31

PIR1	orf19.220	Putative cell wall protein of the PIR family	0.19
Dehydrogenase			
ADH1	orf19.3997	Alcohol dehydrogenase	0.47
ADH2	orf19.5113	Alcohol dehydrogenase I	0.53
ADH5	orf19.2608	Probable alcohol dehydrogenase	0.48
FDH11	orf19.7600	Glutathione-dependent formaldehyde dehydrogenase	0.63
GAP1	orf19.6814	Glyceraldehyde-3-phosphate dehydrogenase	0.47
IFE2	orf19.5288	Protein described as an alcohol dehydrogenase	0.47
LYS12	orf19.2525	Homo-isocitrate dehydrogenase	0.66
Drug resistance			
CRD1	orf19.4784	Cu-transporting P1-type ATPase	0.56
CDR4	orf19.5079	Multidrug resistance protein	0.38
QDR1	orf19.8138	Putative antibiotic resistance proteins	0.41
Glycolisis			
CDC19	orf19.3575	Pyruvate kinase	0.56
ENO1	orf19.395	Enolase I	0.54
FBA1	orf19.4618	Fructose-bisphosphate aldolase	0.45
PDC11	orf19.2877	Pyruvate decarboxylase	0.39
PDC12	orf19.4608	Pyruvate decarboxylase I	0.56
PFK1	orf19.3967	6-phosphofructokinase Alpha subunit	0.59
PFK2	orf19.6540	6-phosphofructokinase Beta subunit	0.43
TPI1	orf19.6745	Triose phosphate isomerase	0.58
TPS2	orf19.3038	Threalose-6-phosphate phosphatase	0.47
Hexose transport			
HGT8	orf19.2021	Sugar transporter	0.30
HGT6	orf19.2020	Sugar transporter	0.35
HGT7	orf19.2023	Sugar transporter	0.16
Oxidase/peroxidase			
GPX2	orf19.85	Glutathione peroxidase	0.47
SOD22	orf19.7111.1	Superoxide dismutase	0.61
Transcription factor			
CPH2	orf19.1187	Transcriptional activator of hyphal growth	0.53
CTA24	orf19.4054	Transcriptional activator	0.64
FCR1	orf19.6817	Zinc cluster transcription factor	0.59
HAC1	orf19.2432	Putative transcription factor	0.65
TYE7	orf19.4941	Basic helix-loop-helix transcription factor	0.28
ZCF20	orf19.11621	Predicted zinc-finger protein of unknown function	0.64
Transporter			
ATP1	orf19.6854	F1F0-atpase complex F1 alpha subunit	0.47
ENA22	orf19.6070	P-type ATPase involved in Na^+ efflux	0.54
IFC1	orf19.3746	Oligo-peptide transporter	0.56
PHO84	orf19.655	High-affinity inorganic phosphate/H^+ symporter	0.49
PMA1	orf19.5383	Plasma membrane H^+-transporting atpase 1	0.58
Other function			
BEL1	orf19.6906	Protein of the 40S ribosomal subunit	0.58
CMK1	orf19.5911	Ca^{2+}/calmodulin-dependent ser/thr protein kinase	0.56
COX1	CaalfMp08	Subunit I of cytochrome c oxidase	0.58
CRH11	orf19.2706	Probable membrane protein	0.58
DAK2	orf6.1104	Dihydroxyacetone kinase	0.35
DDI1	orf19.7258	Response to DNA alkylation by homology	0.65
DED1	orf19.7392	RNA helicase	0.64
DEF1	orf19.7561	Protein required for filamentous growth	0.40
ERG251	orf19.12101	C-4 sterol methyl oxidase	0.46
ERO1	orf19.4871	Required for protein disulfide bond formation in the ER	0.59
HSP30	orf19.4526	Heat shock protein	0.49
IPF18732	orf19.8330	Histidine-rich glycoprotein precursor	0.43
IPF2400	orf19.6816	Putative aldehyde reductase	0.58
IPF85	orf19.5334	Similar to *Saccharomyces cerevisiae* Tis11p	0.48
IPF8806	orf19.9844	6-phosphofructose-2-kinase	0.55
IPF9955	orf19.10993	Regulated by Tsa1p	0.21
OSM1	orf19.6882	Similar to *Saccharomyces cerevisiae* Osm1p	0.40
RHR2	orf19.5437	DL-glycerol phosphatase	0.47
RIB3	orf19.12693	3,4-dihydroxy-2-butanone 4-phosphate synthase	0.56
SAP9	orf19.6928	Aspartyl proteinase 9	0.63
SKS1	orf19.3669	Serine/threonine kinase	0.27
THI4	orf19.5986	Thiazole biosynthetic enzyme precursor	0.52

UCF1	orf19.1354	Transcriptionally regulated by iron or by yeast-hyphal switch	0.60
URA2	orf19.9896	Multifunctional pyrimidine biosynthesis protein	0.61
XYL2	orf19.7676	Protein described as similar to D-xylulose reductase	0.59
YHB1	orf19.3707	Flavohemoglobin	0.34
Unknown finction			
14 genes			

Genes involved in carbohydrate metabolism and energy generation

Because polyamine catabolism has been reported to be involved in the regulation of energy and glucose metabolism in a mouse model [368], the analysis of the microarray data was directed towards discovering whether polyamine is involved in the energy and glucose metabolism of a *C. albicans odc* mutant. During the yeast-mode of growth, numerous genes involved in glycolysis, gluconeogenesis and carbohydrate transport (*HGT8, HGT6* and *HGT7*) were down-regulated. Oral administration of spermidine has been reported to result in the precocious expression of a carbohydrate-transporter mRNA and protein in the proximal and distal regions of the small intestine [505]. The findings reported here are the first results reported regarding the effect of polyamines on the expression of glucose transporter–related genes under low putrescine levels in *C. albicans*. However, a highers percentage of genes from the glycolytic pathway, encoding transcription factors and transporters showed a greater than 1.5-fold decrease during the yeast mode of growth including down-regulation of the hexose transporters (as mentioned above), small-molecule transporters (*ATP1, PHO84* and *PMA1*).

FRE5, a predicted ferric reductase that is induced by the CCAAT-binding factor, along with the induction of Hap43p [15,29,259], of carbohydrate transporters (*JEN1* and *HGT14*) and of a membrane transporter (*orf19.7336*) homologue of *S. cerevisiae, AZR1* were the main up-regulated genes. *ENA22*, a homologue of *ScENA5,* which appears to encode a transporter involved in the uptake of sodium [504], was down-regulated. Our results suggest that lower levels of polyamines caused a significant and marked shift in the down-regulation of genes involved in metabolism and energy generation during the yeast-form growth.

Regulation of other genes categories

As shown in Table 4.1, the gene expression level was increased in four genes encoding components of histones, *HTB1, HTA1, HHF21* and *HHF2.* The expression of genes involved in RNA processing including *SBP8* homologue of *S.*

cerevisiae LSM1 [314], IPF12381 homologue of *S. cerevisiae SGN1* [509] and *MRS4*, an RNA-splicing protein that plays a role in chromosome rearrangements [275,327], was also up-regulated during the yeast mode of growth. Only one transcription factor was up-regulated during the yeast-form growth -*GCN4*, described as the primary regulator of the transcriptional response to amino acid starvation [192]. However six transcription factors -*CPH2*, *CTA24*, *FCR1*, *HAC1*, *TYE7* and *ZCF20*- were down-regulated. Cph2p regulates hyphal development in *C. albicans*, because the *cph2/cph2* mutant strain showed medium-specific impairment of both hyphal development and induction of hypha-specific genes [262]. The other differentially expressed genes encoding for cell wall proteins (e.g *ALS1, BMT4, HYR1*, *PGA26*, *PGA31, PIR1*) (among them *PGA10, PGA45, PGA7*, *PRA1* and *RBT5* were up-regulation), dehydrogenases (*ADH1, FDH11, IFE2* and *LYS12*), drug resistance controlling proteins (*CDR1, CDR4* and *QDR1)* and oxidase/peroxidises (*GPX2* and *SOD3)*. These results suggest that polyamine level controls a broad range of gene functions.

Transcriptome of *odc* mutant during the yeast-hypha transition

The effect of low and high levels of polyamines was analized during the yeast-hypha transition. Our analysis showed 1981 genes to be altered by comparison of both levels of putrecine, with 653 genes (32.96%) having unknown functions. Genes expressed differentially are listed in Supplementary Tables 4.3 and 4.4. The genes with the highest- and lowest-fold expression during the three time periods of hyphal transition (15, 60 and 180 min) were *SPE2* and *ECE1*, encoding for an adenosylmethionine decarboxylase precursor and cell-elongation protein, respectively. Approximately 21 genes involved in the production of cell wall proteins and 15 genes controlling cell polarity and filament formation showed lower accumulation of their transcripts during the above-mentioned three times of yeast-hypha transition, whereas 7 genes encoding the cell wall proteins and none related to cell polarity and filament formation showed an increase in their transcript levels during the same period (Table 4.2).

Table 4.2 Distribution of differentially expressed genes during the three times (15, 60 and 180 min) of yeast-hypha transition into functional categories in the *C. albicans odc/odc.* Growth was carried out in Lee medium supplemented with 0.01 mM and 10mM of puterescine.

Gene Name	Accession no	Description (CandidaDB)	Fold regulation		
Down-regulated genes			**15 min**	**60 min**	**180 min**
Acetyl-coenzyme					
ACC1					
orf19.7466	Acetyl-coenzyme-A	carboxylase	0.41	0.25	0.59
ACS1	orf19.1743	Acetyl-coenzyme-A synthetase	0.47	0.36	0.37
ACS2	orf19.1064	Acetyl-coenzyme-A synthetase	0.56	0.28	0.56
POT13	orf19.2046	Acetyl-CoA C-acyltransferase	0.43	0.58	0.56
Adenine biosynthesis					
ADE13	orf19.3870	Enzyme of adenine biosynthesis	0.48	0.21	0.44
ADE17	orf19.492	Enzyme of adenine biosynthesis	0.26	0.21	0.36
Amino acid permease					
AGP1	orf19.8784	Asparagine and glutamine permease	0.35	0.36	0.63
CAN2	orf19.111	Amino acid permease	0.56	0.42	0.62
CAN5	orf19.3641	Amino acid permease	0.61	0.60	0.55
HNM3	orf19.2587	Choline permease	0.47	0.51	0.33
IPF4580	orf19.6522	Putative allantoate permease	0.52	0.58	0.50
MAL31	orf19.3981	Maltose permease	0.63	0.44	0.50
Cell Wall					
AAF1	orf19.7436	Adhesion mediating surface antigen	0.36	0.37	0.41
ALS3	orf19.2355	Agglutinin like protein	0.04	0.02	0.0
BGL22	orf19.7339	Putative glucanase	0.64	0.42	0.33
CHS5	orf19.807	Putative chitin biosynthesis protein	0.55	0.65	0.63
DPM1	orf19.5073	Protein with similarity to *S. cerevisiae* Dpm1p	0.37	0.28	0.46
ECE1	orf19.3374	Cell elongation protein	0.02	0.01	0.13
ECM17	orf19.4099	Putative sulfite reductase	0.63	0.57	0.62
ECM33	orf19.3010.1	Cell wall biogenesis	0.40	0.39	0.54
FGR6	orf19.5315	Protein lacking an ortholog in *S. cerevisiae*	0.51	0.38	0.55
GLC3	orf19.13067	1.4-glucan branching enzyme	0.60	0.36	0.44
HYR1	orf19.12440	Hyphally regulated protein	0.35	0.07	0.18
HWP1	orf19.1321	Hyphal wall protein	0.03	0.01	0.05
IPP1	orf19.3590	Inorganic pyrophosphatase (hypha cell wall)	0.59	0.20	0.36
PGA26	orf19.2475	Putative GPI-anchored protein	0.49	0.29	0.29
PGA36	orf19.5760	Putative GPI-anchored protein	0.31	0.27	0.26
PGA48	orf19.6321	Putative GPI-anchored protein	0.53	0.58	0.49
PGA53	orf19.12120	Putative GPI-anchored protein	0.49	0.45	0.59
PGA54	orf19.2685	Putative GPI-anchored protein	0.20	0.08	0.33
PGA59	orf19.2767	Putative GPI-anchored protein	0.43	0.24	0.44
RBT1	orf19.1327	Repressed by *TUP1* protein 1	0.35	0.22	0.34
SUN41	orf19.3642	Putative cell wall beta-glucosidase	0.24	0.18	0.25
Cell polarity and filament formation					
ACT1	orf19.5007	Actin (by homology)	0.44	0.24	0.53
ARF3	orf19.1702	GTP-binding protein of the ARF family	0.47	0.35	0.50
ARP3	orf19.2289	Actin related protein	0.35	0.35	0.58
BET3	orf19.5817	Fusion of ER to Golgi transport vesicles	0.45	0.39	0.46
CDC12	orf19.3013	Septin	0.62	0.39	0.57
CDC23	orf19.6437	A anaphase-promoting complex component	0.37	0.52	0.49
CHC1	orf19.3496	Clathrin heavy chain	0.54	0.54	0.54
COF1	orf19.953.1	Protein described as cofilin	0.50	0.40	0.48
GDI1	orf19.7261	GDP dissociation inhibitor	0.62	0.34	0.46
IPF1022	orf19.4579	Similar to Saccharomyces cerevisiae Erv29p	0.20	0.29	0.60
MHP1	orf19.461	Protein similar to *S. cerevisiae* Mhp1p	0.25	0.51	0.56
RDI1	orf19.5968	Rho GDP dissociation inhibitor	0.40	0.34	0.52
SEC24	orf19.12194	Component of COPII coat of ER-Golgi vesicles	0.34	0.33	0.52
TEM1	orf19.10519	GTP-binding protein of the RAS superfamily	0.56	0.52	0.36
TPM2	orf19.6414.3	Tropomyosin	0.43	0.24	0.40
Dehydrogenase					
ADH1	orf19.3997	Alcohol dehydrogenase	0.08	0.06	0.13
ADH2	orf19.5113	Alcohol dehydrogenase I	0.15	0.18	0.27
ADH5	orf19.2608	Probable alcohol dehydrogenase	0.42	0.17	0.14
GAP1	orf19.6814	Glyceraldehyde-3-phosphate dehydrogenase	0.19	0.04	0.06

HOM6	orf19.2951	Homoserine dehydrogenase	0.58	0.36	0.50
IDH1	orf19.4826	Isocitrate dehydrogenase	0.61	0.39	0.45
IDP1	orf19.5211	Isocitrate dehydrogenase	0.49	0.32	0.57
IDP2	orf19.3733	Isocitrate dehydrogenase	0.30	0.22	0.64
URA1	orf19.4836	Dihydroorotate dehydrogenase	0.38	0.29	0.39
Ergoestrol biogenesis					
DAP1	orf19.489	Regulation of ergostreol biosynthesis	0.44	0.28	0.41
ERG1	orf19.406	Squalene epoxidase	0.42	0.44	0.51
ERG24	orf19.1598	C-14 sterol reductase	0.31	0.27	0.42
ERG251	orf19.12101	C-4 sterol methyl oxidase	0.34	0.16	0.29
Glycolysis					
CDC19	orf19.3575	Pyruvate kinase	0.27	0.12	0.28
ENO1	orf19.395	Enolase I	0.16	0.06	0.19
FAB1	orf19.9088	Phosphatidylinositol 3-phosphate 5-kinase	0.57	0.45	0.56
FBA1	orf19.4618	Fructose-bisphosphate aldolase	0.10	0.06	0.16
GPM1	orf19.903	Phosphoglycerate mutase	0.37	0.16	0.30
HXK2	orf19.8176	Hexokinase II	0.44	0.38	0.36
PDC11	orf19.2877	Pyruvate decarboxylase	0.20	0.11	0.14
PDI1	orf19.5130	Protein disulfide-isomerase precursor	0.31	0.34	0.53
PFK1	orf19.3967	6-phosphofructokinase Alpha subunit	0.23	0.26	0.33
PFK2	orf19.6540	6-phosphofructokinase beta subunit	0.20	0.16	0.19
PGK1	orf19.3651	Phosphoglycerate kinase	0.11	0.06	0.15
PGM2	orf19.10359	Phosphoglucomutase	0.49	0.23	0.16
TPI1	orf19.6745	Triose phosphate isomerase	0.30	0.16	0.27
Hexos transport					
HGT6	orf19.2020	Sugar transporter	0.57	0.30	0.39
HGT7	orf19.2023	Sugar transporter	0.45	0.46	0.57
HGT12	orf19.3668	Hexose transporter	0.57	0.44	0.46
Lysine biosynthesis					
LYS1	orf6.1158	Saccharopine dehydrogenase	0.49	0.38	0.42
LYS12	orf19.2525	Homo-isocitrate dehydrogenase	0.47	0.23	0.35
LYS7	orf19.4449	Chaperone of superoxide dismutase Sod1p	0.60	0.38	0.57
LYS9	orf19.7448	Lysine biosynthesis	0.66	0.30	0.41
Mitochondria					
CPR3	orf19.1552	Peptidylprolyl isomerase Mitochondrial	0.66	0.32	0.41
GCV3	orf19.5006	Glycine decarboxylase Subunit H	0.47	0.18	0.43
HRD3	orf19.1191	Involved in HMG-COA reductase degradation	0.54	0.61	0.53
MAE1	orf19.3419	Mitochondrial malic enzyme	0.23	0.40	0.56
MDM12	orf19.6900	Involved in mitochondrial inheritance	0.39	0.47	0.39
MIS11	orf19.2364	Mitochondrial C1-tetrahydrofolate synthase	0.56	0.30	0.51
POR1	orf19.1042	Mitochondrial outer membrane porin	0.58	0.13	0.22
YHB1	orf19.3707	Flavohemoglobin (by homology)	0.19	0.18	0.22
Respiration and electron transport					
ATP1	orf19.6854	Protein similar to alpha subunit of ATP synthase	0.39	0.16	0.29
COX13	orf19.1467	Cytochrome-c oxidase chain via	0.58	0.43	0.54
COX3A	CaalfMp04	Subunit III of cytochrome c oxidase	0.39	0.47	0.38
COX3B	CaalfMp15	Subunit III of cytochrome c oxidase	0.33	0.50	0.38
COX4	orf19.1471	Cytochrome-c oxidase	0.56	0.43	0.51
CUP5	orf19.5886	Vacuolar H^+-ATPase	0.57	0.60	0.60
IPF6566	orf19.6873.1	Electron carrier activiry	0.53	0.58	0.56
NAD6	CaalfMp02	Subunit 6 of NADH:ubiquinone oxidoreductase	0.47	0.57	0.65
PMA1	orf19.5383	Plasma membrane H^+-transporting ATPase 1	0.47	0.52	0.40
STF2	orf19.2107.1	ATP synthase regulatory factor	0.34	0.11	0.13
VMA1	orf19.1866	Vacuolar ATPase V1 domain subunit G	0.61	0.36	0.53
VAM6	orf19.1567	Vacuolar carboxypeptidase Y	0.26	0.36	0.38
TCA cycle					
LSC1	orf19.3358	Succinate-coa ligase / synthetase	0.63	0.28	0.46
LSC2	orf19.1860	Succinate-coa ligase beta subuni	0.61	0.30	0.49
OSM1	orf19.6882	Flavoprotein subunit of fumarate reductase	0.16	0.18	0.33
Transcription factor					
GCF1	orf19.400	Protein that binds the regulatory region *HWP1*	0.58	0.53	0.59
IPF29	orf19.5975	Putative transcription factor	0.29	0.28	0.36
IPF9826	orf19.11621	Predicted zinc-finger protein of unknown function	0.34	0.40	0.35
TIP120	orf19.6729	TBP-binding transcription regulator	0.61	0.54	0.47
TYE7	orf19.4941	Basic helix-loop-helix transcription factor	0.49	0.52	0.48
ZCF4	orf19.1227	Putative transcription factor	0.51	0.44	0.44

Transport					
CTR1	orf19.3646	Copper transport protein	0.50	0.49	0.31
FET54	orf19.4215	Protein similar to multicopper ferroxidase	0.34	0.20	0.35
HOL4	orf19.12021	Member of major facilitator superfamily	0.46	0.49	0.52
PHO84	orf19.1172	Inorganic phosphate transport protein	0.22	0.49	0.41
Other function					
55 genes					
Unknown function					
33 genes					
Up-regulated genes					
Cell wall					
BMS1	orf19.2504	Membrane protein involved in bud site selection	2.18	3.75	2.36
ECM1	orf19.5299	Involved in cell wall biosynthesis	1.79	3.32	2.00
MNN11	orf19.2927	Mannosyltransferase complex component	2.65	2.73	2.59
MNN4	orf19.10399	Protein required for mannosylphosphorylation	2.83	2.48	1.79
PGA31	orf19.5302	Cell wall protein; putative GPI anchor	2.62	1.98	1.78
PHR2	orf19.13500	Ph-regulated protein 2	2.57	2.18	2.23
PIR1	orf19.220	Putative cell wall protein of the PIR family	3.52	2.29	1.71
DNA-directed RNA polymerase					
RPB5	orf19.6340	DNA-directed RNA polymerase I, II, III	2.06	2.62	3.25
RPB8	orf19.6314	DNA-directed RNA polymerase I, II, III	1.69	1.82	1.77
RPC25	orf19.443	DNA-direcred RNA polymerase III	2.11	2.15	2.22
RPC40	orf19.3564	RNA polymerase	2.29	2.42	2.89
RPC53	orf19.2715	DNA-directed RNA polymerase III	1.75	2.43	2.00
RPC82	orf19.2847	DNA-directed RNA polymerase III	2.26	2.32	2.16
PAF1	orf19.3613	DNA-directed RNA polymerase II	1.96	1.80	2.43
Mitochondrial ribosomal protein					
RSM7	orf19.4018	Putative mitochondrial ribosomal protein S7	2.11	1.72	1.85
MRPS12	orf19.2438	Putative mitochondrial ribosomal protein S12	1.91	1.62	1.81
MRP1	orf19.1662	Mitochondrial ribosomal protein	2.20	1.77	1.96
MRP10	orf19.2650.1	Mitochondrial ribosomal protein	1.92	1.62	1.60
MRP17	orf19.947	Mitochondrial ribosomal protein	1.77	1.74	2.02
MRP20	orf19.3350	Similar to *Saccharomyces cerevisiae* Mrp20p	1.67	1.66	2.09
MRP7	orf19.7203	Mitochondrial ribosomal protein yml2 precursor	2.49	2.39	3.29
MRPL17	orf19.585	Ribosomal protein of the large subunit	1.85	1.64	2.10
MRPL40	orf19.484	Putative mitochondrial ribosomal protein	1.94	1.62	1.68
MRPS28	orf19.2520	Ribosomal protein	2.11	1.89	2.59
MRT4	orf19.12996	Required for mRNA decay	1.92	2.87	2.52
YML6	orf19.7019	Ribosomal protein, Mitochondrial	2.17	2.39	2.19
Nucleolar protein					
ENP1	orf19.5507	Essential nuclear protein	3.82	5.92	3.50
ENP2	orf19.6686	Mutation confers resistance to 5-fluorocytosine	2.68	3.21	2.96
NOP1	orf19.3138	Fibrillarin	2.03	2.63	2.65
NOP14	orf19.5959	Mutation confers resistance to 5-fluorocytosine	2.87	3.56	2.20
NOP15	orf19.7050	A nucleolar ribosome biogenesis factor	3.16	4.17	2.93
NOP2	orf19.501	Nucleolar protein	1.70	3.26	2.92
NUP133	orf19.3552	Nuclear pore protein	2.17	1.65	1.79
NUP84	orf19.1298	Nuclear pore protein	2.21	1.99	1.67
Ribosomal protein					
RPF1	orf19.10184	Maturation of 5.8S rRNAfrom tricistronic rRNA	2.35	2.22	2.15
RPL23A	orf19.3504	Ribosomal protein L23.e	1.94	1.61	2.18
RPN4	orf19.1069	26S proteasome subunit	4.19	5.03	3.50
RPS27A	orf19.413.1	Ribosomal protein S27.e	1.96	1.67	1.89
RRP42	orf19.5039	rRNA processing protein	2.27	2.62	2.69
RRP6	orf19.58	Involved in 5.8S rRNA processing	1.64	2.93	2.09
RRP8	orf19.3630	Similar to *S. cerevisiae* RRP8	2.16	2.91	2.68
RRP9	orf19.2830	U3 small nucleolar ribonucleoprotein	3.20	4.40	3.41
RRS1	orf19.6014	Regulator for ribosome synthesis	1.99	2.27	2.57
IMP3	orf19.7488	U3 small nucleolar ribonucleoprotein	1.83	2.36	2.06
RNA helicase					
DBP2	orf19.171	ATP-dependent RNA helicase of DEAD box family	1.81	3.52	2.51
DBP3	orf19.12334	ATP-dependent RNA helicase	2.26	5.22	2.55
DBP8	orf19.6652	DEAD box protein ATP-dependent RNA helicase	1.77	2.24	1.81
DBP9	orf19.3393	Dead box helicase	2.09	3.60	2.73
CHR1	orf19.3756	DEAD-box ATP-dependent RNA helicase	2.29	2.89	1.96

DRS1	orf19.7635	ATP dependent RNA helicase	2.07	2.54	1.85
HAS1	orf19.11444	ATP-dependent RNA helicase	1.79	2.67	2.15
MRH4	orf19.3481	Putative ATP-dependent RNA helicase	1.65	2.18	2.58
MTR4	orf19.8915	RNA Helicase	1.56	1.90	2.00
RRP3	orf19.7546	RNA-dependent ATPase, Helicase	1.79	2.13	1.64
SUV3	orf19.4519	ATP-dependent RNA helicase	2.26	2.41	2.10
Transcription factor					
ACE2	orf19.6124	Putative transcription factor	2.38	1.92	1.72
BCR1	orf19.723	Transcription factor	2.10	3.03	2.92
BRE1	orf19.976	Putative transcription factor	1.65	1.57	1.71
CAP1	orf19.9191	Transcriptional acivator	1.78	2.65	1.78
CTA8	orf19.4775	Activates transcription in 1-hybrid assay	1.81	2.68	1.76
DAL81	orf19.3252	Transcriptional activator	1.79	2.14	1.65
GCN4	orf19.1358	Transcriptional activator	3.20	3.72	3.64
INO2	orf19.7539	Transcriptional activator	2.26	2.33	1.83
IPF6857	orf19.1757	Putative transcriptional regulator	1.93	2.46	1.94
NRG1	orf19.7150	Transcriptional repressor	2.59	3.44	1.86
RMS1	orf19.10177	Putative transcriptional regulator	2.83	3.47	2.28
SFU1	orf19.4869	Transcriptional regulator of iron-responsive genes	2.12	1.90	1.98
TAF19	orf19.5174	TBP-associated factor by homology	1.77	1.73	1.82
TBP1	orf19.1837	Transcription initiation factor	1.89	2.29	2.67
WOR2	orf19.5992	Transcriptional regulator of white-opaque switch	1.83	2.00	2.00
ZCF3	orf19.1168	Predicted zinc-finger protein of unknown function	2.72	3.10	2.90
ZPR1	orf19.3300	Zinc finger protein	1.87	4.28	3.80
Translation initiation factor					
CDC95	orf19.9378	Translation initiation factor 6	2.23	3.42	3.14
GCD11	orf19.4223	Translation initiation factor eIF2	1.70	2.88	1.84
GCD7	orf19.825	Translation initiation factor eiIFb subunit	1.82	2.03	2.05
NIP1	orf19.4635	Translation initiation factor subunit	1.90	2.25	1.96
SUA5	orf19.7088	Translation initiation protein	1.69	2.20	1.94
SUI1	orf19.8867	Translation initiation factor 3	2.35	1.68	2.61
SUI3	orf19.7161	Translation initiation factor eIF2 beta subunit	1.66	1.84	2.38
TIF1	orf19.3324	Translation initiation factor	3.15	2.86	2.22
Other Function					
104 genes					
Unknown Function					
115 genes					

Consistent with the phenotipic pattern of mutant cells during the yeast-hypha transition at low levels of polyamine (0.01 mM), as reported previously by Herrero et al [188], several hypha-specific genes were down-regulated. Among these, we highlight the down-regulation of *ECE1* [32], *RBT1* [37], *HWP1* [419], *DDR48* [261], *PHR1* [370], *HYR1* [370] and *ALS3* during the three times of yeast hypha transition. We have also identified down-regulation of several other genes encoding cell wall proteins, putative GPI-anchored proteins (*PAG26, PAG36, PAG48, PAG53, PAG54* and *PAG59*) and up-regulation of *MNN11, MNN4, PGA31, PHR3* and *PIR1*. Other categories of down-regulated genes at the three times of yeast-hypha transition represented functional molecules as diverse as genes involved in acetyl coenzyme A (*ACC1, ACS1, ACS2* and *POT13*), adenine biosynthesis proteins (*ADE13* and *ADE17*), amino acid permeases (e.g. *AGP1*, *CAN2* and *CAN5*), proteins controlling cell polarity and filament formation (*ACT1, ARF3* and *ARP3*), dehydrogenases (*ADH1, ADH2* and *HOM6*), proteins of lysine biosynthetic pathway (*LYS1, LYS7* and *LYS12*) and ergosterol biogenesis (*ERG1, ERG24* and *ERG251*) (Table 4.2).

Also we found down-regulation of genes such as *GCF1,* IPF29*, TIP120, TYE7, ZCF4* and *ZCF20,* coding for transcriptional factors.

Expression of metabolic genes

About 13 genes involved in the glycolytic pathway includindig genes encoded enzymes that are required for both glycolysis and gluconeogenesis showed a decline in their transcript levels during the three times of yeast-hypha transition, whereas none of the genes of this pathway were up-regulated (Table 4.2). It is therefore probable that glycolisis and gluconeogenesis are down-regulated in the odc mutant.

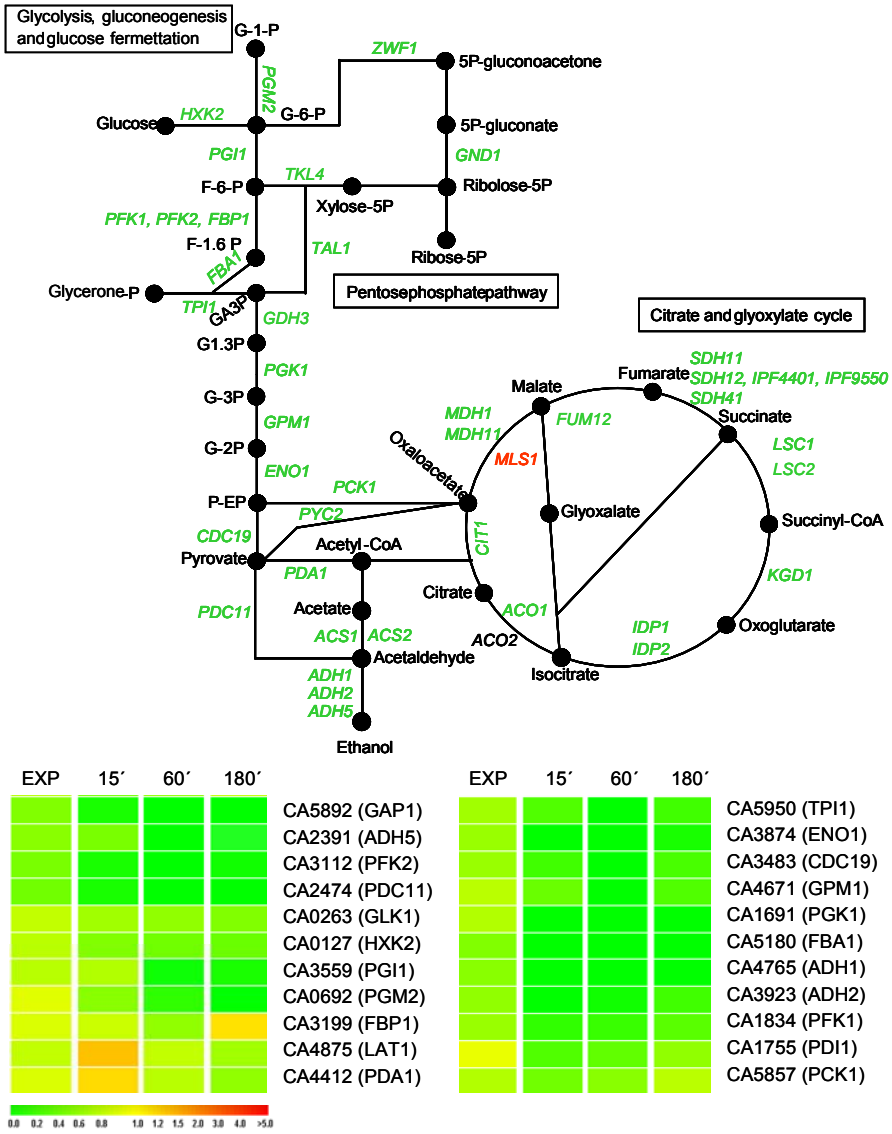

Figure 4.2 Transcriptional regulations of genes involved in carbohydrate metabolism by *C. albicans odc/odc* mutant during yeast mode of growth and during the yeast-hypha transition. Representative genes responsible for specific cellular functions are grouped and their regulation at different times (15, 60 and 180 min) of hypha development are listed. Red (up-regulated genes), green (down-regulated), and black (un-changed genes) names represent the CandidaDB

Under hypha-inducing conditions, numerous genes having predicted roles in mitochondria functions showed increased expression. These included a large number of proteins with structural roles in mitochondrial organisation and biogenesis, such as mitochondrial ribosomal proteins (Table 4.2). Figure 4.3 shows the expression patterns of genes involved in electron transport and respiration during the first, second and third periods of incubation and include a several of enzymes required for the tricarboxylic acid cycle (Table 4.2) and components required for electron transfer were down-regulated.

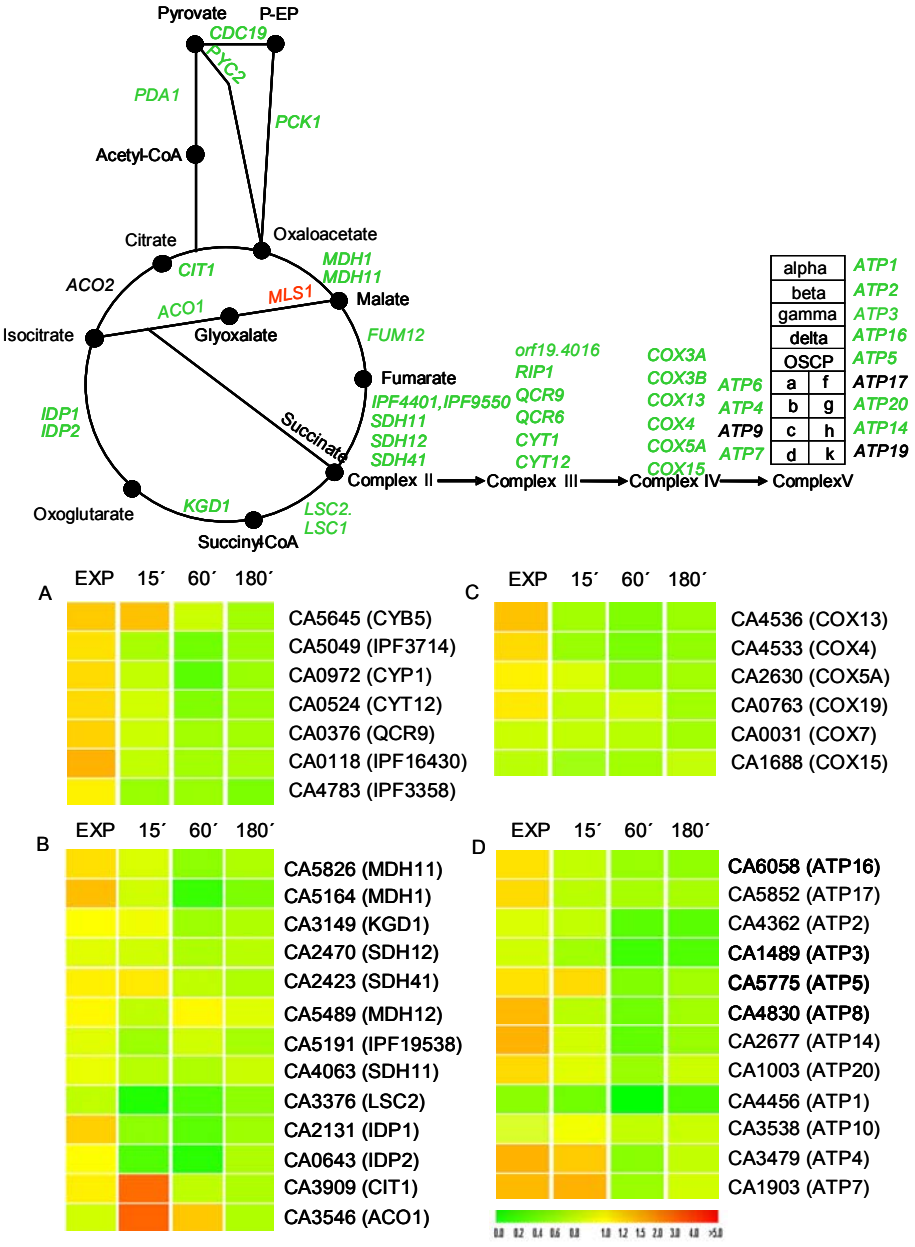

Figure 4.3 Transcriptional regulations of genes involved in respiration by *C. albicans odc/odc* mutant during yeast mode of growth and during the yeast-hypha transition in Lee medium. Representative genes responsible for specific cellular functions are grouped and their regulation at different times (15, 60 and 180 min) of hypha development are listed. Red (up-regulated genes), green (down-regulated), and black (un-changed genes) names represent the CandidaDB.Transcript levels of corresponding complex III (**A**), TCA pathway (**B**), complex IV (**C**) and complex V (**D**).

Overall, these results suggest that electron transport and respiration are repressed in the *odc* mutant strain during the yeast-hypha transition. Lower metabolic activity of the mutant cell at the three times of yeast-hypha transition due to down-regulation of genes involved in carbohydrate metabolism (glycolysis and hexose transport) and amino acid permease (*AGP1, CAN2, CAN5, HNM3,* IPF4580 and *MAL31*) can reflect a lower respiratory activity. Our results give new clues to understand the role of polyamines in *C. albicans* and further work is needed to characterise the effect of polyamines on electron transport and respiration in *C. albicans*. Inhibition of both ODC activity and putrescine uptake causes mitochondrial damage and loss of mitochondria in *Leishmania promastigote* cell [483], which is in agreement with the down-regulation of electron transport and energy generation-related transcripts at low polyamine levels.

Other transcripts

The transcriptional profiling analysis showed up-regulation of 17 of genes encoding for transcription factors during the three times of yeast-hypha transition (Table 4.2). These genes were *ACE2,* a putative transcription factor involved in the regulation of morphogenesis producing its deletion a hyperfilamentous phenotype [232]; *BCR1,* a transcription factor required for biofilm formation but not for hyphal growth [226,354,377]; *BRE1,* a putative transcription factor with the C_3HC_4 zinc-finger DNA-binding motif, transposon mutation of which affects filamentous growth [354,477]; *CAP1,* a transcription factor of the AP-1 family, involved in oxidative stress response and multidrug resistance [3,4,494,495,536]; Cta8p, a protein that activates transcription in one-hybrid assay in *S. cerevisiae* and is transcriptionally activated by Mnl1p under weak-acid stress [228,377]; *DAL81*, a predicted zinc-finger protein of unknown function that has similarity to *S. cerevisiae* Dal81p, which is involved in the regulation of nitrogen-degradation genes [298,544], *GCN4,* a transcriptional activator of general amino acid control response [472]; *INO2,* a transcriptional activator that may be involved in the regulation of ribosomal protein genes [198]; CA2262, a putative transcription factor with the zinc-finger DNA-binding motif, which transcription is up-regulated in clinical isolates from HIV+ patients with oral candidiasis [354,529]; *NRG1,* a transcriptional repressor involved in regulation of hyphal genes [38,330]; *TAF19,* a transcription factor involved in regulation of transcription during the G1 phase of mitotic cell cycle [518]; *TBP1,* a

transcription initiation factor that binds to the TATA box sequence [273]; *WOR2,* a transcriptional regulator of the white-opaque switching that is required for maintenance of opaque state [545]; *ZCF3*, a predicted zinc-finger protein of unknown function [298]; *ZPR1*, a protein with putative zinc finger that is regulated by Gcn4p [390]; and *SFU1,* a transcriptional regulator of iron-responsive genes [259].

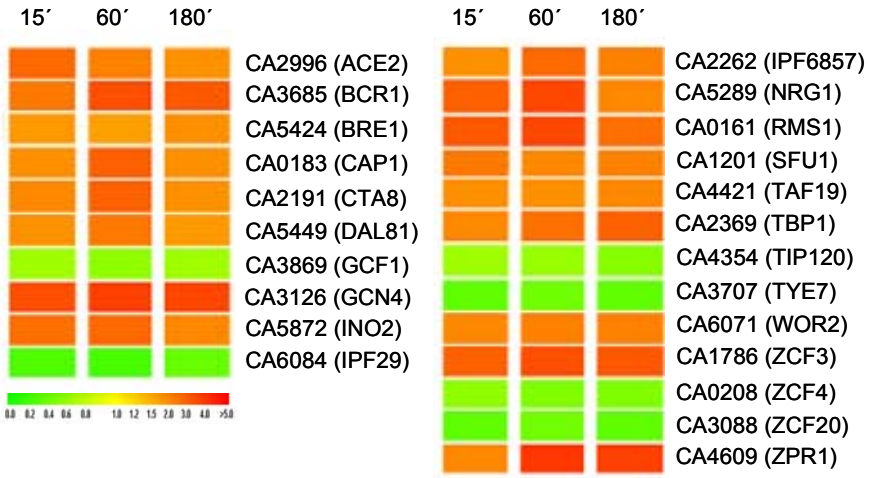

Figure 4.4 Transcriptional regulations of genes encoding transcription factors during three times of incubation by *C. albicans odc/odc* mutant.

Among the down-regulated transcription factors, we have found *GCF1,* a protein that binds to the regulatory region in the *HWP1* promoter and regulates a hypha-specific gene [235]; CA6084, a putative transcription factor; CA3088, a predicted zinc-finger protein of unknown function; *TIP120,* a TBP-binding transcription regulator; *TYE7,* a basic helix-loop-helix transcription factor; and *ZCF4,* a putative transcription factor (Figure 4.4).

Other up-regulated genes included proteins such as DNA-directed RNA polymerase (e.g *RBP5, RBP8* and *RPC25*) (Figure 4.5A), nucleolar proteins (e.g *ENP1, ENP2* and *NOP1*) (Figure 4.5B), ribosomal proteins (e.g *RPL23, RPS27A* and *RRS1*) (Figure 4.5C), RNA helicases (*DBP2, DBP3* and *DBP8*) (Figure 4.5D) and translation initiation factors (*CDC95, GCD11* and *GCD7*) (Figure 4.5E). We also detected that the expression of several genes encoding for mitochondrial ribosomes were increased in all the three times of hypha transition (Figure 4.5F). Currently, very little information regarding the behaviour of these genes is available; nevertheless, the regulation of these groups of genes by polyamines is interesting, because it supports our hypothesis about the possibility of polyamines being involved in the global regulation of gene expression.

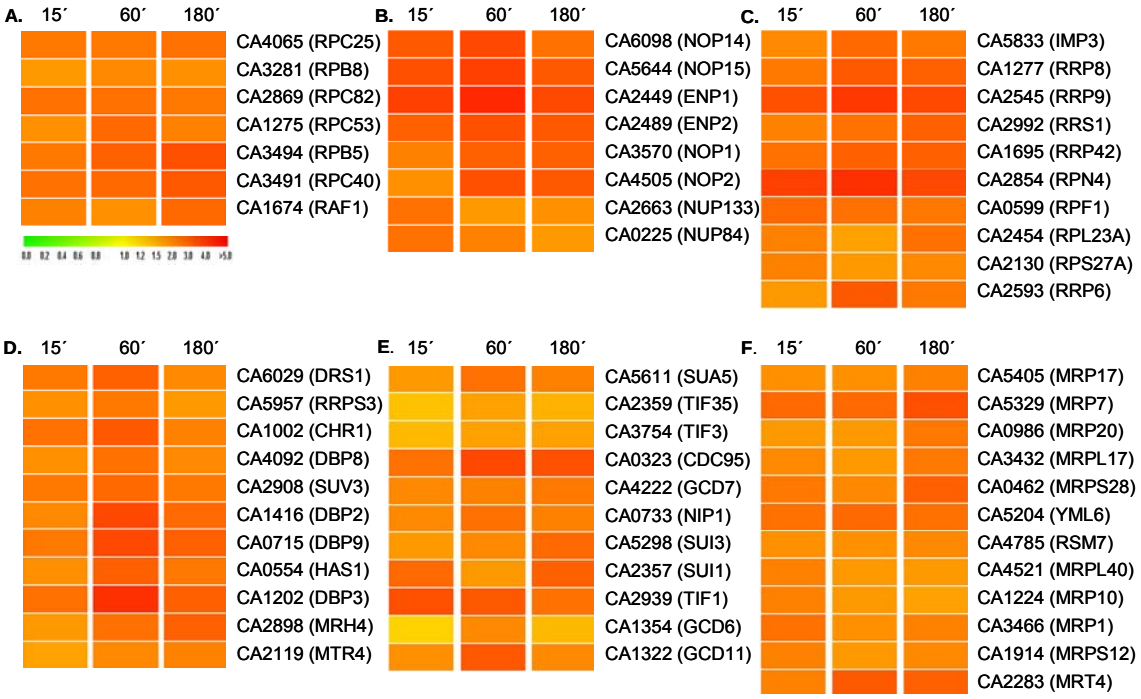

Figure 4.5 Transcriptional regulation by temperature of genes involved in DNA-directed RNA polymerase (**A**), nucleolar proteins (**B**), ribosomal proteins(**C**), RNA helicase (**D**), translation initiation factors (**E**) and mitochondrial ribosomes (**F**) Representative genes were grouped by gene families.

Common up- and down-regulated genes at 15 and 60 minutes of induction

The analysis of the microarray data of cells obtained during the hyphal transition showed a total of 188 genes to be differentially expressed commonly between 15 and 60 min of induction: 92 genes were up-regulated and 96 were down-regulated (Supplementary Tables 4.3 and 4.4).

Among the up-regulated genes, we highlight the induction of genes encoding the rRNA processing protein (*FAL1, RPP1* and *HCA4*), proteins involvd in glutamate metabolism (*GDH3, GNP1, PUT1* and *PUT2*), genes encoding stress-response proteins (*HSP70, HSP78, HSP90, SSA2, SSC1, SSE1* and *STI1*) and four transcription factors (*MIG1,* a transcriptional repressor that regulates genes for utilisation of carbon sources [139,330]; *SNF2,* a protein involved in transcriptional regulation, orthologue of *S. cerevisiae* Snf2p, which is the catalytic subunit of the SWI/SNF chromatin remodelling complex that interacts with Swi1p [301]; *STP4,* a putative transcription factor with the zinc-finger DNA-binding motif; induced in core caspofungin response [91,140,354]; and *TEC1,* a TEA/ATTS transcription factor involved in regulation of hypha-specific genes and required for biofilm formation [29,261,262,354]). Among the down-regulated genes, we have detected four

groups of genes involved in the following functional categories. Genes involved in the secretory pathway; *ARF21*- an ADP-ribosylation factor that is involved in intracellular transport [107,263]; *CLC1* -similar to *S. cerevisiae CLC1* a subunit of the major coat protein, involved in intracellular protein transport and endocytosis [425]; *COP1*- similar to *S. cerevisiae COP1* Alpha subunit of the COPI vesicle–coatomer complex, which surrounds the transport vesicles in the early secretory pathway [145]; *ERD2*- similar to *S. cerevisiae ERD2*, an integral membrane protein that both binds to the HDEL motif in proteins destined for retention in the endoplasmic reticulum and that has a role in the maintenance of normal levels of ER-resident proteins [178,366,412]; *ERV25* -orthologue of *S. cerevisiae ERV25,* a protein that forms a heterotrimeric complex with Erp1, Erp2p, and Emp24, members of the p24 family involved in endoplasmic reticulum to the Golgi transport [25,329]; *ERV46* a protein localised in COPII-coated vesicles that forms a complex with Erv41p involved in the membrane-fusion stage of transport [71]; *SEC16,* orthologue of *S. cerevisiae SEC16*, a COPII-vesicle coat protein required for ER transport-vesicle budding and autophagosome formation (Sec16p is bound to the periphery of ER membranes and may act to stabilise initial COPII complexes [150,420,459]); *SEC18,* a functional homologue of *S. cerevisiae* Sec18p, which acts in protein transport [347]; *SEC23,* orthologue of *S. cerevisiae SEC23,* component of the Sec23p-Sec24p heterodimeric complex of the COPII vesicle coat, involved in ER to Golgi transport and autophagy [111,217]; *SEC26,* orthologue of *S. cerevisiae SEC26,* an essential beta-coat protein of the COPI coatomer, involved in ER-to-Golgi protein trafficking and maintenance of normal ER morphology [112]; *SEC31,* orthologue of *S. cerevisiae SEC31,* essential phosphoprotein component (p150) of the COPII coat of secretory pathway vesicles [398]; and *SLY1,* orthologue of *S. cerevisiae SLY1* a hydrophilic protein involved in vesicle trafficking between the ER and Golgi [243] (Figure 4.6). The second set contains the genes involved in O-directed glycosylation (*PMT2, PMT1* and *PMT4*). The third set of genes is implicated in the mitotic cell cycle and cell cycle control (*CDC1, CDC11, CDC42* and *RAS1*) (Figure 4.6). And finally, three transcription factors including *CAS4,* a protein of RAM cell-wall integrity-signalling network with transcription factor activity that is required for hyphal growth [26,46,439]; *TFC1,* a protein with RNA polymerase III transcription factor activity [297]; and *UME6,* a transcription factor that is required for wild-type hyphal extension [22].

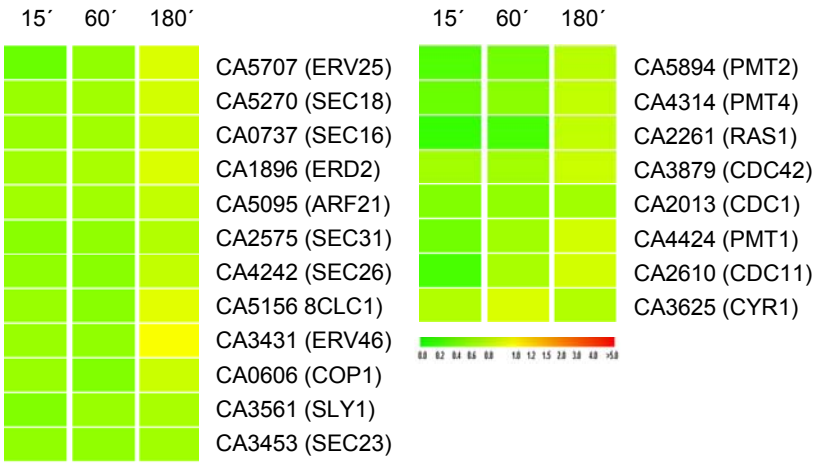

Figure 4.6 Transcriptional regulations of genes involved in secretory pathway and signal transduction during the three times of incubation by *C. albicans odc/odc* mutant.

Common up- and down-regulated genes at 60 and 180 minutes of induction

Of the total up- and down-regulated genes between 60 and 180 min of incubation, we observed common differential expression of 267 genes; 130 were up-regulated and 137 genes were down-regulated.

Of the 130 ORFs, four genes (*MRPL23A, MRPL36, MRPL49* and *MRPS5*) encode proteins involved in mitochondrial ribosomal proteins, five (*GCD2, GCD14, PRT, TIF5* and *YIF2*) encode proteins involved in translation initiation factors, three genes (*RPA12, RPA49* and *RPB4*) code for proteins involved in DNA-directed RNA polymerase II and 10 genes (IPF12778, IPF7998, *NIP7, NOP4, NOP58, RPL15B, RRP1, RRP45, RSA2* and *SIK1*) encode proteins involved in ribosomal protein. We detected four genes encoding proteins involved in transcription factors, namely, *CAS5* -a putative transcription factor that regulates response to cell wall damage and is required for wild-type transcriptional response to caspofungin [46,354]; *HAP41* -a protein with transcription activator activity that is transcriptionally activated by Mnl1p under weak acid stress [39]; *SUA70* -a predicted transcriptional regulator that is down-regulated during planktonic growth [196,334]; and *TAF60*-a transcription factor of the TFIID complex with general RNA polymerase II transcription factor activity [518]. Among the down-regulated genes, we have identified a number of genes involved in electron transport (*ATP14, ATP16, ATP2, ATP3, ATP5, ATP8, COX5A, CYT12,* IPF3358, *MCR1, QCR6 QCR9* and *VMA2*) (Supplementary Table 4.4 and Figure 4.3), cell polarity (*ABP1, ARC40, ARP2,*

CRN1, PFY1 and *TUB2*), tricarboxylic acid pathway (*FUM12, LSC2, MDH1* and *MDH11*) (Figure 4.3) and cell wall formation (*BGL21, CHT2, PGA4, PGA56* and *PHR1*). We observed down-regulation of three transcriptional factors, including *CTA24* -a putative transcriptional activator that is down-regulated by Efg1p [108]; *UME7* -a putative transcription factor with zinc-cluster DNA-binding motif, similar to *S. cerevisiae* Ume6p, which is involved in the regulation of meiotic genes [298,354,544]; and *TUP1* -a transcriptional corepressor [330].

Sets of genes regulated only at one incubation time

A total of 289 genes were expressed differentially at 15 min of hypha transition, among which 124 and 165 genes were up- and down-regulated, respectively.

Expression of some categories of up-regulated, genes include those involved in nitrogen, sulphur and selenium metabolism (*TRR1, UGA11, NFU1, ARG1* and *GLN1)*, two aconitate hydratases (*ACO1* and *ACO2*), several multidrug resistance factors (*CDR1, CDR2* and *QDR2*) and ribosomal proteins *MRPL32, MRPL11, RSM18*, *RPS10, MRPL3, MRPL24* and *MRPL33*.

The main categories of down-regulated genes correspond to those encoding cell wall proteins (*ALG8, ALS2, GAA1, IFF5, MNN2, MNT2*, *MNT3, PGA37* and *TOS1*) and genes involved in metabolism of porphyrins (*HEM2, HEM12* and *HEM15*). We have detected regulation of eight transcription factors including *CTA24,* a member of a family of telomere-proximal genes (a putative transcriptional activator that is down-regulated by Efg1p [108,482,529]); *GAL4,* a putative transcription factor with a zinc cluster DNA-binding motif [306,544]; *HAC1,* a putative transcription factor that is involved in unfolded protein response and control of cell morphology [354,508,539]; IPF14284, a putative transcription factor with a zinc-finger DNA-binding motif, similar to bacterial DnaJ, which is transcriptionally regulated by iron [297,354]; IPF7646, a predicted transcriptional regulator with a bZip domain [110]; and two predicted zinc-cluster proteins of unknown function *ZCF1, ZCF32* [298,364] (Supplementary Tables 4 .3 and 4.4). Most of these genes didn't change thier expression after 60 and 180 min of induction.

The expression of 388 genes was affected by a factor of ≥1.5 at low levels of polyamines compared with growth at high polyamine levels at 60 min; among these, 196 and 192 genes were up- and down-regulated, respectively. The main

categories of up-regulated genes were genes belong to ribosome biogenesis (*PET56, MAK21, RRN3, RRP4* and *RRP43*), translation initiation (*GCD6, TIF35* and *TIF3*), RNA helicase (*DBP2, DBP6, DED1, ECM16* and *MSS116*), and DNA-directed RNA polymerase (*RPA135, RPA43* and *RPO31*). Five transcription factors -*ARG81,* a putative transcription factor with zinc-cluster DNA-binding motif, with similarity to *S. cerevisiae* Arg81p involved in the regulation of arginine-responsive genes [298,354,544]; *CRZ1,* a putative transcription factor and similar to *S. cerevisiae* calcineurin-regulated transcription factor Crz1p [354,360]; *MRR1,* a zinc-finger protein and regulator of *MDR1* transcription, mutations of which cause up-regulation of *MDR1* (which encodes the plasma membrane multidrug efflux pump) [113,326]; *SEF2,* a putative zinc-cluster protein, whose expression is repressed by Sfu1p under high-iron conditions [294,298]; and *ZMS1,* a putative transcription factor with zinc-finger DNA-binding motif [354]- were also up-regulated. Genes involved in electron transport (*ATP20, ATP4, ATP7, COB ,COQ2, COX15, CTM1, CYT1,* IPF12564, *NAD1*, *NCP1,* RIP1*, TFP1, TFP3, VMA4, VMA7* and *VMA8*), TCA cycle (*FUM12, SDH11* and *SDH12*) (Figure 4.3 and Supplementary Table 4.4), ribosomal protein (*RNH1, RPL28, RPL30, RPL33, RPL39, RPL9B, RPS19A, RPS21B* and *RPS25B*), adenine biosynthesis (*ADE1, ADE6, ADE12* and *APT1*), cell cycle and cell polarity (*BUD2, BUD6* and *CDC10*) and two transcription factors -*SUC1,* a putative transcriptional regulator with N-terminal zinc finger that regulates alpha-glucosidase in *C. albicans* and *S. cerevisiae* [233,298]; and *ZCF39,* a putative transcription factor with a zinc-cluster DNA-binding motif [298,354]- were down-regulated.

The expression of 288 genes was affected by a factor of ≥ 1.5 after 180 min, with 181 and 107 genes being up- and down-regulated, respectively. The overrepresented categories of up-regulated genes after 180 min were genes encoding mitochondrial ribosomal proteins (*IMG1,* IPF3698, *MRPL15*, *MRPL16, MRPL28, MRPL31, MRP2 MRPL39, MRPL7*, *MRPS8* and *MRPL9*) and proteins involved in mitochondrial transport (*OXA1, TOM20, TIM9, TIM44, TIM54* and *YMC1*). Other group of genes include those involved in response to exposure to the cadmium ion exposure (*GSH1, ZRC1* and *MET14*) and three transcription factors -*HAC1, TAF25,* similar to *S. cerevisiae TAF10,* involved in RNA polymerase II transcription initiation and chromatin modification [163]; and *TFA1,* a protein with polyglutamate motifs and abundant Ser/Thr residues, described as a subunit of

TFIIE, which is a basal transcription initiation factor of RNA Polymerase I [141]- also were up-regulated. Some of the down-regulated genes encode proteins involved in electron transport (*COX19, COX7, CYB5, GDH3* and *GLT1*), histones (*HHF21, HTA1* and *HTA3*) and thiamine biosynthesis (*THI4, THI6* and *THI13*).

Genes involved in signal transduction

Because it has been reported that *C. albicans odc* null mutant shows defects in hyphal transition at low polyamine levels [188] and that the cAMP signal pathway is a regulating system involved in the hyphal transition [16,17,218,403], our analysis was focussed on genes involved in signal transduction. In our data, seven differentially expressed genes involved in signal transduction were affected and all were down-regulated at the first and second time points of hyphal transition. The transition from yeast-hypha of *C. albicans* is influenced by temperature and this signal-transduction cascade is modulated by *RAS1,* which controls the cAMP pathway and MAP-kinase cascade in *C. albicans* [265,388]. Because adenylate cyclase is a component of the cAMP pathway and it is involved in the growth of signals, *RAS1* activates both cell growth and hyphal transition of *C. albicans* [65,388]. Down-regulation of signal transduction transcripts, such as those of *RAS1,* and decrease in expression of *CYR1* (Figure 4.6) at two time periods of hyphal transition supplemented with 0.01 mM of putrescine, suggested that adequate levels of polyamines are necessary for activation of the RAS-signal pathway.

Discussion

Polyamines are essential for normal cell growth, as has been shown in several studies with mutants and inhibitors of the polymine metabolic pathway [50,59,95,101,170,195,225,493]. However, the effect of polyamine starvation has not yet been clearly established. Some authors have reported that elongation of proteins and nucleic acids diminishes and that translation fidelity is impaired [188]. In addition, polyamines have a distributed charge, whose spacing may allow them to interact more flexibly with phosphates, DNA and RNA [188]. The biological function of polyamines in *C. albicans* has been reported previously by our group by both deletion of the *ODC* gene and analysis of the consequences of this deletion on growth. The effects of low (0.01 mM) and high (10 mM) polyamine

concentrations on the yeast-hypha transition and on several phenotypes associated with cell-wall defects have been shown [188]. The null *odc* mutant of *C. albicans* behaves as a putrescine auxotroph, which is in agreement with the results described for other fungi, such as *S. cerevisiae* [133], *Neurospora crassa* [100] and *U. maydis* [170]. Global transcriptome profiles for *C. albicans odc* mutant exposed to low and high polyamine levels were generated and compared both during the yeast mode of growth and during the yeast-hypha transition. Several studies have compared the effect of polyamines on yeast and mammalian cells [58,88,171,300,475]. Our transcriptome analysis showed that low polyamine levels affect the level of expression of 2019 genes during yeast mode of growth and during the hypha-transition conditions. The K-means clustering approach illustrated that the expression of 16.86% of these genes was similarly affected during the yeast-form growth and yeast-hypha transition (data not shown).

This suggests that cells may have developed common transcription regulatory mechanisms to accommodate all the biological functions under low polyamine levels during both yeast growth and hyphal transition. During the hyphal transition, numerous genes involved in DNA-directed RNA polymerases, nucleolar proteins, RNA helicases (a large group of enzymes that function in virtually all aspects of RNA metabolism), ribosomal proteins and translation initiation factors were up-regulated at all three time points of incubation, suggesting polyamines play a role in the regulation of genes involved in protein synthesis and RNA metabolism. It has been reported that spermidine regulates protein synthesis (by stimulation at low concentrations and inhibition at high concentrations) [423]. There is a common theme in eukaryotic polyamine biology: the effect of polyamines on protein synthesis and the regulation of polyamine synthesis by translational mechanisms. Clearly, polyamines have many functions in the cell; however, protein synthesis may be particularly sensitive to the intracellular polyamine environment. Protein synthesis is more rapidly affected by polyamine depletion than RNA or DNA synthesis [197]. Over-accumulation of polyamines has been associated with an inhibition of protein synthesis [184,185], leading to decreased cell growth and eventual cell death. Increase in expression of *SPE2* (adenosylmethionine decarboxylase precursor), genes involved in translation initiation factors and ribosomal proteins under low levels of polyamine after the three time periods of incubation suggests that polyamines play a role in control of mRNA translation

initiation and ribosomal leaky scanning in *C. albicans*. Up-regulation of a number of genes that code for mitochondrial ribosomal proteins at the first, second and third time periods of incubation suggests that one of the metabolic changes caused by low polyamine levels in cells is the increase in expression of genes encoding protein synthesis, especially of mitochondrial protein synthesis. Ribosome production may exploit more than 50% of the synthetic effort of rapidly growing eukaryotic cells [142]. Therefore, by increasing ribosome synthesis, cells may be able to redirect these resources towards the defence against the post-translational effects of low polyamine levels. Among the down-regulated genes after the three time points of induction, we identified a number of genes that code for cell wall proteins, putative GPI-anchored proteins and genes have been detected previously as hypha-specific genes [130,342,356], suggesting that polyamines may mediate trancriptional control of cell wall–related genes. As mentioned above, the down-regulated genes encode cell wall proteins that are known to be associated with filamentous growth in *C. albicans* [18,32,37,131,261,419], thereby confirming the present results and are in agreement with the phenotype of the mutant strain in low levels of polyamine during hyphal transition as described previously [188]. It will be of interest to understand how and why polyamines lead to a drastic reduction in their transcripts. Several studies have compared the effects of polyamines on the transcriptome in yeast and mammalian cells. There has, however, been little overlap between the responsive genes reported in these studies and those presented in the current report. This may be because our experiment is a unique study that was carried out to investigate the role of polyamines in the dimorphism of *C. albicans*. Transcriptome analysis showed that the expression of genes involved in carbohydrate metabolism including genes involving in glycolysis and glucose transport were down-regulated during the yeast hypha transition. In yeast, low glucose causes a decrease in the level of cAMP, with concomitant low PKA activity [222,457,546]. Furthermore, there was a decrease in the expression of the maltase gene *MAL31* (maltose permease), which metabolises maltose into glucose [85,320]. The combination of decrease in sugar production and transport and decrease in expression of *TYE7* may lead to a glucose level that would limit PKA activity. This will ultimately lead to blocking of hypha outgrowth. In addition, we observed down-regulation of most of the genes encoding glycolysis enzymes during the yeast mode of growth, which is in agreement with the growth-rate pattern

of mutant cell in low level of polyamine as previously reported [188]. Among the other transcripts that showed down-regulation, genes involved in the biosynthesis of acetyl coenzyme A, amino acid permeases, cell polarity and filament formation, dehydrogenases, ergosterol biogenesis, respiration and electron transport (Table 4.2) were the predominant ones. However, down-regulation of most of the genes involved in cell polarity and filament formation, respiration and electron transport categories were observed after one and two times of hypha development. These results suggest that low levels of polyamines cause a significant and marked reduction in gene expression, especially for the genes categories denoted previously and that, this down-regulation is connected with the morphogenetic switch.

The mutant cells show decreased respiration during hyphal transition, which is accompanied by down-regulation of genes encoding subunits III of cytochrome c oxidase, NADH ubiquinone oxidoreductase and isocitrate dehydrogenase transcripts at the second and third time periods of hyphal transition, indicating a low metabolic status of the *C. albicans* mutant cells during the yeast-hypha transition. Overall, these results provide strong evidence that polyamines control the metabolic status, especially the carbohydrate metabolism (glycolysis), in *C. albicans*, which may contribute significantly towards maintaining homeostasis, electron transport and production of ATP.

Consistent with the post-translational role attributes of polyamines, several transcripts involved in the secretory pathway and genes related to protein-o-glycosylation were down-regulated at 15 and 60 min of hyphal transition (Figure 4.6). The changes observed in both the cellular metabolism and intracellular transport in the mutant cells are obviously not the results of a single mode of action of polyamines. However, it is clear that transcription/translation of a number of genes is coordinated with polyamine-mediated changes in the metabolite profiles and other transcriptional profiles in the mutant cells at 0.01 mM of putrescine, suggesting that activation and sustenance of transcription is one mode by which polyamines regulate *C. albicans* metabolism during both growth in the yeast form and during the yeast-hypha transition.

In animal cells, higher polyamine concentrations regulate transcription either by acting as a switch between different coactivators without altering the state of the chromatin [295] or by affecting histone acetylation/deacetylation [194].

Furthermore, a post-transcriptional role for polyamines has been shown in the down-regulation of animal genes [276], whereas a certain group of *Escherichia coli* genes is controlled by the diamine putrescine at the translational level [528]. In the latter case, it was suggested that putrescine enhances transcription of a group of genes by increasing the level of transcription factors. Polyamine-mediated modulation of protein synthesis may involve, among other things, structural changes in RNA, stimulation of ribosomal assembly, tRNA formation [215] or a frameshift during translation into protein, which is dependent on a Shine-Dalgarno-like sequence in *E. coli* [190].

Regulation of gene expression by polyamines can also occur through their effects on the structure of eIF5A or oligomerisation of nucleosomal arrays [94]. It is of interest to note here that the levels of eIF5 transcripts (*TIF5*) and other translation initiation factors as mentioned above were increased at all the three time points of incubation. Analyses of differentially expressed genes during the yeast-hypha transition identified the down-regulation of several genes encoding enzyme-mediated signal transduction that may be involved in regulation of hyphal transition. These include *CDC42* (control of cell division), *RAS1* (GTP-binding protein), *RHO3* (GTP-binding protein of the rho family), *SAR1* (GTP-binding protein of the ARF family) and *RSR1* (GTP-binding protein) at the first and second time points of incubation. Decreases in expression of *RAS1* and of *CYR1* genes (Figure 4.6) during hyphal transition suggest that inactivation of PKA due to low cAMP levels will ultimately lead to the blocking of hyphal outgrowth. The PKA pathway coordinates post-translational regulation of a variety of proteins, such as the key enzymes of glycolysis and gluconeogenesis [303,345], suggesting that pathways involved in the sensing and transduction of signals are preferentially affected during hyphal transition.

Analysis of *Candida albicans* mutant lacking the histone acetyltransferases, *GCN5* and *HAT1*

Abstract

The *HAT1* gene of *Candida albicans* was disrupted in a *gcn5* background to investigate the role of the two histone acetyltransferases, *GCN5* and *HAT1*, in growth, morphogenesis, and pathogenicity. When cultured in liquid medium Lee pH 6.8 at 37ºC, the *gcn5, hat1* double mutant failed to form hypha on all liquid and solid media assayed, except in embedded medium. Other phenotypes observed included sensitivity to Congo red, Hygromycin, Fluconazole, Itraconazole, and Caffeine. Whole-genome microarray analysis of the *gcn5, hat1* double mutant was used to identify differential expression of genes during the yeast mode of growth. The expression of a total of 521 genes was found to be altered in which 247 genes were upregulated and 274 were down-regulated. The main categories of differentially expressed genes were those corresponding to oxidative stress response, oxidase/peroxidases, dehydrogenases, transport activity, cell wall proteins, amino acid metabolism, ergoestreol biosynthesis, glycolysis pathway, and aerobic respiration. About 32% of the differentially regulated genes included genes encoding products not yet classified for the functional attributes.
Last, an attenuated virulence of the *gcn5, hat1* mutant under the usual statistical infection protocol was shown.

Introduction

Candida albicans, the most frequently isolated fungal pathogen in humans, is dimorphic; it switches between the budding yeast form and the filamentous hyphal or pseudohyphal form. This transition is induced by many different environmental cues including high temperature, neutral pH, serum, etc. Dimorphism is thought to contribute to the virulence of *Candida* [79,148,210,408,421,445]. Histone acetyltransferases (HATs) are a class of enzymes regulating histone acetylation, which has been identified in several cancers. In particular, imbalance in histone acetylation can lead to changes in chromatin structure and transcriptional misregulation of genes that are involved in the control of proliferation, cell-cycle progression, and differentiation [47]. The histone acetyltransferases, *GCN5* and *HAT1*, are proteins with important roles in the regulation of the gene expression of *C. albicans*, although their functions have not yet been widely investigated. In the

present work we show that deletion of the *GCN5* and *HAT1* as double mutant like both simple mutants is associated with a difference between the growth rate of the double mutant and CAI4 liquid medium at 28ºC. Morphologically the double mutant, like both simple mutants, is very different to the CAI4 strain. The main phenotype of the double mutant is the inability to switch from the yeast to the filamentous form in liquid media containing serum or at 37ºC and in all the solid media assayed.

Materials and methods

Strains, medium and growth conditions

Table 5.1 strain used in this study

Strains	Genotype	Parental strain	Reference
***C. albicans* strains**			
SC5314	Clinical isolate		[149]
CAI4	*ura3::imm434/ura3::imm434*	CAF2-1	[132]
CAMR	*ura3::imm434/URA3*	CAI4	(Rodriguez and Dominguez, 2005)
RGL4	*ura3::imm434/ura3::imm434 hat1::hisG/hat1::hisG*	CAI4	(Degano and Dominguez, 2000)
AHM1	*ura3::imm434/ura3::imm434 gcn5::hisG/gcn5::hisG hat1::hisGURA3hisG /HAT1*	RDG4	This work
AHM2	*ura3::imm434/ura3::imm434 gcn5::hisG/gcn5::hisG hat1::hisG/HAT1*	AHM1	This work
AHM3	*ura3::imm434/ura3::imm434 gcn5::hisG/gcn5::hisG hat1::hisGURA3hisG/hat1*	AHM2	This work
AHM4	*ura3::imm434/ura3::imm434 gcn5::hisG/gcn5::hisG hat1::hisG/hat1::hisG*	AHM3	This Work
HAV1	*ura3::imm434/URA3 gcn5::hisG/gcn5::hisG hat1::hisG/hat1::hisG*	AHM4	This work
RDG4	*ura3::imm434/ura3::imm434 gcn5::hisG/gcn5::hisG*	CAI4	Degano and Dominguez, 2000)
***E. coli* strain**			
DH5α	*F-Φ80lacZΔM15, Δ(lacZYA-argF), U169, deoR, recA1, endA1, hsdR17,.phoA, supE44, (rk-, mk+), gyrA96, Δ-thi-1, gyrA96 relA,*		[175]
Plasmid			
pJBR3	*Hat1* promoter and terminator flanked in *URA3*-marked pSNC1 vector		(Degano and Dominguez, 2000)

Escherichia coli strain was grown at 37ºC in LB medium supplemented with 100 μg of ampicillin per ml for plasmid selection. *C. albicans* strains used in this study, listed in Table 5.1, were cultured at 28ºC in YEPD. The solid and liquid media used were as have been described in Chapter III for inducing the yeast-hypha transition.

Media for integrative transformation

Gene deletion was performed using the "Ura-blaster" protocol as previously described [132]. Ura^+ transformants were streaked on MM plates; single colonies were picked and grown in 5 ml YNB medium. Genomic DNA was extracted for Southern analysis and diagnostic PCR. Counter selection of Ura^- segregants was done on YNB medium containing 5-fluoro-orotic acid (5-Foa) and 0.2 mM uridine.

Table 5.2 Primes used in this study

Primer	Sequence 5´-3´	Primer	Sequence 5´-3´
URA1	GGATACTATCAAACAAGAGG	**RD17**	GGTGACAAACTAGTTCAATGC
URA2	AATGCTGGTTGGAATGCTTA	**RD18**	GCTTATCAAGCTTTGGAAG
IP2	TTACAATCAAAGGTGGTCC	**RD32**	GGCAATCACATCAAGACT
IP3	GGTACAGTTGTTCCTCACA	**RD33**	TGTTCAACTTCAAGTCTC

The DNA manipulations, disruption of the *HAT1* gene and analysis of nucleic acids have been carried out as described in Chapter III.

Results

Construction of the *C. albicans gcn5, hat1* double mutant

Ura-blaster cassette was used to construct a *CaHAT1* deleted strain in a *gcn5* mutant background (Figure 5.1). The pJBR3 plasmid used in this study was constructed by Degano and the *C. albicans gcn5* mutant was transformed using the protoplast method. After transformation the *C. albicans* RDG4 (*gcn5-Ura*$^-$) strain with pJBR3, four colonies were obtained. PCR analysis (not shown) indicated that two transformants contained a proper deletion of one allele of the *CaHAT1* gene. The positive transformants were streaked on 5-Foa medium. The colonies that grew on these plates were checked by PCR for the absence of the *C. albicans URA3* gene. One of the positive colonies was transformed again with the pJBR3 construct. In this second round of transformation, five colonies were screened, and two *hat1* transformants were found with both alleles deleted as is shown by Southern blot (Figure 5.1.B).

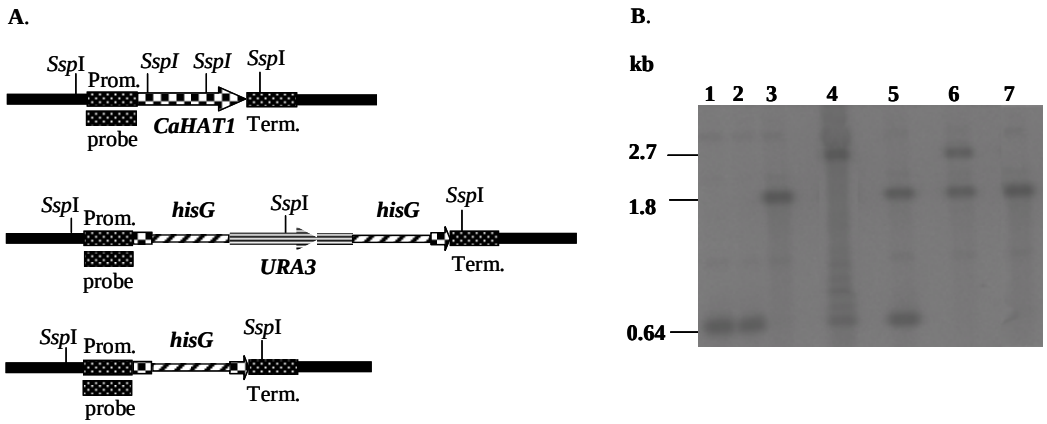

Figure 5.1 Deletion of *CaHAT1* alleles (A) Structure of different alleles.
The *HAT1* gene and the alleles disrupted by the *hisG-URA3-hisG* cassette or by *hisG* alone are shown. **(B)** Southern blot analysis of genomic DNA was performed with the following strains digested with *Ssp*I. **Lane 1**, CAI4 (*GCN5/GCN5; HAT1/HAT1*)
Lane 2, RDG4 (Δ*gcn5::hisG/*Δ*gcn5::hisG;HAT1/ CaHAT1*)
Lane 3, RGL4 (*GCN5/GCN5;*Δ*hat1::hisG/*Δ*hat1::hisG*)
Lane 4, AHM1 (Δ*gcn5::hisG/*Δ*gcn5::hisGHAT1/*Δ*hat1:hisGURA3hisG*)
Lane 5, AHM2 (Δ*gcn5::hisG/*Δ*gcn5::hisGHAT1/*Δ*hat1::hisG*)
Lane 6, AHM3 *(*Δ*gcn5::hisG/*Δ*gcn5::hisG*Δ*hat1::hisGURA3hisG/*Δ*hat1::hisG*)
Lane 7, AHM4 (Δ*gcn5::hisG/*Δ*gcn5::his* Δ*hat1::hisG/*Δ*hat1::hisG*)
The 459 bp, fragment was used as a probe.

Phenotype of the double mutant cells

Morphologically, the double mutant like both simple mutants is very different from CAI4 strain as show in Figure 5.2. The culture of the *gcn5, hat1* double mutant in YEPD liquid medium like both simple mutants is mainly composed of a mixture of spherical cells and short chains of elongated, aberrantly shaped cells attached to one another.

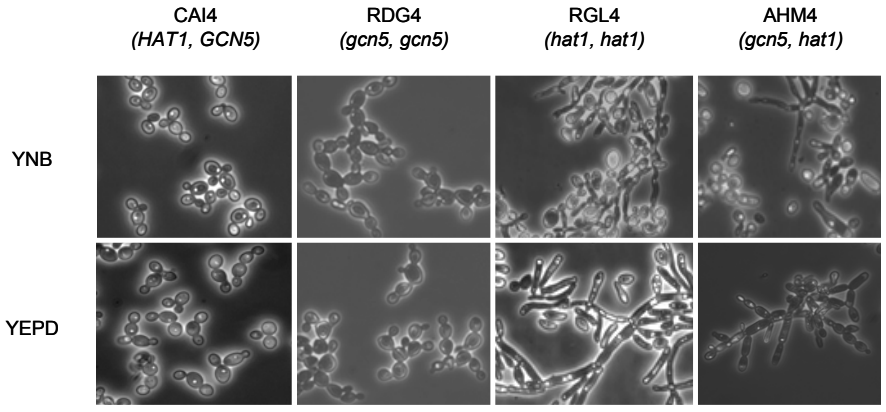

Figure 5.2 Cellular morphology of CAI4, RDG4, RGL4 and AHM4 strains in YNB and YEPD medium

The growth rate of the *gcn5, hat1* strain was 1.5-fold slower than that of CAI4 strain (Figure 5.3). This result suggested that deletion of *hat1* compensates partially the growth defect of the *gcn5* mutant under standard conditions. Another distinctive

feature of the *gcn5, hat1* mutant was the color; it was whiter than the parental and CAI4 strains (data not show).

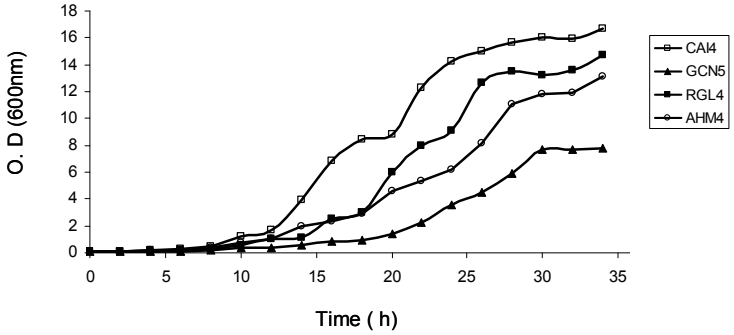

Figure 5.3 Growth curves of the mutant strains. Strains were pre-grown on rich medium containing 1% glucose and inoculated at an O.D at 600nm of 0.1 YEPD. Growth was monitored every two hours over a period of 34 h by measuring the optical density of the cultures.

Behavior of the double mutant strain in solid media

In order to determine whether the absence of *C. albicans* Gcn5p and Hat1p is involved in hyphal morphogenesis, we examined the ability of CAI4, double and both single mutants to undergo hyphal transition under several conditions on solid media.

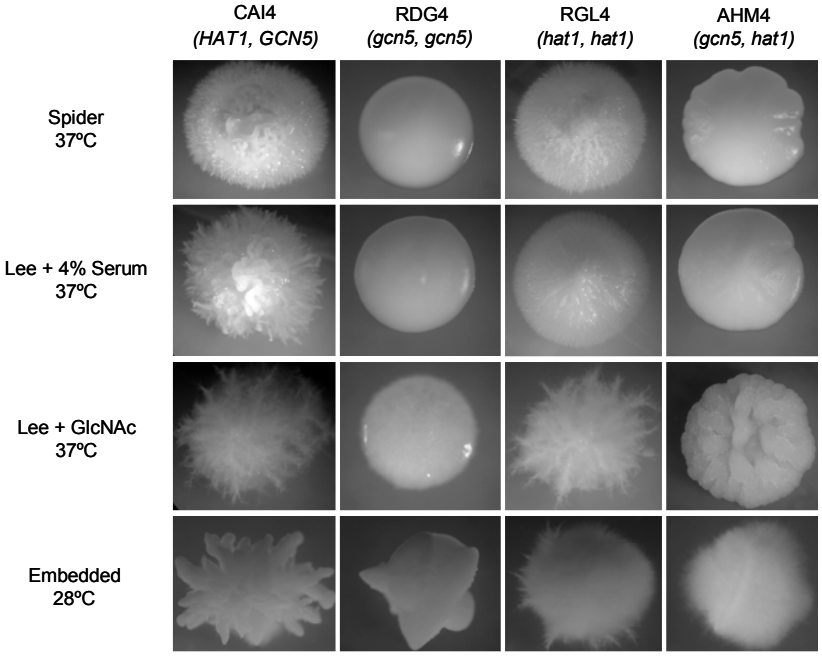

Figure 5.4 Phenotypes of the CAI4, RDG4, RGL4 and AHM4 grown on solid media which induce hyphal development. The plates were incubated for 5 days at 37C.

Cells of the simple and double mutants as well as CAI4 strain were plated at the 50 ufc in solid media plates. Solid media normally induces filamentous growth after 3 days. The CAI4 strain displayed normal filamentous growth, with long chains of

hypha growing outward from the colony. In contrast, the *gcn5, hat1* mutant like the *gcn5* single mutant was completely defective for filamentous growth in all media assayed except in embedded agar medium at 28ºC (Figure 5.4); in this medium, the *gcn5, hat1* strain showed hyperfilamentation like the *hat1* simple mutant.
This fact suggests cooperation of the Hat1p in the suppression of an alternative morphogenetic signalling pathway. It has been reported that deleting the APSES (Asm1, Phd1, Sok2, Efg1 and StuA family) protein genes, *EFG1* and *EFH1*, leads to an increase in filamentation under embedded hypoxic conditions [108]. The present results are very similar to those reported for the *efg1* deletion strain under microaerophilic condition.

Behavior of the *C. albicans gcn5, hda1* mutant in liquid media

The behavior of the double mutant strain was next investigated by assessing hyphal formation in Lee (pH 6.8, 37ºC) or in serum inducing media. For that purpose, *C. albicans* CAI4, and both simple mutant strains as well as the *gcn5, hat1* mutant were grown on different hypha-inducing media. As can be seen in Figure 5.5, the *gcn5, hat1* like the both simple mutants failed to form true hypha in the presence of calf serum at 37ºC. The culture was heterogeneous, being composed of a mixture of enlarged spherical cells and short chains of elongated, aberrantly shaped cells attached to one another. This result demonstrated that morphologically *gcn5* showed a dominant phenotype in the double mutant cells.

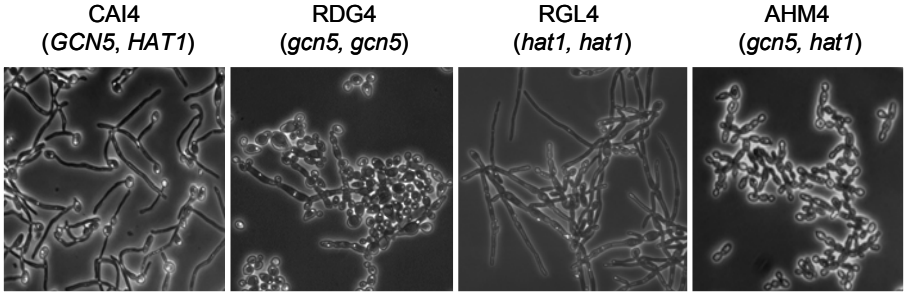

Figure 5.5 Morphology of CAI4, RDG4, RGL4 and AHM4 strains during the yeast-hypha transition in Lee media + 4% serum at 37ºC

Behavior of the *C. albicans gcn5, hda1* in chlamydospore formation

To examine the association of both the histone acetyltransferase genes with chlamydospore formation, cornmeal agar was used to compare the phenotypes of the CAI4, both simple and the *gcn5, hat1* strains. The *hat1* simple mutant

apparently formed chlamydospores like CAI4 strain, whereas the double mutant and the *gcn5* simple mutant as showed in Figure 5.6 were defective in the chlamydospore formation.

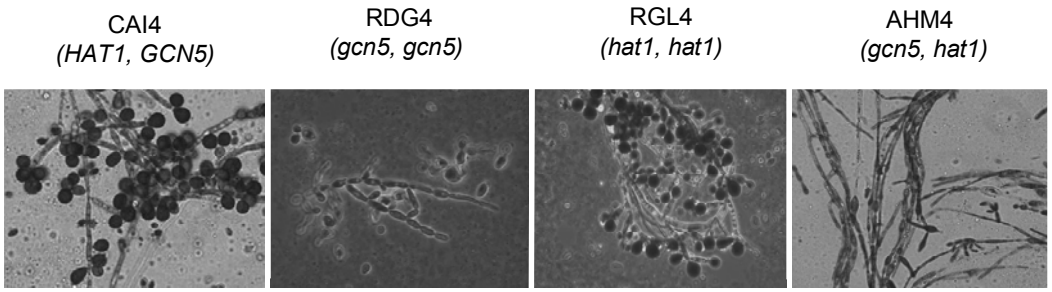

Figure 5.6 A chlamydospore formation of *C. albicans* is defective in *gcn5, hat1* double mutant strain

Virulence

The observation that the *C. albicans GCN5* gene is required for hyphal emergence or elongation *in vitro* and virulence led to the examination of whether *gcn5, hat1* double mutant affects virulence of *C. albicans*. Mice were injected intraperitoneally with a reconstructed *URA3* in *gcn5, hat1* mutant, and the mouse survival was monitored over a period of 30 days. After three days of infection with 5×10^8 stationary phase cells, mice injected with CAMR (CAI4-*URA3*$^+$) strain showed signs of systemic disease, including weight loss (data not show). Infection with CAMR strain resulted in rapid mortality. No difference in morility was found between mice infected with cells of *hat1* [378] and *gcn5, hat1* double mutant. Forty percent of mice injected with 5×10^8 cells of the *gcn5, hat1* double mutant survived for the observed period (Figure 5.7). In chapter III, we descrived that 80% of mice injected with *gcn5* mutant cells survived for same period. This result demonstrated again that *hat1* compensates partially phenotype of the *gcn5* mutation in mice model.

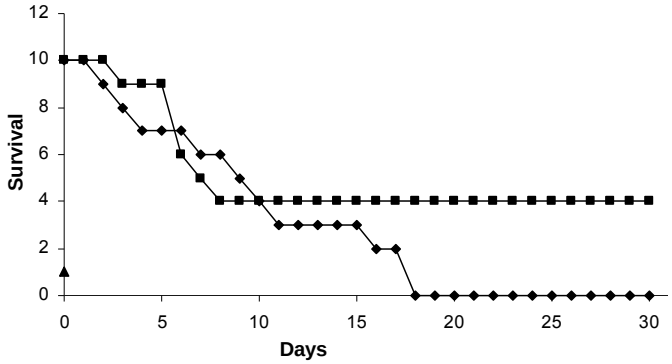

Figure 5.7 Virulence assays. Survival curves for mice (n=10 for each *C. albicans* strain at each inoculation dose) infected with 5×10^8 cells of *C. albicans*.♦ CAMR (CAI4-*URA*+). ■ HAV1 (*gcn5, hat1-URA*+).

Additional phenotypic changes

The double mutant cells appeared to be more resistant than both *hat1* and *gcn5* simple mutants to Zymolyase sensitivity test (Figure 5.8) supporting the idea that Gcn5p and Hat1p jointly control a set of genes involved in the cell wall architecture.

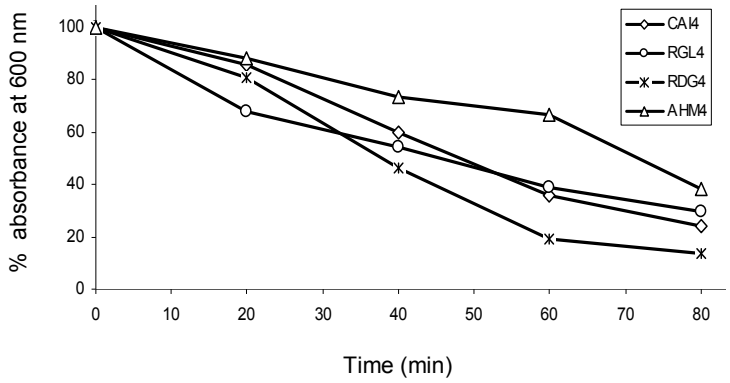

Figure 5.8 Resistance to a cell wall-degrading enzymatic complex of *C. albicans*
The CAI4 (◊), RGL4 (○), RDG4 (*) and AHM4 (Δ) were grown until the exponential phase in YEPD, at 28ºC. Aliquots (2 ×10^7 cells) were resuspended in 10 mM Tris-Hcl pH 7.5 and treated with 10 µg of Zymolyase per ml. The decrease in optical density (percentage of resistant cells) is monitored every 20 minutes.

Sensitivity to CFW, Congo red, Hygromycin, Itraconazole, Caffeine, Floconazole, NaCl, and SDS

In order to assess the consequences of deletion of *GCN5* and *HAT1* on the robustness of the cell wall, both single, double mutants, and CAI4 strain were tested for sensitivity to Calcofluor white, NaCl, Congo red and to SDS in plate assays (Figure. 5.9). Deletion of *GCN5* increased sensitivity to Calcofluor White, Congo red and SDS, whereas deletion of *HAT1* did not (except Congo red). However, the double *gcn5, hat1* mutant was more sensitive than both single mutants to Congo red and was also sensitive to Calcofluor white. Deletion of *HAT1* gene restored SDS and NaCl (1 M) tolerance in the *gcn5* mutant.

These findings prompted us to carry out a further four phenotypic tests (Hygromycin, Fluconazole, Itraconazole, and Caffeine sensitivity) in order to bether characterize and define the *gcn5, hat1* simple and double mutants. The double mutant like the *gcn5* simple mutants was more sensitive to Itraconazole, Fluconazole, Caffeine and Hygromycin than the *hat1* mutant and CAI4 strain.

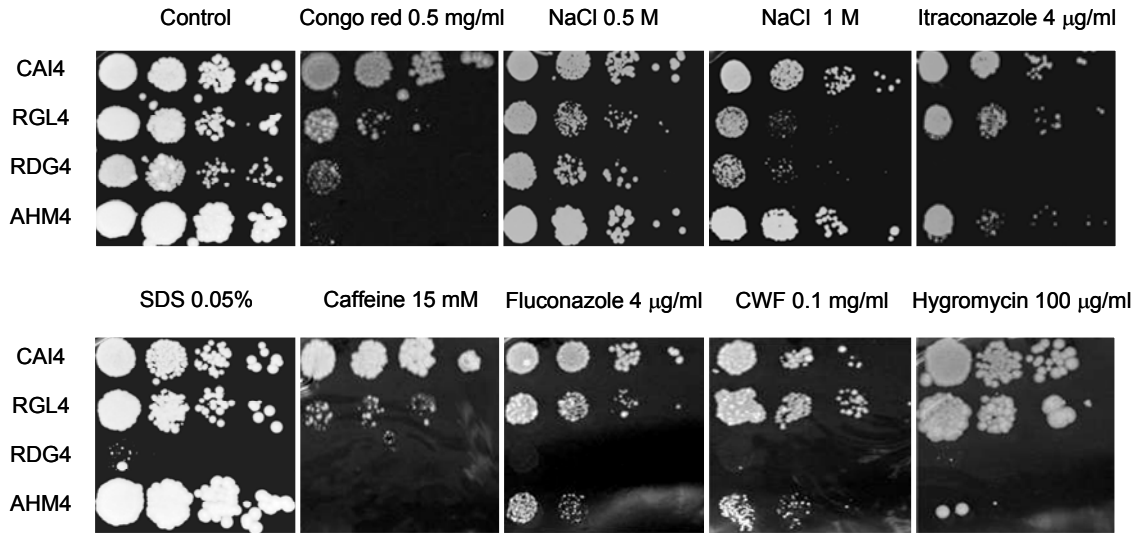

Figure 5.9 Sensibility assays of CAI4, RDG4 (*gcn5*), RGL4 (*hat1*) and AHM4 (*gcn5, hat1*) to different agents. All strains were grown in liquid YEPD. The optical density at 600nm of the cultures was adjusted to 0.4 with the same medium and 5 µl aliquots from the cultures and from 10-fold serial dilutions was spotted onto YEPD plates supplemented with different agents. Plates were incubated at 28ºC for 4 days.

Alcian blue binding assay

The level of Alcian blue binding for CAI4 was determined both for simple and double mutant strains of *C. albicans* using the method previously described [189]. The *gcn5* has been reported to be increased in Alcian blue binding whereas deletion of *HAT1* did not. However, the double *gcn5, hat1* mutant showed a significant increase in Alcian blue binding with respect to *hat1* mutant and CAI4 strain and decreased with respect to *gcn5* mutant (Figure 5.10).

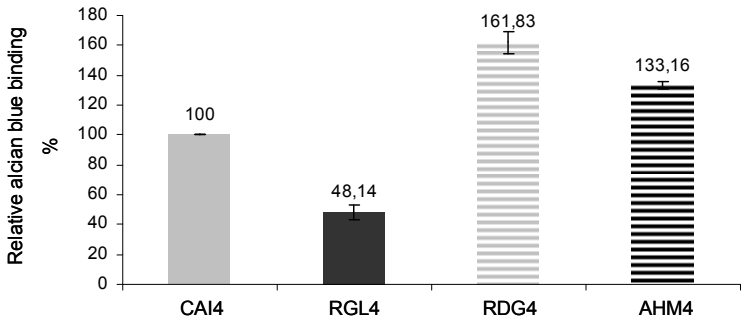

Figure 5.10 Cell surface charge in AHM4 (*gcn5, hat1)* strain. Alcian blue binding assays. Relative dye binding was calculated as the percentage of dye bound compared with results for the parental strains, CAI4, RDG4 (*gcn5*) and RGL4 (*hat1*).

The results suggest that deletion of *HAT1* gene slightly compensate phenotype of *gcn5* mutant.

Results of transcription profile

Transcript profiling was used to further examine the roles of the histone acetyltransferases (*HAT1* and *GCN5*) in *C. albicans*. cDNA microarrays carrying an almost complete set of *C. albicans* ORFs were used for these experiments, in which the transcriptomes of *gcn5, hat1* mutant were compared with wild-type cells under identical growth conditions. Genes that displayed reproducible and statistically significant changes in expression relative to wild-type cells were identified after exposure to noninducing growth conditions. Lists of all genes that displayed significant regulation by double mutant are provided as supplemental data (Supplementary Tables 5.1 and 5.2).

To explore the functions of the histone acetyltransferases (*GCN5* and *HAT1*) under conditions that promote growth in the yeast form, the transcriptomes of the CAI4 and double mutant strains in YNB medium were compared at 28ºC. The double deletion of *GCN5* and *HAT1* altered the expression of 521 genes by ≥1.5-fold, with 247 genes being up-regulated and 274 being down-regulated. Only 52 and 283 genes are affected by the deletion of *HAT1 and GCN5*, respectively. Five genes (*FDH3, GRP2, HGT6, HGT8* and IPF3964) were commonly affected in both single and double mutants. The simultaneous deletion of *HAT1* and *GCN5* showed a transcriptional pattern that overlapped partially with that of *gcn5* single mutant (58 genes with 48 genes up- and 10 genes down-regulated). However, a new subset of 453 genes was affected in the *gcn5, hat1* double mutant that was not affected in any of single mutants. This result suggested that Gcn5p and Hat1p jointly controlled the regulation of a broad range of genes. Distinct groups of genes were either up- or down-regulated during the yeast mode of growth in the double mutant.

Categorization of the regulated genes according to their functional categories of the MIPS database revealed that double deletion of *gcn5, hat1* had a striking effect on carbon metabolism (Table 5.3). Transcript levels for almost all glycolytic genes (e.g., *PFK1, PFK2* and *ENO1*) and for genes involved in the metabolism of reserve sugars trehalose and glycogen (e.g., *TPS2, TPS3* and *GSY1*) were down-regulated in the double mutant strain. Transcript levels for some citric acid cycle enzymes (e.g., *ACO1* and *FUM12*), dehydrogenases, glycine (*MIS11*), and formate catabolic enzymes (*FDH1, FDH3* and *FDH4*) were up-regulated (Supplementary Tables 5.1 and 5.2). Almost the same transcriptional profile for glycolytic genes, for genes involved in the metabolism of reserve sugars trehalose and glycogen, acid cycle

enzymes and formate catabolic enzymes has been reported for *efg1* mutant strain under noninducing conditions [108].

Moreover, Similar to the *gcn5* mutant [106], we detected varation of expression several Gcn5p-dependent genes in the double mutant including the up-regulation of genes involved in stress response (*ASR1, ASR3, DDR48, FUN12, HSP104, HSP12, HSP31, SSA4* and *RTC3*), in cell wall proteins (*PGA13, PGA56, PIR1, RHD3, YWP1, HPF1* and *IPF885*), transporter (*CFL4, HGT19, PHM7* and *PTR21*) and encode for transcription factors (*NRG1, TBP1* and *STP4*) and the down-regulation two genes encode for ribosomal proteins (*RPL10A* and *RPL20B*), two genes encode for cell surface proteins (*ALS2* and *ALS4*) and three genes involved in energy pathways (*ACS2, MIS11* and *TPI1*).

Table 5.3 Functional classification of differentially genes during the yeast mode of growth in the *gcn5, hat1* doubles mutant cells

Gene name	Accession no	Description (CandidaDB)	Fold regulation
Down-regulated genes			
Cell wall			
AAF1	orf19.7436	Adhesion and aggregation mediating surface antigen	0.59
ALS3	orf19.2355	Agglutinin like protein	0.58
ALS2	orf19.2122	Agglutinin-like protein	0.58
ALS4	orf19.4556	Agglutinin-like protein	0.54
BGL22	orf19.7339	Glucanase; induced during cell wall regeneration	0.61
ECM21	orf19.12351	Involved in cell wall biogenesis	0.63
FGR12	orf19.5479	Protein lacking an ortholog in S. cerevisiae; transposon	0.60
IFF5	orf19.10397	Putative GPI-anchored protein of unknown function	0.62
KRE9	orf19.5861	Cell wall synthesis protein	0.50
MNT2	orf19.1663	Alpha-1,2-mannosyl transferase	0.26
PGA38	orf19.10273	Putative GPI-anchored protein of unknown function	0.57
PGA45	orf19.2451	Cell wall protein; putative GPI-anchor	0.55
PGA50	orf19.1824	Putative GPI-anchored protein of unknown function	0.62
Dehydrogenase			
ADH1	orf19.3997	Alcohol dehydrogenase	0.66
ADH5	orf19.2608	probable alcohol dehydrogenase	0.57
GPD1	orf19.1756	Glycerol-3-phosphate dehydrogenase	0.54
GPD2	orf19.691	Glycerol 3-phosphate dehydrogenase	0.56
URA1	orf19.4836	Dihydroorotate dehydrogenase	0.18
Aerobic Respiration			
ABC1	orf19.10842	ubiquinol--cytochrome-c reductase	0.57
DLD2	orf19.6755	D-lactate ferrycytochrome C oxidoreductase	0.64
DLD3	orf19.5805	D-lactate ferrycytochrome C oxidoreductase	0.58
COX8	orf19.5213	Cytochrome C Oxidase	0.61
COX9	5868	Cytochrome C Oxidase	0.64
IPF16549	orf19.4273	Aerobic respiration	0.57
RIB3	orf19.12693	3,4-dihydroxy-2-butanone 4-phosphate synthase	0.53
SHY1	orf19.4841	SURF homologue protein	0.44
Secratory pathway			
BET3	orf19.5817	Targeting and fusion of ER to Golgi transport vesicles	0.62
BET5	orf19.302	Targeting and fusion of ER to Golgi transport vesicles	0.60
Transport			
BPT1	orf19.6383	Membrane transporter of the ATP-binding cassette	0.62
DUR31	orf19.781	Urea transport protein	0.63

HAK1	orf19.6249	Potassium transporter	0.56
HGT7	orf19.2023	Hexose transporter	0.48
IFC1	orf19.3746	Oligopeptide transporter	0.44
IFC3	orf19.3749	Oligopeptide transporter	0.38
IPF11077	orf19.3432	Membrane transporter	0.58
NAS2	orf19.4550	Membrane transporter	0.63
OPT1	orf19.2602	Oligopeptide transporter	0.10
ZRT2	orf19.1585	Zinc transport protein	0.37
Fatty acid metabolism			
ERG1	orf19.406	Squalene epoxidase	0.59
ERG251	orf19.12101	C-4 sterol methyl oxidase	0.65
ERG9	orf19.3616	Farnesyl-diphosphate farnesyltransferase	0.62
Amino acid metabolism			
ARO4	orf19.4060	3-dehydro-deoxyphosphoheptonate aldolase	0.63
CHA11	orf19.7404	L-serine/L-threonine deaminase	0.47
FEN2	orf19.12981	Allantoate permease transporter	0.61
FUR4	orf19.313	Probable uracile or allantoin permease	0.61
HOM3	orf19.1235	Aspartokinase	0.35
MET13	orf19.288	Methylene tetrahydrofolate reductase	0.47
SAM2	orf19.657	S-adenosylmethionine synthetase 2	0.51
Glycolysis			
CDC19	orf19.3575	Pyruvate kinase	0.40
ENO1	orf19.395	Enolase I	0.63
FK26	orf19.9844	6-phosphofructose-2-kinase	0.44
PFK1	orf19.3967	6-phosphofructokinase alpha subunit	0.45
PFK2	orf19.6540	6-phosphofructokinase beta subunit	0.45
PGI1	orf19.3888	Glucose-6-phosphate isomerase	0.65
PGK1	orf19.3651	Phosphoglycerate kinase	0.59
PGM2	orf19.10359	Phosphoglucomutase	0.60
Up-regulated genes			
Oxidative stress response			
CTA1	orf19.6229	Catalase A. peroxisomal	3.59
GAD1	orf19.1153	Glutamate decarboxylase	3.18
GRP2	orf19.4309	Reductase	2.23
GRP4	orf19.10660	Putative reductase	1.63
GRP5	orf19.6868	Dihydroflavonol-4-reductases	1.60
MCR1	orf19.3507	NADH-cytochrome-b5 reductase	1.57
PST2	orf19.3612	1,4-benzoquinone reductase	2.32
TRP99	orf19.8054	Thioredoxin peroxidase/alkyl hydroperoxide reductase	1.73
TSA1	orf19.7417	Protein of TSA/alkyl hydroperoxide peroxidase	2.26
Cell wall			
PGA13	orf19.6420	Putative GPI-anchored protein	2.39
PGA39	orf19.6302	Putative GPI-anchored protein of unknown function	2.15
PGA4	orf19.4035	Putative GPI-anchhored protein	1.63
PGA56	orf19.1105.2	Putative GPI-anchor	2.53
PGA6	orf19.12229	Putative GPI-anchored cell-wall protein	2.02
PIR1	orf19.220	Putative cell wall protein of the PIR family	2.39
RBT5	orf19.5636	GPI-anchored cell wall protein involved in hemoglobin utilization	1.69
RHD3	orf19.5305	GPI-anchored protein that localizes to the cell wall	1.57
TOS1	orf19.1690	Putative Anchor subunit of a-agglutinin	2.20
YWP1	orf19.3618	Putative cell wall protein	1.56
Oxidase/Peroxidase			
CFL1	orf19.1263	Ferric reductase	1.69
CFL4	orf19.9488	Ferric reductase	1.72
CFL5	orf19.1930	Ferric reductase	1.95
IPF6231	orf19.10802	Prolin-methionine-R-oxid reductase	1.55
IPF3358	orf19.4016	Ubiquinol-cytochrome-c reductase	1.63
IFG3	orf19.944	Probable d-amino acid oxidase	1.90
PUT1	orf19.4274	Proline oxidase	1.57
TRP99	orf19.8054	Protein described as thioredoxin peroxidase	1.73
TSA1	orf19.7417	Protein of TSA/alkyl hydroperoxide peroxidise	2.26
Dehydrogenase			
ALD5	orf19.13228	Aldehyde dehydrogenase (NAD+)	2.03
ALD6	orf19.742	Putative aldehyde dehydrogenase	1.68

CSH1	orf19.4477	Putative aryl-alcohol dehydrogenase	2.82
EBP5	orf19.3234	NADPH dehydrogenase	1.62
FDH1	orf19.1774	Putative formate dehydrogenase	1.53
FDH3	orf19.7600	Glutathione-dependent formaldehyde dehydrogenase	1.80
FDH4	orf19.1774	Formate dehydrogenase	2.25
IFD1	orf19.8650	Putative aryl-alcohol dehydrogenase	1.93
IFD6	orf19.1048	Putative aryl-alcohol dehydrogenase	1.87
IPF5389	orf19.5565	3-hydroxyisobutyrate dehydrogenase	1.59
LPG20	orf19.771	Putative oxidoreductase	1.71
PUT2	orf19.3974	1-pyrroline-5-carboxylate dehydrogenase	2.69
Transporter			
ACR1	orf19.3931	Succinate-fumarate transporter	1.93
CCC1	orf19.6948	Transmembrane Ca^{2+} transporter	2.13
HGT11	orf19.4527	Hexose transporter	3.37
HGT12	orf19.3668	Hexose transporter	3.44
HGT17	orf19.4682	Putative glucose transporter	1.69
HGT19	orf19.5447	Putative glucose/myo-inositol transporter	2.37
HGT6	orf19.2020	Sugar transporter	1.52
HGT8	orf19.2021	Sugar transporter	1.57
HIP1	orf19.4940	Histidine permease	1.62
JEN2	orf19.12767	Carboxylic acid transporter protein	3.02
PTR21	orf19.6937	Peptide transporter	1.86
SGE11	orf19.4779	Putative transporter	2.26
STL1	orf19.13176	Sugar transporter	2.01

We have detected regulation genes encoding, for cell wal proteins including the up-regulation *PGA39, PGA4, PGA56, PGA6, RBT5*, *TOS1* and down-regulation *ALS3, ALS2, BGL22, ECM21, FGR12, IFF5, KRE9, MNT2, PGA38, PGA45* and *PGA50* in the double mutant during the yeast mode of growth. We detected the up-regulation many genes involved in oxidative stress response (*CTA1, GAD1, GRP2, GRP4, GRP5, MCR1* and *PST2*), transporters (*ACR1, CCC1, JEN2*, *HIP1, PTR21* and *SGE11*), ferric reductases (*CFL1* and *CFL5*), oxidases/perxodases (*PF6231, PF3358, IFG3, PUT1, TRP99* and *TSA1*), dehydrogenases (*ALD5, ALD6, EBP5, IFD1,* IPF5389 and *PUT2*). Among the up-regulated genes, four genes (*HGT11, HGT12, HGT17* and *STL1*) were related to glucose transport. This result is in strong contrast to those reported in the *gcn5* and *hat1* simple mutant [106], in which these groups of genes are not regulated during yeast mode of growth. Further groups of genes regulated jointly by Gcn5p and Hat1p include genes involved in components of the respiratory chain (*ABC1, DLD2, DLD3, COX8, COX9,* IPF16549*, RIB3* and *SHY1*), biosythesis of ergoestrol (*ERG1, ERG251* and *ERG9*), amino acid metabolism (*ARO4, CHA11, FEN2, FUR4, HOM3* and *MET13*) and genes encoding for transporters (*BPT1, DUR31, HAK1, FC1, IFC3,* IPF11077*, NAS2* and *ZRT2*).

Discussion

Previous analysis has shown that members of the histone acetyltransferase family (*GCN5* and *HAT1*) are required for hypha formation in *C. albicans* in liquid and solid media. The present phenotypic analysis indicates that growth of the *gcn5, hat1* double mutant is associated with a detectable deficiency in the rate of colony formation on rich mediua plates at 28ºC. Morphologically, the *gcn5, hat1* double mutant and both simple mutant strains have shown a phenotype very different to CAI4 strain. Thus, under our experimental conditions, Gcn5p and Hat1p appear as important proteins for the regulation of the genes that are critical for cell growth and morphology. The most prevalent phenotype is the inability of the *gcn5, hat1* strain to switch from the yeast to filamentous form in liquid media containing serum at 37ºC and in all the solid media assayed except in embedded medium. In embedded medium, the *gcn5, hat1* like the *hat1* strain was hyperfilamentous when compared to the wild type. In contrast, the *gcn5* simple mutant strain has failed to form filamentation in embedded medium in agreement with those reported previously by Doedt et al [108]. Other phenotypes observed include sensitivity of the double mutant like *gcn5* simple mutant to Congo red, Calcofluor white, and Caffeine suggesting a remodelling of cell wall composition in the double mutant. No difference in sensitivity was found towards SDS and NaCl when compared to CAI4 strain (Figure 5.9) suggesting that deletion of *HAT1* gene restored SDS and NaCl (1 M) tolerance in the *gcn5* mutant.

The study of chlamydospore formation has been performed to determine whether members of histone acetyltransferases that block hyphal development also have a function in the formation of chlamydospores. The *gcn5, hat1* strain like *gcn5* mutant strain is defective for chlamydospore formation. However, similar to CAI4 strain, *hat1* mutant has competently formed chlamydospores [106]. These results demonstrate that Gcn5p like Efg1p is needed for chlamydospore formation, and that Gcn5p produces the predominant phenotypes in double mutant cells.

Upon comparing our results and those reported in the both *gcn5* and *hat1* simple mutant [106], we observed that expression several of genes involved in the glucolytic patway, oxidative estress, amino acide metabolism, glocuse transport, genes encoding for cell wall proteins were regulate jointly by Gcn5p and Hat1p. We detected expression 58 genes were regulated commonly in the double mutan and *gcn5* simple mutant. Of those, genes involved in stress response, energy

pathways, encoding for cell wall proteins, transcription factors and transportes were the predominant ones. There was, however, little overlap between the regulated genes reported in these studies and those presented in the *hat1* mutant [106] that is consistent with dominant phenotype of *gcn5* simple mutant.

Down-regulation of a number of genes involved in ergosterol biosynthesis has been observed. Ergosterol genes were mainly upregulated after exposition to antifungal agents [231,281]. This result is consistent with the results showing that the double mutant grown as yeast exhibited azole sensitivity.

Introducción

El reino de los hongos incluye numerosas especies importantes desde diversos puntos de vista como son el ecológico, el económico o el médico. Las especies fúngicas al descomponer la materia orgánica contribuyen a la continuidad del ciclo de nutrientes dentro de los ecosistemas, la mayoría de plantas vasculares no podrían crecer sin los hongos simbióticos asociados a sus raíces. Algunos hongos proporcionan de forma directa alimento al hombre y otros son utilizados para dar determinadas características a productos consumidos por el hombre, como el pan. Resulta de especial relevancia médica la capacidad de algunas especies de este reino de sintetizar y secretar al medio antibióticos. Por otro lado, algunas especies fúngicas causan infecciones en animales y plantas que resultan difíciles de tratar, debido a que las células fúngicas son células eucariotas que realizan sus procesos metabólicos de forma similar a las células de organismos superiores. Los hongos son organismos ubicuos encontrándose especies fúngicas en casi todos los hábitats. Muchas de estas especies son capaces de adaptarse a diferentes ambientes, desarrollando en un corto periodo de tiempo una respuesta transcripcional y fisiológica determinada ante un cambio de las condiciones del medio [451].

Candida albicans

Características generales. Posición taxonómica

El género *Candida* engloba unas doscientas especies que comparten características como reproducción asexual por gemación y ausencia de pigmentos carotenoides. Las especies del género *Candida* se incluyeron inicialmente dentro de la clase *Deuteromycetes*, grupo que incluye los comúnmente llamados "hongos imperfectos" y que corresponden con especies no relacionadas cuya principal característica es la ausencia de ciclo sexual conocido. Actualmente el género *Candida* se ha incluido dentro de los ascomicetos de acuerdo con la siguiente clasificación [51]:

Reino: Fungi
Phyllum: *Ascomycota*
Clase: *Hemiascomycetes*
Orden: *Saccharomycetales*

Familia: *Candidaceae*

Género: *Candida*

El cambio de clasificación se ha debido a varios factores. Datos de comparación de secuencias de diferentes genes indican que filogenéticamente las especies con las que están más relacionadas las especies del género *Candida* son ascomicetos. Además, todas las especies de *Candida* presentan características que comparten con los ascomicetos y las diferencian de los basidiomicetos, como es la gemación de una yema por locus, en lugar de la gemación sucesiva de varias yemas a partir de un mismo locus, propia de los basidiomicetos [51]. *C. albicans* es la especie más estudiada de su género ya que es el patógeno fúngico oportunista de mayor incidencia en humanos. Los rasgos más significativos de *C. albicans* son su capacidad de formar tubos germinativos y clamidosporas, seguida de su patrón de asimilación de azúcares. Se han establecido varios biotipos dentro de la especie basándose en similitudes fenotípicas y de DNA [358].

El genoma de *C. albicans*

C. albicans es un organismo diploide. Desde el año 2000 se dispone de la secuencia completa de su genoma. La secuenciación fue llevada a cabo por el Stanford Genome Center partiendo de DNA de la cepa SC5314. Dicha estirpe fue elegida por ser un aislado clínico usado habitualmente en análisis moleculares y ensayos de virulencia, y poseer un cariotipo estándar. Se ha estimado que el genoma haploide de *C. albicans* comprende aproximadamente 16 Mb, siendo por tanto un 30% mayor que el de *S. cerevisiae*. Se han realizado sucesivos ensamblajes de las secuencias que han sido publicados en la página web *http://www sequence.stanford.edu/group/candida/download.html.* Recientemente se ha publicado el ensamblaje 20, en el que se han podido anillar casi en su totalidad las secuencias de los supercontigs del ensamblaje 19 en los 8 cromosomas de *C. albicans*. Se ha realizado un mapa físico de todos los ORFs que se encuentra disponible en la página *http://albicansmap.ahc.umn.edu/* (University of Minnesota y Biotechnology Research Institute de Montreal). El DNA mitocondrial de *C. albicans* ha sido también secuenciado, estimándose un tamaño total de 40,420 pares de bases (Número de acceso GeneBank: AF285261). A partir del ensamblaje 19 varias organizaciones llevaron a cabo una anotación de las posibles secuencias de marcos de lectura abierta (ORFs) presentes en el genoma de *C. albicans.* Así, por

primera vez se hizo pública la base de datos CandidaDB (*http://www.pasteur.fr Galar_Fungail/CandidaDB*) creada por el consorcio europeo Galar Fungail Group, del cual forma parte nuestro grupo de investigación. Se consideraron ORFs relevantes para la anotación aquellas que codificaran una secuencia de más de 150 aa, que tuvieran un homólogo en las bases de datos públicas o tuvieran una probabilidad significativa de codificar una proteína según la predicción del programa GeneMark. Se identificaron 6244 ORFs, 130 de ellas codifican tRNAs. Se les dió nombre y función a aquellos ORFs que correspondían con genes previamente caracterizados o que por su elevado porcentaje de identidad con un gen de *S. cerevisiae* probablemente codificaran su homólogo funcional en *C. albicans*.

Características de las diferentes morfologías

C. albicans es un organismo polimórfico, dada su capacidad de crecer en forma de hifa, levadura, pseudohifa o dar lugar a la formación de clamidosporas. Las levaduras o blastosporas tienen forma elipsoidal con un diámetro aproximado de 5 μm, se dividen por gemación y son más abundantes durante el crecimiento saprófito del hongo [358]. Las hifas tienen forma cilíndrica con un diámetro aproximado de 2 μm, las paredes laterales se mantienen paralelas a lo largo de todo el filamento y no presentan constricciones en los septos [451]. Las pseudohifas consisten en cadenas de células alargadas que se originan mediante un proceso de gemación en el que no se llegan a separar la célula madre y la célula hija. A diferencia de las hifas, las paredes laterales de las pseudohifas no se mantienen paralelas, por lo que muestran valores de diámetro variable, son más gruesas (el diámetro mínimo se encuentra alrededor de 2.8 μm) y presentan constricciones en los septos [358,451]. Las clamidosporas son esporas asexuales con una pared gruesa que se forma mediante el recubrimiento de las células preexistentes, son redondeadas con un tamaño aproximado de 8 a 12 μm de diámetro y aparecen en posiciones laterales o terminales de hifas y pseudohifas, sobre células suspensoras alargadas. *In vivo* rara vez se detectan estas estructuras [70]. En el laboratorio (*in vitro*) su formación se induce en medios sólidos pobres en nutrientes, suplementados con detergentes, en condiciones microaerófilas y a temperaturas entre 20ºC y 30ºC, mientras que su formación se reprime en presencia de glucosa [51,353]. La capacidad de formar clamidosporas es una característica casi exclusiva de *C. albicans* solo compartida por *C. dubliniensis* y

algunas cepas de *C. tropicalis* [51,440]. En ocasiones las hifas y las pseudohifas son estructuras similares que resultan difíciles de diferenciar. Una forma clara de determinar si se trata de una u otra morfología consiste en visualizar el primer septo formado entre la levadura madre y el filamento. En el caso de las hifas este primer septo se forma dentro del tubo germinativo, mientras que en las pseudohifas se forma en el cuello de la célula madre [156,497]. Esta diferencia se debe a que en las hifas la elongación del tubo germinativo ocurre antes del inicio del ciclo celular, marcado por la síntesis de DNA, mientras que en las levaduras y pseudohifas la gemación depende del inicio del ciclo celular [182,451]. Las células de *C. albicans* también pueden dar lugar a la formación de biopelículas, se trata de comunidades de células organizadas en estrecha relación con la superficie sobre la cual se forman. En el caso de *C. albicans,* los biopelículas están constituidos por levaduras, hifas, pseudohifas y material extracelular (polisacáridos y proteínas) que se disponen de forma ordenada en capas. Generalmente las capas internas están constituidas mayoritariamente por levaduras mientras que en las más externas se encuentran principalmente formas filamentosas. El estudio de los biopelículas de *C. albicans* posee gran interés médico dada su aparición en epitelios, catéteres y prótesis y su marcada resistencia a antifúngicos [109,139].

Factores de virulencia de *C. albicans*

El espectro de afecciones causadas por *C. albicans* es superior al de la mayoría de patógenos oportunistas, lo que es atribuible a su capacidad para sobrevivir en diferentes tejidos del hospedador; cada uno de ellos asociado a un conjunto diferente de presiones ambientales, y a un amplio repertorio de factores de virulencia [53]. Se conoce poco acerca de la biología de *C. albicans* como comensal, tan solo se sabe que pequeñas variaciones en el sistema inmune del hospedador determinan la forma de crecimiento, por lo que la línea que separa la forma comensal de la patógena parece ser realmente delgada. El método en el que se han basado la mayor parte de los investigadores para considerar una determinada propiedad como factor de virulencia consiste en demostrar que las cepas carentes de dicha actividad no son capaces de establecer infecciones, pero si son capaces de vivir como comensales. Las etapas sucesivas del proceso de infección y los diferentes tipos de patologías causados por *C. albicans* están condicionadas por diferentes combinaciones de estos factores de virulencia [337].

Entre los factores de virulencia atribuidos tradicionalmente a *C. albicans* se encuentran la presencia en la superficie celular de proteínas de adhesión a tejidos del hospedador, la secreción de enzimas líticas y la capacidad de llevar a cabo la transición dimórfica [81]. El denominado cambio fenotípico espontáneo o *"switching"*, la capacidad de asimilación de hierro a partir de tejidos del hospedador y propiedades como el tigmotropismo y el mimetismo molecular constituyen otros posibles factores de virulencia de *C. albicans* aún poco caracterizados [53,135,337,498].

Mecanismos de regulación transcripcional

Características generales

La regulación de la expresión génica se realiza a diferentes niveles que se encuentran relacionados entre sí. En los promotores de todos los genes se encuentran secuencias reguladoras comunes que son reconocidas por la maquinaria de la RNA polimerasa II, tales como las secuencias situadas en el extremo 5´ denominadas "upstream activating sequences" (UAS) y las secuencias TATA. Además, es necesaria para la transcripción la presencia del elemento iniciador, que determina el punto donde comienza la transcripción y que se encuentra siempre en el extremo 3´ de la caja TATA, pero para el cual no se ha establecido una secuencia consenso. En determinados promotores se encuentran sitios de reconocimiento para factores de transcripción específicos, que activan o reprimen la transcripción en respuesta a diferentes estímulos. Uno de los mecanismos de regulación de la transcripción consiste en la modulación de la actividad y concentración de las proteínas que actúan como activadores y represores de la transcripción, produciéndose como consecuencia la expresión diferencial de los genes diana de estos reguladores. Muchos activadores actúan sobre las histonas y el DNA provocando fenómenos de reposicionamiento o desensamblaje de los nucleosomas, que afectan a la accesibilidad de la maquinaria de transcripción general.

La cromatina

El DNA es la molécula biológica que contiene la información genética de los organismos, y en las células eucariotas se aloja en el núcleo. Dadas las dimensiones del DNA, que es una molécula muy larga, es necesario plegarlo

previamente para almacenarlo en el espacio limitado del núcleo. Para ello las moléculas de DNA se asocian con diferentes proteínas compactándose en una estructura organizada y jerárquica denominada cromatina [176,503]. Esta es una estructura inherentemente represiva que dificulta los procesos en los que se requiere acceder a la secuencia de DNA. Las proteínas mayoritarias de la cromatina son las histonas, unas proteínas de baja masa molecular, ricas en aminoácidos básicos. Las histonas están muy conservadas en todos los organismos eucariotas [465], lo que sugiere que la estructura fundamental de la cromatina debió aparecer en un antecesor común, posiblemente con el objetivo de resolver el problema del empaquetamiento del DNA dentro del núcleo [429]. La cromatina no es una estructura estática y cerrada en la que histonas y otras proteínas se asocian al DNA con una función únicamente estructural, sino que estas proteínas son sometidas a diversos tipos de modificaciones y alteraciones, lo que se traduce en una relajación de la estructura de la cromatina. Así pues, la cromatina es una estructura dinámica que puede adoptar diferentes estados de compactación regulando el acceso a la información contenida en el DNA, y permitiendo la ejecución de procesos celulares pre-programados como son el ciclo celular, la diferenciación celular o la adecuación de la respuesta transcripcional para la adaptación a situaciones ambientales cambiantes. Procesos fisiológicos como la replicación, la transcripción o la reparación del DNA, también precisan que se pueda regular el grado de compactación de la cromatina. Las histonas internas son proteínas muy conservadas, pequeñas y ricas en lisinas y argininas, lo que les confiere un carácter fuertemente básico. Las masas moleculares de las histonas de *S. cerevisiae* son: H2A 14.28 kDa; H2B 14.24 kDa; H3 15.37 kDa y H4 11.37 kDa. Desde el punto de vista estructural, las histonas tienen dos dominios: el dominio globular de la región C-terminal y un dominio no estructurado de la región N-terminal. El dominio globular contiene el motivo 'histone fold', formado por unos 65 aminoácidos que se estructuran en una hélice α corta seguida de un giro/hoja *β*, una hélice α larga, otro giro/hoja β, y por último otra hélice α corta. Las tres hélices del dominio 'histone fold' de una histona encajan con las tres hélices del dominio 'histone fold' de otra histona. Este dominio participa en interacciones histona-histona y también en interacciones histona-DNA en el nucleosoma. Los extremos N-terminales también participan en la interacción con nucleosomas adyacentes [291]. Algunos de los residuos de las histonas son susceptibles de

sufrir modificaciones postraduccionales, tales como acetilación, metilación, fosforilación, ubiquitinación y sumoilación. Aunque generalmente se ha aceptado que estas modificaciones afectan principalmente a residuos localizados en los dominios N-terminales, recientemente se han identificado residuos de los dominios C-terminales que también sufren modificaciones postraduccionales [531]. Todas estas modificaciones participan en la regulación de la actividad de la cromatina. Además de las histonas internas, la cromatina también contiene la histona H1, que se dispone en el exterior de los nucleosomas. Existen diferentes variantes de la histona H1 que constituyen una compleja familia de proteínas y que pueden ser específicas de tejido o del momento del desarrollo [234]. Las variantes de H1 son parcialmente redundantes entre sí y pueden regular la actividad de la cromatina estabilizando la fibra de 30 nm y la posición de los nucleosomas, o alterando la accesibilidad de factores reguladores [49,136]. Además de todos estos participantes, también interviene una amplia variedad de proteínas no histona, poliaminas e iones metálicos divalentes en el establecimiento y regulación de la estructura de la cromatina.

Regulación de la actividad de la cromatina

Como se ha descrito anteriormente, la cromatina esta formada por la asociación de proteínas al DNA, lo que da lugar a una estructura de naturaleza represiva que inhibe procesos como la transcripción, la reparación y la replicación del DNA. Sin embargo la cromatina no es una estructura estática, sino variable y dinámica, que participa en la regulación de todos estos procesos. Existen diferentes tipos de complejos proteicos que modulan la estructura de la cromatina: complejos que modifican histonas químicamente, complejos que remodelan cromatina mediante el empleo de la energía del ATP, y complejos que modifican el DNA. Además la constitución de nucleosomas con variantes de histonas, diferentes de las histonas canónicas, confiere diferentes propiedades estructurales a la cromatina.

Modificaciones postraduccionales de las histonas

Las primeras modificaciones postraduccionales identificadas sobre las histonas se localizaron en residuos de los extremos N-terminales. Se ha asignado, por ello, una especial atención a estas regiones, relacionándolas con la regulación de la actividad de la cromatina. Sin embargo, recientemente se han identificado diversas

modificaciones postraduccionales sobre residuos localizados en la región globular de las histonas [136,531]. La acetilación es la modificación postraduccional de histonas más estudiada, ya que es la que se conoce desde hace más tiempo. La acetilación de lisinas neutraliza la carga positiva del aminoácido y desde su descubrimiento se ha pensado que esto podría disminuir la fuerza de su interacción con el DNA, afectando así a la estructura de la cromatina. La acetilación está catalizada por enzimas histona acetiltransferasa (HAT) y puede ser eliminada por histona desacetilasas (HDAC).

Acetilación

La acetilación de lisinas es la modificación postraduccional de histonas de la que se han realizado un mayor número de estudios en una gran diversidad de organismos eucariotas, desde la levadura hasta plantas y humanos. Desde siempre, se ha pensado que cuando las lisinas no están acetiladas y por tanto tienen una carga positiva, se favorece la interacción de los extremos N-terminales con el DNA generándose una cromatina más compacta y reprimida. La acetilación de lisinas, al eliminar la carga positiva, debilitaría la interacción de los extremos N-terminales con el DNA provocando la relajación de la estructura de la cromatina. Por tanto, aunque no siempre es cierto, generalmente se ha relacionado la acetilación de histonas con la expresión génica, de manera que las histonas hiperacetiladas se encuentran asociadas con regiones genómicas transcripcionalmente activas, mientras que la desacetilación de histonas da lugar a la represión y al silenciamiento [252,474]. Sin embargo, actualmente se sabe que la acetilación de histonas está relacionada con muchos otros procesos fisiológicos, además de la regulación de la transcripción, como la progresión del ciclo celular, recombinación y reparación del DNA, apoptosis, elongación de la transcripción o el ensamblaje de nucleosomas [252,304].

Histona acetiltransferasas

En la cromatina de los organismos eucariotas el DNA se organiza alrededor de octámeros de histonas, dando lugar a la estructura nucleosomal. Las histonas sufren una serie de modificaciones postraduccionales entre las que se encuentra la acetilación de residuos de lisina, situados en los extremos N-terminales de estas proteínas. Esta modificación reversible, catalizada *in vivo* por las histona

desacetilasas (HD) y las histona acetiltransferasas (HAT), ha sido implicada durante muchos años en la regulación de diversos procesos celulares de gran importancia. Recientemente, el papel de la acetilación de histonas en la regulación de la transcripción ha cobrado gran importancia con el descubrimiento de que proteínas con actividad HAT y HD son reguladores transcripcionales.

En la levadura *Saccharomyces cerevisiae* se han identificado genes que codifican para cuatro proteínas con actividad acetiltransferasa*, GCN5, HAT1, ESA1* y *TAF130* que ya se habían detectado previamente por métodos bioquímicos

La acetiltransferasa de histona de *C. albicans GCN5*

El coactivador transcripcional de levadura *GCN5* forma parte de un gran complejo multimérico que es requerido para el remodelamiento de la cromatina y la activación transcripcional. *GCN5* está conservado en las especies de *Candida* y en la mayoría de las regiones homólogas forman parte del dominio HAT y el bromodominio (Figura 1.7 y 1.8). Muchos genes de levadura codifican proteínas acetiltransferasas de histonas y desacetilasas de histonas en *C. albicans* y su fenotipo relativo es totalmente desconocido. En nuestro laboratorio se han diseñado un análisis fenotípicos de cepas de levadura que contienen deleciones en *GCN5* y otros genes que codifican para miembros de las familias HATs y HDACs de *C. albicans.* El genoma de *C. albicans* contiene seis ORFs que potencialmente codifican proteínas desacetilasas de histonas conteniendo el dominio HDACs y cinco acetiltransferasas de histonas. Una búsqueda en la base de datos de la NCBI (*http://www.ncbi.nlm.nih.gov/BLAST/*) indica que una proteína de *Candida albicans* llamada *Gcn5* (orf19.705) comparte la mayor similitud con *Pichia guilliermondii* ATCC 6260 (81%), *Pichia stipitis* (78%), *Lodderomyces elongisporus* (70%), *Debaryomyces hansenii* (73%) y *Saccharomyces cerevisiae* (70%) (Figura 1.8). El número de acceso en el GeneBank para la secuencia nucleotídica de la *GCN5* de *C. albicans* es NW_139602.

La proteína Gcn5p de levadura, ancestro de la familia de las acetiltransferasas de histona (HAT), transfieren un grupo acetilo desde el acetil-coenzima A al grupo ε-amino de la cadena lateral de residuos de lisina específicos de histonas H3 y H4 [392,516]. Gcn5p no actúa por cuenta propia, sino como la subunidad catalítica de dos complejos separados y altamente conservados llamados ADA y SAGA (Spt-Ada-Gcn5-acetiltransferasa). Las proteínas Spt se encuentran exclusivamente en

el complejo SAGA, Spt20p es necesaria para el mantenimiento de la integridad y la función del complejo entero [517]. La acetilación de histonas por la proteína Gcn5p está implicada en el desplazamiento de los nucleosomas de los promotores durante la activación transcripcional y también en ayudar al reclutamiento de la proteína de unión TATA, la RNA polimerasa II y coactivadores [154]. La acetilación por lo tanto facilita la formación de una estructura de cromatina "abierta" correspondiente al genoma transcribiendose [99,251,449]. Durante la división celular, la remodelación de la cromatina se extiende a lo ancho de las regiones cromosomales, produciendo grandes longitudes de compactación y recondensación a lo largo de todo el genoma en cada división celular [93,489]. Perturbar el equilibrio entre la HAT y la desacetilasa de histona altera las actividades de las proteínas a una escala celular, lo cual conduce a varias enfermedades, incluyendo el cáncer. Varios trabajos han resaltado la participación de Gcn5p sola o en combinación con otras HATs tales como Sas3p en el ciclo celular [199,223] y en la regulación transcripcional de un grupo de genes requeridos al final de la telofase [247]. En células de mamíferos, la pérdida del homólogo Gcn512p es letal durante la embriogénesis, induce un alto nivel de apoptosis [519], y afecta la transición G2/M en mutantes nulas de células madre en embriones de ratón [277]. En levaduras, el nucleosoma centromérico especializado, el cual contiene la proteína Cse4p variante de la histona H3 [318], es necesaria para el ensamblaje y las interacciones de los componentes internos del cinteocoro en el centrómero [72] y para la correcta fijación de los cromosomas al huso en la metafase. Mutaciones en los componentes del centrómero/cinetocoro o modificaciones epigenéticas de esta estructura pueden dar lugar a una incorrecta segregación del cromosoma y en el retraso de G2/M. En la levadura la Gcn5p está físicamente relacionada con el centrómero. Se ha descrito regulación epigenética dependiente de Gcn5p en el centrómero/cinetocoro en mitosis [488].

Histona desacetilasas

Las histona desacetilasas (HDACs) son enzimas hidrolíticas capaces de revertir la acetilación, de manera que los niveles estacionarios de acetilación de las histonas depende del balance de las actividades HAT y HDAC. Al igual que para las HATs, también se ha descrito que las HDACs pueden desacetilar proteínas no histona como p53, E2F, α-tubulina y MyoD [209,521]. La clasificación de los diferentes

tipos de HDACs es confusa, pero generalmente se aceptan cuatro clases de HDACs: las tipo Rpd3p o clase I; las tipo Hda1p o clase II; las tipo Sir2p dependientes de NAD^+ y las tipo HD2 [292]. Rpd3p y Hda1p son desacetilasas de acción global que afectan a muchos promotores y regulan así la transcripción de muchos genes [386]. Las proteínas Hos1p y Hos2p (*HDA one similar*) de levaduras pertenecen a la subfamilia de enzimas tipo Rpd3p. Hos3p es una HDAC que a pesar de haberse identificado en diferentes organismos, no encaja exactamente ni en el grupo de HDACs tipo Rpd3p ni en el tipo Hda1p. Por este motivo unas veces se ha clasificado como una HDAC de clase II y otras se ha situado entre las dos clases, aunque en realidad no parece correlacionar bien con ninguna de las dos categorías. Característico de estas enzimas a la resistencia a inhibidores como la Tricostatina A (TSA), que afectan a HDACs de clase I y II [55].

Las histona desacetilasas participan en la regulación de la transcripción génica, pero también están implicadas en la regulación de otros procesos como el ciclo de división y la proliferación celular y el desarrollo de cáncer, lo que ha dado lugar a una búsqueda y caracterización exhaustiva de inhibidores de HDACs [414]. Al igual que otros complejos implicados en la regulación de muchos procesos celulares vitales, las actividades HDACs se encuentran finamente reguladas por múltiples mecanismos. La actividad desacetilasa de las proteínas HDAC depende, entre otras cosas, de interacciones proteína-proteína específicas, de manera que las subunidades catalíticas requieren otras subunidades o cofactores para ser activas (la mayoría de las enzimas recombinantes son inactivas). Además las HDACs son reguladas por modificaciones postraduccionales, a nivel de expresión y por disponibilidad de cofactores. Por último cabe resaltar la importancia de la regulación a nivel de localización subcelular.

En relación con la desacetilación específica, se ha descrito que el complejo *HDA1* de *S. cerevisiae* es dirigido a través del represor general ScTup1p al promotor del gen *ScENA1,* gen que codifica una ATPasa de membrana involucrada en el transporte de sodio desde el citoplasma al exterior celular. *In vitro,* se ha demostrado que ScTup1p interacciona con ScHda1p y ScHda3p pero no con ScHda2p. El anclaje de ScTup1p a secuencias específicas del promotor de *ScENA1* (URS, *upstream repressor sequences*) es llevada a cabo a través de las proteínas de unión a DNA ScSko1p y ScMig1p [515]. La actividad de ScHda1p en el promotor de *ScENA1* causa la desacetilación local de la cromatina en una región

que incluye los dos nucleosomas situados aguas abajo del sitio de unión del complejo [515]. Otro de los genes cuya expresión se encuentra alterada en el mutante nulo *Schda1* es *ScFLO11,* gen que codifica una proteína de la superficie celular relacionada con adhesividad y formación de pseudohifas. La represión de este gen depende de su propio promotor y de la posición que ocupa en el genoma, dado que su inserción en otra región resulta en la pérdida de la regulación. ScHda1p podría relacionarse con la regulación a nivel del promotor de este gen, teniendo en cuenta que ScTup1p es dirigida al promotor de *ScFLO11* por medio de la proteína de unión a DNA ScSfl1p y se ha demostrado que ScTup1p puede interaccionar físicamente con ScHda1p. Basándose en estas observaciones se ha propuesto que ScSfl1p podría dirigir a ScTup1p junto a ScHda1p al promotor de *ScFLO11* [173]. Además se ha sugerido que ScTup1p podría dirigir a ScHda1p a promotores de otros genes necesarios para los procesos de conjugación, utilización de glucosa y oxígeno, respuesta a estrés osmótico y a daños en el DNA [524]. En *S. cerevisiae* se ha estimado que aproximadamente el 30% de los genes reprimidos por ScTup1p son reprimidos parcialmente por ScHda1p [165]. Como se ha indicado anteriormente, ScTup1p puede llevar a cabo su papel como represor por otros mecanismos diferentes a la interacción con histona desacetilasas, como la interacción directa con la maquinaria de transcripción o la modificación de la posición de los nucleosomas. Estos mecanismos parecen solaparse en algunos genes, dado que se ha demostrado que al combinarse las mutaciones en genes que intervienen en cada uno de los tres procesos se detecta desrepresión de genes que no se encontraban en los mutantes sencillos y que si se encuentran desreprimidos en ScTup1p [165,515,538].

Desacetilación global por ScHda1p.

Al igual que en el caso de otras HDACs, se ha demostrado que ScHda1p participa en la desacetilación de regiones amplias de la cromatina y en la desacetilación inespecífica a lo largo de todo el genoma [252].

La utilización de microarrays de acetilación ha permitido definir dominios subteloméricos del genoma de *S. cerevisiae* que se ven afectados por la actividad de ScHda1p y que se han denominado regiones HAST (Hda1p-affected subtelomeric domains) [386]. Estos dominios son regiones continuas de cromatina (entre 4 y 34 kb) que se encuentran cerca de los extremos de la mayoría de los

cromosomas, adyacentes a la heterocromatina telomérica [252].

Las regiones subteloméricas afectadas por ScHda1p contienen una elevada proporción de genes relacionados con el metabolismo de carbohidratos, especialmente genes que intervienen en gluconeogénesis y crecimiento en condiciones adversas como escasez de nutrientes, choque osmótico o baja concentración de oxígeno en el medio. Dichos grupos de genes se encuentran habitualmente reprimidos en medios ricos por mecanismos que involucran a ScTup1p y su expresión se libera en condiciones de estrés nutricional [118,252,386,524,525].

Dentro de las regiones HAST se encuentra el gen *ScFLO11*, que como indicábamos anteriormente, es un gen cuya represión requiere ScHda1p. La relación entre ScHda1p y la expresión de *ScFLO11*, además de explicarse a través de interacciones con ScTup1p y con el promotor del gen, podría explicarse a través de la acción de ScHda1p sobre todo el dominio HAST. Esta observación está de acuerdo con el hecho de que el silenciamiento de *ScFLO11*, además de ser dependiente del promotor es dependiente de su localización en el genoma [173].

Se ha observado así mismo que la deleción de *ScHDA1* causa un incremento de acetilación en una región de 4.25 kb que incluye el gen *ScPHO5*, a cuyo promotor ScHda1p no esta específicamente dirigida. En ausencia de la histona desacetilasa ScHda1p, el gen *ScPHO5*, una vez que desaparecen las condiciones que inducen su expresión, muestra una cinética de reversión al estado de represión mas lenta que la cepa silvestre, por lo que se ha propuesto que una de las posibles funciones de la desacetilación global por ScHda1p es permitir una rápida conversión al estado de represión [489].

Por último, ScHda1p interviene en la desacetilación de histonas a lo largo de todo el genoma, acción global que se ha sugerido podría ser importante para mantener los niveles de expresión basal [489,524]. Se ha observado que ScHda1p se une a regiones codificantes que están preferentemente hipometiladas en el residuo lys 4 de la histona H3 y se ha demostrado que existe correlación entre la metilación de estos genes y la actividad transcripcional, por lo que se ha propuesto que la metilación en estas regiones podría favorecer la transcripción al impedir la unión de los complejos de desacetilación [31]. Por otro lado, se ha observado en diferentes especies que la desacetilación del aminoácido lys 9 de la histona H3,

llevada a cabo por la proteína homóloga a ScHda1p, estimula la metilación de DNA, favoreciendo la formación de la heterocromatina, donde la transcripción se mantiene silenciada [341,524].
Dado que *in vitro* ScHda1p no tiene actividad histona desacetilasa por si misma, es probable que también ScHda2p y ScHda3p intervengan en los procesos de desacetilación global.

Poliaminas

Las poliaminas putrescina, espermidina y espermina son compuestos nitrogenados alifáticos que actualmente se consideran como reguladores del crecimiento y desarrollo tanto en plantas, animales y microorganismos por su efecto demostrado sobre el crecimiento, la división y la diferenciación celular a bajas concentraciones. Por su carácter policatiónico pueden unirse a moléculas cargadas negativamente tales como ácidos nucleicos, proteínas o fosfolípidos, alterando la expresión génica y la actividad de ciertos enzimas, así como variando la fluidez y la permeabilidad de las membranas biológicas. En algún caso, las poliaminas actúan como reserva de nitrógeno, constituyendo la única fuente del mismo.
La participación fisiológica de las poliaminas ha sido conocida mediante la realización de estudios en células incapaces de biosintetizar poliaminas, empleando inhibidores de la síntesis de estos compuestos y usando animales de laboratorio, como ratones transgénicos.[491]. Con estas investigaciones se ha comprobado que las poliaminas son compuestos indispensables para el crecimiento y el buen funcionamiento celular. Las propiedades de las poliaminas se deben principalmente a que cuando se encuentran en pH fisiológico los grupos amino están cargados positivamente, lo que les permite interactuar con grupos aniónicos de las proteínas, de los ácidos nucleicos y de los fosfolípidos, para proporcionarles estabilidad en su estructura [66]. La ornitina descarboxilasa (ODC), es una enzima presente en todos los organismos, participa en procesos de transición dimórfica, de diferenciación celular y crecimiento celular [170,221]. El análisis de su secuencia de aminoácidos muestra zonas altamente conservadas en organismos eucarióticos incluyendo a los hongos, es la enzima clave del metabolismo de las poliaminas y está altamente regulada transcripcional, traduccional y postraduccionalmente [468]. El inhibidor 1,4-diamino-2-butanona (DAB) es ampliamente utilizado para abatir la actividad de la ODC inhibiendo la

transición dimórfica levadura-hifa. Estos datos han sido reportados para *Candida albicans*, *Mucor rouxii*, *Yarrowia lipolytica*, *Paracoiccidiodes brasiliensis* y *Ustilago maydis* [170,310,402]. La delecion del gen que codifica para la ornitina descarboxilasa *ODC1* en *C. albicans* habia sido previamente realizado en nuestros laboratorio [188]. En el nuestro trabajo se demostro que en ausencia de Odc1p en *C. albicans* se puede modular la transición levadura-hifa modulando nivel de poliaminas bajo 0.01 mM o alto 10 mM [188].

Objetivos

La plasticidad morfológica de *C. albicans* constituye un importante factor de virulencia de este patógeno fúngico. La transición levadura-hifa es además un proceso de especial interés para constituir un modelo de diferenciación celular. Las diferentes rutas de señalización que regulan la formación de hifas en *C. albicans* han sido ampliamente estudiadas. Sin embargo, no se ha determinado aún como se producen los cambios en la expresión génica en el contexto de la cromatina durante la transición dimórfica, así como tampoco se ha podido establecer claramente la unión entre las rutas de transducción de señales y los procesos de elongación celular. Considerando que los procesos de acetilación y desacetilación de histonas constituyen uno de los mecanismos de regulación de la expresión génica en organismos eucariotas, nos planteamos los siguientes objetivos:

1. Analizar el perfil de transcripción de la cepa CAI4 durante la transición levadura-hifa.

2. Determinar bajo diferentes condiciones de inducción si la histona acetiltransferasa Gcn5p y la histona desacetilasa I Hda1p de *C. albicans*, son necesarias para el desarrollo de procesos morfogenéticos tales como son la formación de hifas, pseudohifas y clamidosporas.

3. Analizar el papel de Gcn5p y Hda1p el doble mutante en la regulación de la expresión de genes que codifican factores de transcripción claves en el proceso de transición levadura-hifa.

4. Analizar el perfil de transcripción del mutante *odc1/odc1* durante el crecimiento de forma de levadura y durante la transición levadura-hifa a bajas (0.01 mM) y altas (10 mM) concentraciones de putrescina.

5. Determinar bajo diferentes condiciones de inducción si las histonas acetiltransferasas de *C. albicans*, CaGcn5p y CaHat1p, son necesarias para el desarrollo de procesos morfogenéticos, como son la formación de hifas, pseudohifas y clamidosporas.

6. Analizar el perfil transcripcional del mutante doble *gcn5, hat1* durante el crecimiento de forma de levadura.

Materiales y Métodos

Medios de cultivo y condiciones de crecimiento

Medio de cultivo para *E. coli*

Las bacterias se crecieron en medio Luria-Bertani o LB 1 % (p/v) bactotriptona, 0.5 % (p/v) extracto de levadura, 1% (p/v) NaCl.

Medios de cultivo para *C. albicans*

Los medios de cultivo empleados para el crecimiento de levaduras fueron: medio rico o YEPD 1% (p/v) extracto de levadura, 2% (p/v) bactopeptona, 1% (p/v) glucosa]; medio mínimo sintético o YNB 0.67% (p/v) Yeast Nitrogen Base sin aminoácidos, 1% (p/v) glucosa. Los medios de cultivo sólidos se prepararon de la misma manera, pero añadiendo agar al 2 % (p/v). Los cultivos en medio líquido de *C. albicans* se realizaron en matraces Erlenmeyer. El volumen del medio fue siempre inferior a un tercio del volumen del matraz. Las incubaciones se llevaron a cabo en agitadores orbitales a 200 rpm y a 28ºC. El crecimiento se determinó midiendo la absorbancia en un espectrofotómetro a una longitud de onda de 600_{nm}, o mediante el recuento de células en una cámara Thoma. De forma rutinaria, para los inóculos se estimó la cantidad de células considerando la correspondencia entre un valor de D.O. de 0.4 con una concentración de 1×10^7 células/ml.

En todos los casos las células en los medios sólidos utilizados para la inducción de crecimiento filamentoso se sembraron a razón de 50 ufc por placa y se incubaron a 37ºC, excepto en el caso del medio embedded en el que la temperatura de incubación fue de 28ºC.

El medio empleado para la inducción de clamidosporas fue cornmeal agar al 1.7% (Difco). Después de disolver y autoclavar el medio se añadió Tween 80 (Sigma) hasta una concentración final del 0.33%. Las células de las diferentes cepas de *C. albicans* se sembraron en estrías, sobre ellas se dispusieron cubreobjetos con el fin de crear condiciones de crecimiento microaerófilas. Las placas se incubaron en oscuridad a 25ºC, durante 14 días. Se tomaron muestras de cada una de ellas a los 7 y 14 días de crecimiento. Para mejorar la visualización de las clamidosporas al microscopio óptico, las muestras se sometieron a una tinción con lactofenol-azul algodón. Las cepas obtenidas y empleadas en el presente trabajo se conservaron mediante resiembras periódicas en placas de medio sólido. Las placas inoculadas se incubaron a 28ºC durante 48 horas en el caso de *C. albicans* y a 37ºC durante

12 horas en el caso de *E. coli*. Posteriormente se conservaron a 4ºC. Con la finalidad de mantener las células durante largos periodos de tiempo, los microorganismos se almacenaron en viales con glicerol al 30% en ultracongeladores a -80ºC. En el presente trabajo se llevó a cabo la transformación de *C. albicans* mediante la adición de DNA lineal o DNA plasmídico a protoplastos de esta levadura, que se obtuvieron de forma previa a cada transformación. Dado que en todos nuestros experimentos de transformación se utilizó el gen *CaURA3* como marcador de selección, las cepas a transformar fueron siempre Ura^- (CAI4 y derivadas).

Detección de secuencias de DNA específicas (Southern-blot)

Una vez digerido el DNA genómico de *C. albicans* (15-20 μg) con las enzimas de restricción adecuadas, los fragmentos de DNA se separaron, junto a un marcador de peso molecular, mediante electroforesis en geles de agarosa al 1%. El DNA separado se visualizó por la incorporación en el gel de bromuro de etidio y se fotografió junto a una regla con el fin de tener una referencia que permitió posteriormente determinar el tamaño de las bandas hibridadas. La hibridación se realizó en una solución de igual composición a la de prehibridación y a la que se añadió la sonda radiactiva previamente desnaturalizada. La membrana se dejó hibridando durante aproximadamente 16 horas a 65ºC. A continuación se realizaron varios lavados de tiempo variable con 100 ml de las soluciones de los lavados. Los filtros se analizaron con el sistema de detección de radiación, captura y análisis de imágenes BAS-1500 Storage Phosphor Imaging System (Fujifilm). Para ello cada membrana se expuso en oscuridad a una pantalla del sistema (cubierta por una capa de fósforo foto-estimulable), durante un período de tiempo que osciló entre 1 y 12 horas. Posteriormente la superficie de la pantalla fue escaneada y se obtuvo una imagen que reveló la impresión causada por la radiación, permitiendo estimar el tamaño de los fragmentos de DNA que habían hibridado específicamente con la sonda.

Análisis de la sensibilidad a compuestos relacionados con estructura de la pared celular

A partir de células cultivadas hasta fase exponencial, se prepararon suspensiones a una $D.O._{600\ nm}$ de 0.4 y a partir de ellas se realizaron tres diluciones seriadas de

1/10, 1/100 y 1/1000. Se tomaron 5 μl de cada una de las diluciones y se sembraron en placas de medio mínimo conteniendo diferentes concentraciones de los compuestos a analizar: Higromicina; Itraconazol, Flocunazol, NaCl, Calcofluor white, Cafeína y SDS. Las diferencias en el crecimiento de las distintas cepas se valoraron después de 72 horas de incubación a 28ºC. El tiempo de incubación se prolongó en determinados casos con el fin de apreciar mejor la variación en el crecimiento.

Ensayo de afinidad a Alcian blue

El Alcian blue es un colorante catiónico no fluorescente que se une específicamente a las cadenas de fosfomanosas presentes en la superficie celular de las levaduras [20,189]. Este ensayo permite detectar cambios en la síntesis y secreción de las manoproteínas que forman parte de la pared celular. Las células recogidas en fase exponencial se lavaron 2 veces con HCl 0.02 N y a continuación se prepararon 3 diluciones de 1 ml a una D.O. de 2.5 de cada una de las cepas. Las muestras se centrifugaron resuspendiendo las células en 1.5 ml de una solución de Alcian blue 0.005%. Tras 10 minutos de incubación a temperatura ambiente, las muestras se centrifugaron 3 minutos a la máxima velocidad y seguidamente se midió la absorbancia a 600_{nm} de los sobrenadantes. La concentración de Alcian blue de cada sobrenadante se determinó por comparación con una recta patrón que permitió relacionar la concentración de colorante con la absorbancia y cuyo coeficiente de correlación fue 1.00. A partir de esta concentración se estimó el porcentaje fijado por cada cepa considerando como 100% la concentración de Alcian blue del sobrenadante de una muestra tratada de la misma forma pero en la que no se incluyeron células.

Ensayo de virulencia

Se prepararon cultivos de cada una de las cepas de *C. albicans* a ensayar en 100 ml de YEPD a una D.O. inicial de 0.03. Se incubaron durante aproximadamente 12 horas a 28ºC con el fin de obtener células en forma de levadura y en fase exponencial de crecimiento. Las células se recogieron, se lavaron dos veces con PBS 1× y se resuspendieron a una concentración de 5×10^8 células/ml. La concentración celular de la suspensión final se verificó en una cámara Thoma y por absorbancia a 600_{nm}. A partir de dicha suspensión se inocularon por vía

intraperitoneal 10 ratones CD1 (machos de aproximadamente 20 g de peso) con 5×10^8 células de *C. albicans* cada uno. A partir del día de infección se realizó un seguimiento diario de la supervivencia de los ratones infectados que se prolongó hasta el día 30.

Comparación de perfiles de transcripción mediante microarrays de cDNA. Aislamiento de RNA total

Las células se recogieron por centrifugación a 3000 rpm durante 5 minutos a temperatura ambiente, los sobrenadantes se eliminaron mientras que el precipitado de células se resuspendió en el volumen residual de medio que quedó en el tubo de centrifugación. Seguidamente las células se congelaron dejando caer gotas de aproximadamente 20 μl en eppendorfs con nitrógeno líquido. Los tubos se mantuvieron en un recipiente con nitrógeno líquido para evitar la descongelación de las muestras, o bien se guardaron a -80ºC hasta su utilización. La lisis celular se llevó a cabo de forma mecánica dentro de una cámara de teflón en la que se introdujo, junto a la muestra, una esfera de tungsteno (7 mm de diámetro) que se sometió a agitación en un Micro-Dismembrator (Braun, Melsungen).

Marcado, síntesis de cDNA e hibridación

Para el marcado con Cianina 3-dCTP o Cianina 5-dCTP (fluorocromos que absorben y emiten fluorescencia a diferentes longitudes de onda) se siguió el método de marcaje directo según el protocolo proporcionado por la casa comercial Eurogentec y en el que se introdujeron pequeñas modificaciones. De forma general puede decirse que se trata de una reacción de retrotranscripción en la cual se incorporan los nucleótidos marcados durante la síntesis de las moléculas de cDNA. En cada reacción se utilizaron 15 μg de RNA total a los que se añadió 1 μl del inhibidor de ribonucleasas RNAsin (Promega) y agua destilada tratada con DEPC hasta completar un volumen de 21 μl. Las soluciones de RNA se dejaron en un baño a 42ºC mientras se mezclaron el resto de componentes de la reacción 5x First-strand buffer (Invitrogen) (8 μl), *C. albicans* specific primer mix (Eurogentec) (0.1 pmoles/μl) (1μl), Oligo dT (12-18) (Sigma) (0.5 μg/μl) (1 μl), Mezcla de dNTPs excepto dCTP (Sigma) (10 mM) (3 μl), dCTP (Sigma) (1 mM) (1 μl), Cianina 3-dCTP o Cianina 5-dCTP (1 mM).(CyDye TM Fluorescent nucleotides Cy3 o Cy5,

Amersham) (1.5 μl), DTT (0.1 M) (Invitrogen) (4 μl). La mezcla se realizó en eppendorfs cubiertos con papel de aluminio para evitar la exposición a la luz de los fluorocromos. La solución de RNA se añadió a la mezcla de reactivos y seguidamente se incubó 5 minutos a 65ºC y 5 minutos a 42ºC. Se añadió 1 μl de transcriptasa reversa (Invitrogen) y se incubó 3 horas a 42ºC, añadiendo 1 μl adicional de enzima transcurrido la mitad del tiempo de incubación. La reacción se detuvo añadiendo 5 μl de EDTA pH 8.0 y 2 μl de NaOH 10 N e incubando la muestra 20 minutos a 65ºC. A continuación, el pH se bajó con la adición de 4 μl de una solución de ácido acético 5 M. La purificación de las moléculas de cDNA sintetizadas se llevó a cabo utilizando el kit de purificación de productos de PCR QIA-kit PCR purification kit (Quiagen) siguiendo el protocolo descrito por la casa comercial. Después de dos pasos de elución con 50 μl de agua destilada cada uno, se cuantificó el cDNA sintetizado, la cantidad de fluorocromo incorporado y la frecuencia de incorporación del mismo según las fórmulas que se indican a continuación: Dada la baja concentración de cDNA que se obtiene, la absorbancia de la muestras se midió directamente, sin diluciones, utilizando ultramicrocubetas (Hellma). Considerando que Eurogentec advierte que no deben usarse muestras con una f.o.i $\leq$ 10 y que los valores óptimos se encuentran entre 20 y 50, en nuestros experimentos decidimos usar solo aquellas muestras con una f.o.i. $\geq$ 16, con el fin de asegurar buenos resultados. El volumen de muestra correspondiente a 65 pmoles de fluorocromo se concentró utilizando filtros microcon-30 (Amicon) hasta conseguir un volumen final de 5 a 10 μl. Para cada hibridación se mezclaron las muestras de cDNA procedentes de cada una de las dos cepas a comparar (marcada una con Cy3 y otra con Cy5) y 5 μl de DNA de esperma de salmón (Sigma-Aldrich) previamente desnaturalizado por calor. El cDNA se desnaturalizó manteniendo la muestra 2 minutos a 95ºC y enfriándola inmediatamente en hielo, seguidamente se añadió tampón de hibridación (Dig Easy Hybridization buffer, Roche) hasta un volumen final de 60 μl. La mezcla se depositó entonces sobre un cubreobjetos de plástico (60×24 mm, Sigma-Aldrich), el microarray se puso en contacto con la muestra por la cara que contenía el DNA y una vez que la muestra se extendió por aproximadamente la mitad del cubreobjetos se dio la vuelta al microarray y se colocó en una cámara de hibridación (Corning), que se sumergió en un baño a 42ºC. Después de 12 horas de hibridación, se retiró el cubreobjetos introduciendo el microarray en una cubeta con la primera solución de lavado (SDS

1%/SSC 2×), se transfirió a un tubo falcon con la misma solución donde se mantuvo 5 minutos a temperatura ambiente con agitación ocasional y protegido de la luz. Para eliminar los restos de SDS, el microarray se pasó sucesivamente por dos tubos falcon con SSC 2×, en el último de ellos se mantuvo 5 minutos con agitación ocasional. Por último el microarray se secó mediante centrifugación a 2000 rpm durante 5 minutos a temperatura ambiente. Los microarrays de *C. albicans* utilizados en el presente trabajo fueron sintetizados por la empresa Eurogentec en colaboración con el consorcio europeo Galar Fungail Group (*www.pasteur.fr/recherche/unites/Galar_Fungail*), al cual pertenece nuestro grupo de investigación. El contenido y la forma de fabricación se describen a continuación y pueden encontrarse en la página *www.eurogentec.com*. La información sobre las secuencias codificantes se obtuvo de la base de datos CandidaDB (*www.pasteur.fr/recherche/unites/GalarFungail/CandidaDB*) desarrollada por el consorcio Galar Fungail Group partiendo de la secuencia completa del genoma de la cepa SC5314 de *C. albicans* publicada por el Stanford Genome Technology Center [96]. A partir de dichas secuencias se diseñaron los oligonucleótidos que permitieron amplificar una región específica de cada ORF, generalmente correspondiente a la región 3´ de la ORF del gen. Se ha llevado a cabo la comparación de los perfiles de transcripción de dos mutantes de *C. albicans* con el de la cepa parental, CAI4, en cuatro condiciones de crecimiento: fase exponencial como levaduras, y a tres tiempos (15, 60 y 180 minutos) de la inducción de la formación de hifas, en medio Lee a 37ºC. Para cada comparación mutante *vs.* CAI4 se han realizado cuatro hibridaciones correspondientes a dos replicados biológicos, en los que el RNA de la cepa mutada procede de cultivos independientes, y dos hibridaciones para cada uno de ellos en los que el cDNA se marcó de forma inversa (*dye swap*). El cDNA de la cepa parental, CAI4, se sintetizó en cada condición a partir de una mezcla de RNA obtenido de diferentes cultivos de esta cepa provenientes de tres colonias. Considerando que para cada comparación se hibridaron 4 microarrays y que cada gen se encuentra depositado por duplicado en cada microarray, para la mayoría de los genes se analizaron 8 datos para cada condición.

Resultados

Respuesta de la cepa CAI4 de *Candida albicans* a la temperatura

Uno de los parámetros involucrados en transición levadura-hifa es la temperatura y su cambio esta implicado a patogénesis.

El análisis transcripcional de *C. albicans* en respuesta a la transición levadura-hifa después de 15, 60 y 180 min de exposición a medio Lee a 37ºC fue llevada a cabo para identificar los genes expresados diferencialmente en la cepa CAI4. Por el método de microarrays de cDNA, se encontraron 341, 405 y 251 genes expresados diferencialmente en cada uno de los tiempos analizados. El nombre y la función de cada uno de ellos se han incluido en las Tablas 2.1-2 del material suplementario. En conjunto se detectó que la expresión de 783 genes (403 sobre- y 380 sub-expresados) es afectada por cambio de la temperatura en al menos uno de los tres tiempos de inducción analizados. La comparación de los genes expresados diferencialmente en cada uno de los tiempos mediante diagramas de Venn señala que los resultados de expresión diferencial varían a lo largo de la inducción (Figura 2.3). Como se ha señalado en le Figura 2.2, se ha detectado claramente la expresion de un número significativo de genes después de 60 minutos, lo que sugiere que la mayoría de las señales y los genes reguladores que participan en la morfogenesis realiza su función rápidamente. Se observó una buena correlación estadística en nuestros resultados 43 genes son comunes a los tres tiempos ensayados (24 sobre- y 19 sub-expresados), 68 genes en los 15 y 60 min (31 sobre- y 37 sub-expresados) y 52 genes en 60 y 180 min. (40 sobre- y 12 sub-expresados). 222 genes (75 sobre- y 147 sub-expresados), 240 genes (155 sobre- y 85 sub-expresados), y 148 genes (74 sobre- y 74 sub-expresados) solo varían en un tiempo de inducción (15, 60 y 180 min. respectivamente). Sin embargo, únicamente 8 genes eran comunes entre los 15 y 180 min lo que muestra la coherencia de nuestro experimento. Los 43 genes comunes a los tres tiempos se describen en la Tabla 2.1. entre ellos se ha detectado la expresión diferencial de varios genes relacionados con pared celular, como *HWP1* [443]; *ECE1* [32]; *PHR1* [131], *RBT1* [434], *YWP1*[159] y *PIR1* [309]. Todos estos genes se han descrito anteriormente como sobre y sub-expresados en niveles similares [226,342] y están reguladas por Efg1p y Tup1p [108,226,434]. En el resto de las categorías funcionales las más representativas son transporte con 9 genes, incluido dos de transporte de oligopéptidos, oxidasa/peroxidasa (4 genes), glicólisis

(3 genes), y dehidrogenases (3 genes). En general, nuestros resultados coinciden con lo descrito anteriormente por varios autores[226,334,342,427], con una sola excepción; no hemos podido detectar la inducción de los genes de la familia de SAP. Esto se debe probablemente a las condiciones que hemos utilizado, mientras que otros autores han utilizado diferentes medios inductores: medio YPD y Lee con suero al 4%. Nuestro resultado está respaldado por el hecho de que el único factor de transcripción que estaba regulado en los tres tiempos es *STP3* [311]. Para analizar el resto de los genes expresados diferencialmente durante la transición dimórfica se agruparon por la similitud de sus patrones de expresión diferencial a lo largo del tiempo, mediante un análisis tipo *Cluster* en el programa GeneSpring 5.0. De esta forma se analizaron por separado aquellos grupos representativos cuya expresión cambiaba al menos en un tiempo de incubación. Se realizaron diferentes distribuciones en un número variable de grupos, y se observó que se podian: pared celular (famila de ALS, glucanasas, glucosidasas y mannosiltransferasas, GPI-anchored proteína), genes implicados en procesamiento de RNA y biogénesis de ribosomas, metabolismo de carbohidratos, transportadores y transportadores de oligopeptidos, resistecia a drogas, oxidasas/peroxidasas, hidrolasas/deshidrogenasas y biosíntesis de tiaminas.

En la Figura 2.4A se ha reflejado la alteración de la expresión de varios genes que codifican proteínas estructurales asociadas de forma preferente a la pared de las hifas como: *ALS1, ALS2, ALS3, ALS5, ALS9, PHR1, HWP1, ECE1,* [32,108,226,279,342,370,443]. En relación con la Figura 2.4B se detectó la sobreexpresión de varios genes implicados en procesamiento de RNA y biogénesis de ribosomas, en la Figura 2.5 se encuentran subexpresados 35 genes relacionados con la ruta de glicolisis. Se ha descrito que la expresión de genes implicados en glicólisis se reduce de forma drástica en el inicio de la transición dimórfica [460] lo que confirma nuestro resultados. En el capitulo II, se ha explicado con más detalles los resultados de este experimento.

Deleción del gen *GCN5* en el mutante *HDA1* de la cepa de NDH4

Nuestro grupo de trabajo, está llevando a cabo el análisis sistemático de la implicación de diferentes histonas desacetilasa e histona acetiltransferasas en la regulación de la transición levadura-hifa y de otros procesos morfogenéticos. En la primera parte de está memoria se han expuesto los efectos en morfogénesis de la

ausencia de una de las probables histonas desacetilasas y de una histona acetiltransferasas de *C. albicans,* Gcn5p y CaHda1p. La interrupción de los dos alelos del gen *GCN5* en la cepa NDH4 (con el gen *HDA1* delecionado) se llevó a cabo siguiendo la estrategia del "Ura-blaster" de forma similar a la descrita en nuestro laboratorio. En la Figura 3.1 se muestra la comprobación del genotipo de las cepas generadas mediante experimentos tipo Southern. El DNA genómico fue digerido con *Mun*I y como sonda se utilizó la secuencia de 465 pb correspondiente a la región 3´ no codificante del gen *GCN5.* Tal y como puede apreciarse en la Figura 3.1, las cepas CAI4, NDH4 y heterocigotas para la deleción del gen presentan la banda de 0.987 kb correspondiente al alelo silvestre de *GCN5.* Esta banda no aparece en las cepas homocigotas para la deleción de *GCN5* (SGM4-3 y SGM4-11) y en su lugar se detectan otras bandas cuyo tamaño se ajusta a la secuencia del cassette de deleción y de las secuencias flanqueantes.

Efecto de la doble deleción *gcn5, hda1* en el crecimiento exponencial

Mediante el seguimiento de la D.O. alcanzada a lo largo del tiempo por cultivos de las diferentes cepas, se detectó un ligero retraso en el crecimiento del doble mutante *gcn5, hda1* y del mutante sencillo *gcn5* respecto a la cepa parental CAI4. Se observó que en fase exponencial el tiempo de generación de las cepas SGM4 y RDG4 era mayor al de la cepa NDH4 y la cepa parental CAI4. Estimamos que en este medio las cepas SGM4 y RDG4 iniciaban la fase exponencial tras aproximadamente 22 horas de crecimiento. En relación con este fenotipo, en *S.cerevisiae* se ha descrito que la cepa homocigota para la deleción del gen *ScGCN5* [461] muestra también defectos de crecimiento en medio rico. Después de inocular en medio mínimo a 28ºC se observó que mientras que en los cultivos de la cepa CAI4 y el mutante simple *hda1* las células crecían únicamente como levaduras en los cultivos del doble mutante *gcn5, hda1* y mutante simple *gcn5* del aparecían pseudohifas y agregados celulares.

Efecto de la doble deleción *gcn5, hda1* en la filamentación en medios sólidos

Se ha analizado el efecto de la doble deleción en la capacidad de filamentación en diferentes medios sólidos y se ha observado que al igual que los dos mutantes simples presenta defectos de filamentación en todos los medios de inducción

ensayados (Figura 3.5).

Efecto de la doble deleción *gcn5, hda1* en la transición dimórfica en medio líquido

Después de inducir la transición dimórfica en medio líquido Lee a 37ºC se observó que el mutante doble *gcn5, hda1,* es incapaz de formar hifas, igual el mutante simple *gcn5*. Ambas cepas forman en lugar de hifas, pseudohifas y agregados celulares aunque el grado de agregación parece menor en el doble mutante que el mutante simple *gcn5*. El doble mutante se comporta así, de diferente forma que el mutante simple *hda1*, ya que en medios líquidos si es capaz de formar hifas. En la inducción en medio Lee a 37ºC con suero observamos el mismo efecto.

Efecto de las deleción de los genes *GCN5* y *HDA1* en la formación de clamidosporas

A continuación se analizó el efecto de la deleción de los genes *GCN5* y *HDA1* en la formación de clamidosporas. Las cepas se incubaron en medio corn meal agar bajo condiciones microaerófilas y a temperatura 25ºC, hemos observado que la cepa doble mutante *gcn5, hda1* no es capaz de formar clamidosporas al igual que el mutante simple *gcn5*.

Análisis de la sensibilidad de la doble deleción *gcn5, hda1* a compuestos que afectan a la pared celular

Uno de los ensayos utilizados para determinar la existencia de posibles alteraciones en pared celular, fue el ensayo de “Alcian blue”. En la Figura 3.10 se muestran los valores del colorante fijado por la cepa CAI4, NDH4, RDG4 y SGM4 de *C. albicans*. Si tomamos como 100% la cantidad de colorante fijado por las células de la cepa silvestre, las células del doble mutante fijan un 185,59%. El porcentaje de colorante por las cepas NDH4 y RDG4 es de un 120,65% y 155,94% respectivamente. Además podemos concluir que, en valor absoluto, el mutante simple hda1 y la cepa CAI4 fijan menos colorante que doble mutante *gcn5, hda1* y mutante simple *gcn5*. Estas diferencias se pueden atribuir a que los mutantes, presentan una mayor cantidad de fosfato en las manoproteínas presentes en la pared celular. En *C. albicans*, la capacidad de adhesión depende de su pared celular [454]. Estudios recientes han revelado que las manoproteínas una vez

glicosiladas forman una interacción directa con el hospedador por lo que defectos en el proceso de glicosilación son importantes para la virulencia [470]. Durante el proceso de transición levadura-hifa existe un rápida remodelación y expansión de la pared celular durante la formación de hifas; por esta razón, mutaciones en genes que intervienen en la construcción de la pared celular presentan defectos de filamentación [189,470]. Además los genes específicos de hifas en *C. albicans* codifican proteínas de secreción de la pared celular. Así que, es de esperar que defectos en cualquiera de las rutas de señalización que regulan el proceso de transición de la levadura-hifa, se vean reflejados en una alteración de algunos componentes de la pared celular. Puesto que las cepas NDH4, RDG4 y SGM4 parecían presentar defectos en la pared celular, decidimos estudiar su sensibilidad a zimoliasa (preparación lítica con actividad de β-1,3 glucanásica y proteásica, que desorganiza y degrada la pared celular y puede ser utilizada para medir cambios en la composición y ensamblaje de la pared celular [375]. Para el ensayo de sensibilidad a zimoliasa, tomamos como referencia estudios previos realizados en nuestro laboratorio [189], en los que se analizó como afectan distintas concentraciones de zimoliasa a la integridad de células CAI4. El resultado obtenido se muestra en la Figura 3.9. Como puede observarse, no se detectó una diferencia significativamente entre la diferencias de la densidad óptica a largo del tiempo en los mutante simple y doble mutante. Por último, decidimos analizar la sensibilidad a otros agentes que afectan a la pared celular: Cafeína, Calcofluor white, NaCl, SDS y Congo red. Como se puede observar en la Figura 3.8, se encontraron diferencias de sensibilidad a estas concentraciones de Calcofluor white entre el doble mutante y el mutante *gcn5* con respecto a la sepa CAI4. El doble mutante *gcn5, hda1* y el mutante sencillo *gcn5* no crecieron en ninguna de las diluciones en placa con Calcofluor white a concentración de 1 mg/ml. Como se pude observar en la Figura 3.8, el doble mutante *gcn5, hda1* y el mutante sencillo *gcn5* se presentan más sensibles con respecto a las cepas CAI4 y el mutante sencillo *hda1*. El doble mutante *gcn5, hda1* y el mutante *gcn5* no crecieron en placa conteniendo cafeína y SDS a una concentración de 15 mM y 0,05% respectivamente. En *S. cerevisiae* la hipersensibilidad a cafeína se ha relacionado con defectos en los mecanismos reguladores de la formación de la pared celular. Todos estos procesos parecen estar alterados en la cepa doble delecionada. También se detectaron ciertas diferencias en la sensibilidad a SDS, un detergente

que altera la membrana plasmática. La cepa doble delecionada parece ser un grado más sensible que la cepa CAI4 y mutante sencillo *hda1.* La hipersensibilidad a SDS se ha relacionado con la integridad de la pared celular, necesaria para la protección de la célula. Además pueden producirse diferencias en la sensibilidad a este compuesto debidas a alteraciones en la composición de la membrana plasmática.

Analisis del transcriptoma del mutante *gcn5, hda1* durante crecimiento de levadura

Al objeto de analizar los posibles genes regulados por procesos de acetilacion y de deacetilacion se analizó el transcriptoma del mutante doble *gcn5, hda1,* utilizando como control la cepa CAI4, durante el crecimiento levadura y durante la transición levadura-hifa. Anteriormente en nuestro laboratorio se han llevado a cabo experimentos en los que se ha analizado el transcriptoma de los mutantes simples *gcn5* y *hda1* tal y como se ha señalado anteriormente, 4 condiciones examinadas, crecimiento exponencial como levaduras (en YNB a 28ºC) y a tres tiempos (15, 60 y 180 minutos) durante la transición dimórfica en medio líquido Lee a 37ºC. A partir de células recogidas en fase exponencial de crecimiento como levaduras, se detectó que se encontraba alterada la expresión de 180 genes por la doble deleción de *GCN5* y *HDA1* durante el crecimiento en forma de levadura. El número de genes que presentan expresión diferencial en el mutante doble *gcn5, hda1* es menor que el detectado en la cepa *gcn5* y mayor que el detectado en la cepa *hda1.* Lo que está de acuerdo con el hecho de que la cepa SGM4 (*gcn5, hda1*) muestra alteraciones del fenotipo similares al del mutante *gcn5.* Se detectaron 3 genes comunes entre los mutantes simples y doble. Entre estos genes, *ALS4* y *ALS2* codifican proteínas de la pared celular, mientras que otro, *BGL22,* codifica una posible endo β 1,3 glucanasa secretada que podría intervenir en remodelación de la pared celular. Estos resultados sugirieren que en condiciones normales estos genes son controlados por separado por Gcn5p y Hda1p. La alteración de la expresión de estos genes permite realizar algunas hipótesis sobre las causas de las diferencias de afinidad a Alcian blue. Las proteínas codificadas por los genes *ALS2* y *ALS4* pertenecen a la familia de aglutininas ALS. El mecanismo por el cual estas proteínas promueven la adhesión a células del hospedador no se conoce aún bien, pero se ha propuesto que podría deberse a la exposición hacia el

exterior de su extremo amino terminal. Este dominio presenta características hidrofóbicas y se encuentra menos glicosilado que la región central [200,202]. De forma general, se ha sugerido que la hidrofobicidad celular viene dada por la exposición de proteínas o regiones proteicas poco glicosiladas y el enmascaramiento de proteínas o regiones hidrofílicas más glicosiladas [183]. La reducción de expresión de *ALS2* y *ALS4* podría suponer así una mayor exposición de proteínas hidrofílicas más glicosiladas, que explicaría también la mayor capacidad de unión a Alcian blue. Además, 47 genes son comunes entre el mutantes simples *gcn5* y doble *gcn5, hda1*. Sin embargo, un subconjunto de 130 genes que se vieron afectados en el doble mutante no sufrió modificaciones en ninguno de los mutantes simples. Estos resultados indican que en la condición de crecimiento de levadura ambos genes regulan conjuntamente un importante grupo de genes. En la Figura 3.12 se ha señalado el número de genes expresados diferencialmente, con un factor de variación de los mutantes simple y doble vs. CAI4 $\geq$ 1.5 en cada condición. El nombre y la descripción de cada uno de los genes en el mutante doble se muestran en los archivos 3.1 y 3.2 del material suplementario. El mutante doble *gcn5, hda1* muestra defectos en el velocidad de crecimiento lo cual podría ser explicado por disminución en la expresión de algunos genes de la ruta de glicólisis (Tabla 3.3). En nuestros experimentos, hemos observado disminución de la expresión de *EFG1*. Se ha reportado subexpression de la mayoría de los genes de la ruta de glicólisis así como en genes relacionados en el metabolismo de azúcares de reserva tales como, tetralosa y glucogeno subexpresados en el mutante *efg1* [108]. Estos resultados sugieren que existe un control indirecto de la expresión de los genes de la ruta de glicólisis en el mutante doble. Hemos encontrado 3 genes sobrexpresados como *CSP37* [416], *PGA13* y *PGA56* que codifican para proteínas de pared celular lo que sugiere una remodelación de la pared celular en el mutante doble. Otro grupo de genes subexpresados fue el de genes que codifican para proteínas ribosomales (13 genes). Se ha reportado que la expresión de 63 genes de 117 (53%) son subexpresados durante el crecimiento en forma de levadura en el mutante simple *gcn5*, esto sugiere que Gcn5 podría ser un regulador de la expresión de genes que codifican para proteínas ribosomales. Se ha detectado dos genes (*CDR1* and *CDR4*) subexpresados, los cuales pertenecen la superfamilia de transportadores de drogas. Se ha reportado la sobreexpresión de *CDR1* en cepas resistentes a

azoles [522]. La subexpresion de los genes *CDR1* y *CDR4*, podría estar relacionada con la sensibilidad del doble mutante a compuestos como anfotericina e higromicina.

Análisis del transcriptoma del mutante *gcn5, hda1* durante la transición levadura-hifa

Se ha detectado alterada la expresión de 664 genes en el mutante doble *gcn5, hda1* al menos en una de las tres condiciones de la transición levadura hifa analizadas. Como puede observarse en la Figura 3.13, la mayor parte de los genes aparecen expresados diferencialmente a los 60 minutos de inducción. Tanto por el número de genes expresados diferencialmente como la comparación de los patrones de expresión, se puede apreciar una marcada diferencia entre los resultados que se han obtenido en los mutantes simples y los tres tiempos del mutante doble. Además durante la inducción, en el mutante doble también se observan diferencias entre los sucesivos tiempos. Estos resultados indican que, estas variaciones pueden deberse a la modulación de la expresión que sufren numerosos genes por ambas proteínas durante la transición dimórfica. 293, 310 y 238 genes se expresaban diferencialmente en cada uno de los tiempos analizados. El nombre y la función de cada uno de ellos se han incluido en las Tablas 3.3 y 3.4 del material suplementario. En conjunto se detectó la expresión diferencial de 664 genes (312 sobre- 352 sub-expresados) en el mutante doble *gcn5, hda1* en al menos uno de los tres tiempos de inducción analizados. Se observó una buena correlación estadística en nuestros resultados, 39 genes comunes en los tres tiempos ensayados (20 sobre- y 19 sub-expresados), 35 genes entre los 15 y 60 min (12 sobre- y 23 sub-expresados) y 33 genes entre 60 y 180 min. (17 sobre- y 16 sub-expresados). 188 genes (72 sobre- y 116 sub-expresados), 203 genes (82 sobre- y 121 sub-expresados), y 135 genes (84 sobre- y 51 sub-expresados) sólo varían en un tiempo de inducción (15, 60 y 180 min. respectivamente). Sin embargo, sólo 31 genes eran comunes entre los 15 y 180 min. Los 39 genes comunes a los tres tiempos se describen en la Tabla 3.4. La categoría principal corresponde a proteínas de pared celular e incluye la sobreexpresión de *RDH3* [61,104,342], *PIR1*, una proteína estructural de pared [309], *KRE1* [36,46] y *CHR11* [6,61], y la subexpresión de genes específicos de la fase hifa, como *ECE1*, [27,32]; *HWP1*, [443]; y dos genes que pertenecen a la familia *ALS* (*ALS1* y *ALS3*). Previamente se ha reportado que estos genes están sobreexpresados o subexpresados durante la transición levadura-hifa [39,342] y la cepa CAI4 (Capitulo II). Se observó que la expresión de los genes *CHR11, RDH3, RBT5,*

PGA62 aumenta en ambos mutantes simples, mutante doble y durante la transición levadura hifa de la cepa CAI4 mediada por la temperatura. Lo que sugiere que la expresión de estos genes es regulada por la temperatura y no por procesos de acetilación y deacetilación. Además se detectó que la expresión de los genes *PHR1 PGA59* y *KRE1* aumenta en la cepa CAI4, en el mutante simple *gcn5* y en el mutante doble, y disminuye en el mutante *hda1* lo que sugiere que es regulada especialmente por el gen *HDA1* (Figura 3.14). Otros genes regulados durante los tres tiempos de incubación pertenecen a las siguientes categorías: transportadores (9 genes), factores de transcripción (5 genes), y biosíntesis de vitaminas (2 genes). Como se mencionó anteriormente, el mutante doble no fue capaz de formar hifas bajo condiciones de inducción, por lo que la subexpresión de estos genes es consistente con nuestros resultados. Además el análisis de los microarrays mostró la sobreexpresion de cinco factores de transcripción, *BDF1* [28,302], *GAL4* [306], *NRG1* [330,331], *TYE7* [177,231,281,342] y *ZCF39* (Tabla 3.4). Hemos encontrado 16 genes (12-sobre y 4-subexpresados) también pertenecientes a esta categoría (factores de transcripción) durante uno o dos tiempos de inducción (Tabla suplementaria 3.3 y 3.4). Siete de estos genes fueron comunes con el mutante simple *gcn5* y el resto podrían estar reguladas por las proteínas Gnc5p y Hda1p. Además hemos observado que 12 genes que codifican para glucanasas, glucosidasas y manosiltransferasas estan regulados a lo largo de la inducción (Tablas suplementaría 3.1 y 3.2 y Figura 3.14). Cinco de estos genes están sobreexpresados (CA5339, *MNN4, MNT2, BGL2* y *GSL22*), y siete subexpresados (*MNT1*, *GSC1*, *GLC3*, *SUN41, MNT3, PMT2 y PMT4*). El cambio en la expresión de estos genes podría estar relacionado con cambios en la composición de la pared celular. Adicionalmente se encontró que algunos de estos genes son comunes entre los mutantes simple y doble. En el capitulo III, se ha explicado con más detalles algunos grupos de genes cuya expresión ha sido modificada por la deleción de *GCN5* y *HDA1*.

Analizar del transcriptoma del mutante *odc1/odc1* durante crecimiento de levadura

En el capítulo IV nos hemos concentrado en el estudio de los patrones de expresión diferencial en la cepa de *C. albicans* (*odc1/odc1)* (agotadas en su reserva interna de poliaminas) suplementando con dos concentraciones de putrescina, concretamente 0.01 mM *vs* 10 mM durante el crecimiento de levadura y la transición levadura-hifa, en medio Lee a 28ºC y 37ºC, respectivamente.

Por el método de microarrays de cDNA, se encontraron 137 genes expresados

diferencialmente en crecimiento de levadura. El nombre y la función de cada uno de ellos se han incluido en la Tabla 4.1 del material suplementario. Dentro de los datos obtenidos en fase exponencial de crecimiento se ha destacado la desregulación de tres grupos de genes. El primero incluye genes relacionados con el metabolismo de carbohidratos (principalmente genes relacionados con ruta de glicólisis), el segundo está constituido por genes de la familia de histonas y el último está formado por genes relacionados con respiración. En relación a este último grupo se ha señalado la subexpresión de los dos genes que codifican para componentes de la cadena de transportadora electrones. En relación con bajo nivel de poliaminas, se detectó la subexpresión de 3 genes que codifican para transportadores de azúcares, Tabla 4.1, *HGT8, HGT6* y *HGT7. C. albicans* posee muchos genes (probablemente 19) que codifican aparentemente para transportadores de azúcares [44,124]. La mayoría de los genes HGT se expresan en *C. albicans* y la función de cada uno de ellos en el transporte de azucares ha sido demostrada [124,484]. La subexpresión de estos genes sugiere que las poliaminas intervienen en la regulación de los transportadores de azúcares en crecimiento de levadura. Además hemos detectado que la subexpresión de un número de genes implicados en ruta de glicólisis sugiere que las poliaminas también intervienen en la regulación de genes de glicólisis durante el crecimiento normal.

Analisis del transcriptoma del mutante *odc1/odc1* durante la transición levadura-hifa

El mismo estudio de microarrays se llevó a cabo durante la transición levadura-hifa. Este análisis mostró 1981 genes alterados en bajas concentraciones de poliaminas, con 599 genes de función desconocida. El nombre y la función de cada uno de ellos se han incluido en las Tablas 4.3 y 4.4 del material suplementario. En conjunto se detectó que la expresión de 1981 genes (1056 sobre- 925 sub-expresados) esta afectada por el cambio en las concentraciones de poliaminas, 0.01 mM respecto a 10 mM. 503 genes son comunes a los tres tiempos ensayados (301 sobre- y 202 sub-expresados), 188 genes son comunes entre los 15 y 60 min (92 sobre- y 96 sub-expresados) y 263 genes entre los 60 y 180 min. (130 sobre- y 133 sub-expresados). 289 genes (124 sobre- y 165 sub-expresados), 388 genes (196 sobre- y 192 sub-expresados), y 288 genes (181 sobre- y 107 sub-expresados) sólo varían en un tiempo de inducción (15, 60 y 180

min. respectivamente). Considerando datos publicados por diversos autores sobre la modulación de la expresión génica durante la transición levadura-hifa, se trató de determinar qué genes implicados en morfogénesis se encontraban alterados en bajo concentraciones de poliaminas en la cepa *Caodc1*. Se han tenido en cuenta para ello datos de tres trabajos independientes publicados por Kadosh y Johnson [226], Lee et al [267] y Nantel et al [342]. Los 505 genes comunes a los tres tiempos se muestran en la Tabla 4.2. entre ellos se ha detectado, la subexpresión de varios genes que habían sido previamente reconocidos como hifa-específicos: *ECE1* [32], *RBT1* [37], *HWP1* [419], *DDR48* [261], *PHR1* [370], *RBT4* [37], *HYR1* [370] y *ALS3*. Las poliaminas parecen actuar sobre la expresión de genes específicos de hifas de *C. albicans*. En los mutantes simple *gcn5* y doble *gcn5, hda1* cinco de ellos, *ECE1, RBT1*, *HWP1, PHR1, HYR1* y *ALS3,* se encuentran subexpresados respecto a la cepa CAI4. Además, Nantel et al [342] mostró que la expresión de estos genes incrementa durante la transición levadura-hifa, lo cual es consistente con los resultados obtenidos para la cepa CAI4, por lo que podrían estar relacionados con la regulación de la morfogénesis, lo que confirma nuestros datos. Como se ha señalado anteriormente en el capitulo IV, se ha detectado la subexpresion de un número de genes relacionados con la pared celular incluidos *AAF1, BGL22, CHS5, DPM1, ECM33, FGR6, GLC3, IPP1, PGA26, PGA36, PGA48, PGA53, PGA54, PGA59 y SUN41.* Seis de estos genes *BGL22, ECM33, FGR6, PGA54, PGA59 y SUN41* han sido identificados como sobrexpresados en la cepa CAI4 durante la transición levadura hifa (capitulo II). Entre los genes sobrexpresados se encuentran 6 genes, *ECM1, MNN11, MNN4, PGA31*, *PHR2* y *PIR1* involucrados en la pared celular a lo largo de la inducción. *PIR1* cuya expresión disminuye e incrementa a largo de la indución en CAI4 y los mutantes *gcn5* y *gcn5, hda1* respectivamente. Estos resultados indican que la proteína Gcn5p y las poliaminas juegan, por tanto, un papel clave en la expresión de genes de pared. Otros grupos de genes subexpresados durante tres tiempos de inducción son: 4 genes que codifican para acetil-coenzimas *ACC1, ACS1, ACS2* y *POT13,* 2 genes implicada a la biosíntesis de adenina *ADE13* y *ADE17*, seis genes de permeasas de aminoácidos *AGP1, CAN2, CAN5, HNM3,* IPF4580 y *MAL31*, 15 genes relacionados con el establecimiento de la polaridad ej. *ACT1, ARF3, ARP3*, *CDC12* y *COF1,* 20 genes relacionados con la ruta de glicólisis y 12 genes relacionados con respiración y transporte de electrones (Tablas 4.3-4 material suplementario y Tabla 4.2).

CaCDC12 codifica para una septina esencial implicada en la formación de septos y tubos germinativos [497]. El homólogo del gen *CaARF3* en *S. cerevisiae* forma

parte de la superfamilia de genes RAS implicados en el desarrollo de la polaridad [207,268]. El homólogo del gen *CaARP3* en *S. cerevisiae* es un componente esencial del complejo Arp2/3, involucrado en endocitosis y polaridad [102,464,478,510]. *COF1* promueve la despolarización del filamento de actina de un modo dependiente del pH. No se han encontrado alteraciones de estos genes relacionados con la polaridad en las mutantes como *gcn5* y *gcn5 hda1*, lo que sugiere que las poliaminas están relacionadas con la regulación de estos genes; controlando de este modo el desarrollo de la transición de levadura-hifa.

Como se ha señalado anteriormente en el capitulo IV, en el mutante nulo *Caodc1* bajo concentración de poliaminas (0.01 mM) se ha detectado la subexpresion de un número significativo de genes relacionados los cuales intervienen en respiración y transporte de electrones, codificados en DNA genómico y DNA mitocondrial *(COX13, CaNDH1, COX3A, COX3B, COX4, NAD6, CUP5, VAM6, STF2, PMA1 y VMA1, ATP20, ATP4, ATP7, COB ,COQ2, COX15, CTM1, CYT1, IPF12564, NAD1, NCP1, RIP1, TFP1, TFP3, VMA4, VMA7, VMA8, ATP14, ATP16, ATP2, ATP3, ATP5, ATP8, COX5A, CYT12,* IPF3358, *MCR1, QCR6 QCR9* y *VMA2*) durante el desarrollo de la transición de levadura-hifa. De acuerdo disminución de la actividad mitocondrial, ruta de glicólisis y ciclo de TCA, esto resulta en una menor producción de ATP. En la cepa delecionada se detectó subexpresión de genes relacionados con obtención de energía y transporte de electrones. Dentro de este grupo se encuentra la mayor parte de genes de la ruta de glicólisis y gluconeogénesis, además de genes implicados en otras rutas de obtención de energía. A bajas concentraciones de poliaminas aparecen subexpresados, en la cepa *Caodc1*, numerosos genes implicados en respiración, reflejando la mayor activación de este proceso en las hifas a altas concentraciones de poliaminas (10 mM). En otros estudios hemos observado la subexpresión un número genes relacionados con la ruta de glicólisis en los mutantes *gcn5* y *gcn5, hda1* durante la transición levadura-hifa. Se ha descrito que la expresión de genes implicados en glicólisis aumenta durante la transición dimórfica en el mutante *hda1* [307]. Estos cambios reflejan diferencias en la modulación de genes de metabolismo durante el desarrollo de pseudohifas e hifas. La regulación de la expresión de genes de glicólisis se ha asociado en diferentes trabajos a la transición dimórfica. Observándose que en el inicio de la transición levadura-hifa la expresión de genes que codifican enzimas de la ruta de glicólisis se reduce

drásticamente y a continuación aumenta progresivamente, llegando a ser mayor en algunos genes, en hifas que en levaduras. Sin embargo, su relación con la morfogénesis no ha sido esclarecida, incluso se ha sugerido que se debe a las variaciones fisiológicas subyacentes al cambio en las condiciones de crecimiento y no al proceso morfogenético en sí [460]. Estos resultados indican que las poliaminas juegan un papel clave en la regulación de los genes necesarios para la respiración, lo que impide el desarrollo de las hifas de *C. albicans*.

Dentro de los grupos de genes subexpresados, se encuentran principalmente genes que codifican histonas (*HHF21, HHF22, HHT21, HHT3, HTA1, HTA3* y *HTB1*) a 60 y 180 min. La subexpresión de estos genes podría estar relacionada en esta condición (0.01 mM) con el retraso de crecimiento que muestra esta cepa respecto a la concentración de 10 mM de putrescina, como se ha sugerido en otros mutantes donde estos genes se encuentran subexpresados y muestran crecimiento más lento [28]. A los 15 y 60 min de inducción, se detecta la subexpresión de genes implicados en la ruta de secreción como *ARF21, CLC1, COP1, ERD2, ERV25, ERV46, SEC16, SEC18, SEC23, SEC26, SEC31* genes implicados en glicosilación como *PMT2, PMT1*, *PMT4* y genes relacionados con mitosis y ciclo celular *CDC1, CDC11, CDC42* y *RAS1. CLC1* codifica para una proteína similar de *S. cerevisiae* Clc1p implicada en el transporte intracelular [425]. Ras1p actúa en el inicio de las rutas de transducción de señales PKA-cAMP y MAPK [128,279,299]. Estos resultados indican que las poliaminas parecen actuar sobre la expresión de genes implicados en diferentes aspectos de control del ciclo celular y ruta de secreción de *C. albicans*. Entre los grupos de genes sobreexpresados a lo largo de la inducción, destacan los genes asociados a pared celular incluidos *BMS1, ECM1, MNN11, MNN4, PGA31*, *PHR2* y *PIR1,* genes que codifican para proteínas involucradas en el DNA-directed RNA polimerasa como *RPB5, RPB8, RPC25, RPC40, RPC53, RPC82* y *PAF1*, genes asociados a procesamiento de rRNA (22 genes), RNA helicasa (11 genes) y factores transcripcionales (17 genes). Se observa la subrexpresión de genes que codifican proteínas ribosomales. En conjunto estos datos demuestran que la ausencia de poliaminas impiden la correcta activación de genes involucrados en el inicio de la filamentación, ruta de secreción, mitosis y ciclo celular, genes que codifican las histonas, genes que intervienen en respiración y transporte de electrones, genes relacionados con la ruta de glicólisis y metabolismo de carbohidratos y genes

relacionados con la pared celular.

Deleción del gene *HAT1* en el mutante *gcn5* de la cepa de RDG4

La interrupción de los dos alelos del gen *CaHAT1* en la cepa RDG4 (con el gen *GCN5* delecionado) se llevó a cabo siguiendo la estrategia del "Ura-blaster" de forma similar a la descrita para la deleción del gen *CaGCN5* en la cepa NDH4. Se partió para ello del cassette de deleción construido para la interrupción del gen *CaHAT1* en la cepa CAI4 [106]. Se trata de un fragmento de 5.1 kb incluido en el vector pJBR3 que se corresponde con la secuencia hisG-CaURA3-hisG flanqueada por dos fragmentos de DNA, de 620 pb y 588 pb, de igual secuencia que el extremo 5´ de la ORF de *CaHAT1* y la región 3´ no codificante del gen, respectivamente. El cassette completo de 5.1 kb fue liberado mediante la digestión del plásmido pJBR3 con las enzimas de restricción *Sac*I y *Kpn*I y utilizado para la transformación de la cepa RDG4. En la Figura 5.1 se muestra la comprobación del genotipo de las cepas generadas mediante experimentos tipo Southern. El DNA genómico fue digerido con *SspI* y como sonda se utilizó la secuencia de 588 pb correspondiente a la región 3´ no codificante del gen *CaHAT1*. Tal y como puede apreciarse en la Figura 5.1, las cepas CAI4, RDG4 y heterozigotas para la deleción del gen presentan la banda de 0.64 kb correspondiente al alelo silvestre de *CaHAT1.* Esta banda no aparece en las cepas homozigotas para la deleción de *CaHAT1* y en su lugar se detectan otras bandas cuyo tamaño se ajusta a la secuencia del cassette de deleción y de las secuencias flanqueantes. Nuestro interés se centra en la regulación de procesos morfogenéticos, por lo que se analizó principalmente la capacidad de las cepas *gcn5, hat1* de llevar a cabo cambios morfológicos en respuesta a las diferentes condiciones del medio. En cada situación se comparó la morfología del doble mutante con la mostrada por la cepa silvestre y los mutantes sencillos *gcn5* y *hat1*. En primer lugar se analizó la morfología celular y la velocidad de crecimiento a 28ºC de la cepa con la doble deleción *gcn5*, *hat1* (AHM4). En la Figura 5.2 y 5.3 se pueden apreciar claramente como el doble mutante AHM4 *(gcn5, hat1)*, al igual que el mutante simple RDG4 (*gcn5*), presenta una morfología celular más agrumada que la cepa CAI4 y forma ramificaciones de células que se mantienen unidas y que acaban formando "rosetas". Mediante el seguimiento de la D.O. alcanzada a lo largo del tiempo por cultivos de las diferentes cepas, se detectó un ligero retraso en el crecimiento del doble mutante *gcn5, hat1* y del mutante sencillo *hat1* respecto a la cepa parental CAI4. Se estimó que entrada en fase exponencial de las cepas AHM4 (16 h) era

mayor al de la cepa parental CAI4 y menor de la cepa RDG4 (*gcn5*). En relación con este fenotipo, en *S. cerevisiae* se ha descrito que la cepa diploide homozigota para la deleción del gen *ScGCN5* muestra también defectos de crecimiento [461].

Efecto de la doble deleción *gcn5, hat1* en la transición dimórfica en medio líquido

Después de inducir la transición dimórfica en medio líquido Lee [271] a 37ºC se observó que el doble mutante forma pseudohifas en lugar de hifas (Figura 5.5), característica fenotípica descrita previamente en el mutante sencill *Cagcn5* [106]. Un resultado similar se obtuvo en medio Lee suplementado con suero.

Efecto de la doble deleción en la filamentación en medios sólidos

Siguiendo con nuestra dinámica de análisis del fenotipo se estudió la capacidad del doble mutante de filamentar en medios sólidos, comparándola con la de los mutantes sencillos. El deleción de *GCN5* causa defectos en la capacidad de filamentar en medio sólido Spider y medio Lee con NAcGlc, donde la miceliación se induce por la fuente de carbono. En la Figura 5.4 puede observarse como las cepas con la doble deleción *gcn5, hat1* al igual que la mutante sencillo *gcn5* presenta también defectos de filamentación en estos medios. Resulta más interesante analizar el efecto de la deleción en el medio embedded donde el mutante sencillo presenta un fenotipo diferente. El mutante simple RGL4 (*hat1*) a diferencia de RDG4 (*gcn5*), es capaz de desarrollar cierta filamentación. La cepa con la doble deleción presenta miceliación en este condicion, mostrando un fenotipo más próximo al mutante simple *hat1* que al de *gcn5*

Efecto de la doble deleción *gcn5, hat1* en la formación de las clamidosporas

La inducción de la formación de clamidosporas se llevó a cabo de forma análoga a la descrita en el análisis del mutante doble *gcn5, hda1*. En el mutante doble, así como en el mutante simple *gcn5* no se detectaron clamidosporas, ni siquiera después de un periodo de incubación prolongado (14 días). Las células de la cepa *hat1* fueron capaces de producir clamidosporas, demostrando con ello que el fenotipo observado se debe a la ausencia de *GCN5.* Se ha reportado, el requerimiento los procesos de remodelación de la cromatina por la formación de clamidosporas mediante la proteína Isw2p [353]. Otro gen cuya alteración causa defectos en la diferenciación clamidosporas es *SCH9,* que codifica una MAP

quinasa que interviene en la regulación de diferentes procesos, entre los que se encuentra el almacenamiento de glucógeno, lo que está en concordancia con la función de las clamidosporas como estructuras de reserva [353]. Sería interesante por tanto determinar si la función de Gcn5p en el metabolismo de este polisacárido se encuentra conservada en *C. albicans,* de forma que pudieran relacionarse los defectos de acumulación de glucógeno con la incapacidad de formar clamidosporas. Se ha sugerido que la existencia en el genoma de *C. albicans* de numerosos genes sin homólogo conocido en otros organismos podría estar relacionada con el desarrollo de este proceso casi específico de *C. albicans*. Son necesarios por tanto nuevos trabajos sobre los mecanismos reguladores que desencadenan la formación de clamidosporas.

Análisis de la sensibilidad de la doble deleción *gcn5, hat1* a compuestos que afectan a la pared celular

Por ultimo, decidimos analizar la sensibilidad a agentes que afectan a la pared celular: Cafeína, Calcofluor white, NaCl, Higromicina (100 μg/ml), Itraconazol, Fluconazol (4 μg/ml), SDS y Rojo congo. Para cada uno de ellos se eligó la concentración máxima que no alteraba la vialidad de la cepa CAI4. Se crecieron las células hasta fase exponencial en medio YEPD y se sembraron distintas diluciones de las mismas en placas que contenían los diferentes compuestos. En la Figura 5.9 puede observarse como la cepa doble delecionada AHM4 muestra mayor sensibilidad a Higromycina, Fluconazole, Itraconazole y Caffeina. Este efecto se observó también en el doble mutante *gcn5, hda1* y del mutante sencillo *gcn5* respecto a la cepa parental CAI4. La cepa doble delecionada no reveló diferencias respecto a la cepa parental CAI4 a SDS y NaCl.

Principales grupos funcionales de genes expresados diferencialmente a 28ºC en el mutante *gcn5, hat1*

Durante el crecimiento como levaduras en medio mínimo YNB a 28ºC, en el mutante *gcn5, hat1* se encuentra alterada la expresión de 521 genes, de los cuales, 247 genes están sobreexpresados y 274 subexpresados, con respecto a la cepa parental CAI4. Dentro del grupo de genes sobreexpresados en la cepa doble delecionada, las categorías y subcategorías estadísticamente significativas son respuesta a estrés oxidativo, metabolismo de carbohidratos, transporte a azúcar y metabolismo de amino ácidos. Dentro del grupo de genes subeexpresados en la cepa doble delecionada, las categorías y subcategorías estadísticamente

significativas son, obtención de energía (principalmente a través de la glicólisis), metabolismo de carbohidratos, transporte de vitaminas y metabolismo de metionina.

Conclusiones

1. Se llevó a cabo el análisis del transcriptoma de la cepa CAI4 durante la transición levadura hifa comparando con células crecidas en forma de levadura. El cambio de temperatura altera la expresión de al menos 783 genes, considerando un factor de variación ≥ 1.5 veces y hemos observado que:

2. La mayor parte de los genes expresado diferencialmente y los mayores niveles de variación se observaron a los 60 minutos, después las diferencias en la expresión génica desminuyen. Este resultado indica que la mayoría de las señales y genes reguladores de morfogénesis ejercen su función rápidamente y posteriormente sólo se expresan genes estructurales y de metabolismo.

3. Durante la transición dimórfica, aparece alterada la expresión de 51 genes correspondientes a proteínas de pared celular. La principal categoría asociada a genes relacionados a la familia de ALS, glucanasas, glucosidasas y manosiltransferasas y proteínas de unión mediante GPI.

4. Se ha llevado a cabo la deleción del gen *GCN5* en la cepa NDH4 (*hda1*). La ausencia de ambas proteínas tiene un efecto en morfogénesis, ya que el doble mutante *gcn5, hda1* presentó la mayoría los defectos acusados por el mutante sencillo *gcn5*: morfología unicamente de pseudohifas cuando crecen a 28ºC, defectos de filamentación, formación de pseudohifas en lugar de hifas en medios líquidos de inducción e incapacidad para formar clamidosporas.

5. El mutante *gcn5, hda1* como en el mutante simple *gcn5* muestra sensibilidad a NaCl, Rojo congo, SDS, Calcoflour white, Fluconazol, Higromicina, Anfotericina y Cafeína.

6. Se ha comparado el transcriptoma del mutante doble *gcn5, hda1* con el de la cepa parental CAI4, observándose que:

En fase exponencial de crecimiento a 28ºC, en la cepa delecionada, se encuentra alterada la expresión de 180 genes, considerando un factor de variación ≥ 1.5 veces. Entre estos genes, aparecen subexpresados genes implicados en ruta de glicólisis y genes implicados en la biosíntesis de ribosomas lo que parece tener relación con el retraso en el crecimiento de la cepa doble delacionada.

Durante la transición dimórfica, aparece alterada la expresión de 664 genes. Las principales categorías asociadas a sobreexpresión son metabolismo de aminoácidos, pared celular, factores de transcripción incluyendo el gen que

codifica el activador transcripcional Gcn4p. La subexpresión de genes que codifican para proteínas ribosomales y otros factores relacionados con funciones generales de crecimiento, refleja una respuesta en el mutante *gcn5, hda1* a retraso en el crecimiento.

7. Se ha observado que el nivel de expresión de los genes relacionados con pared celular como *ALS1, ALS3, ECE1, HWP1, PMT2, PMT4, MNT1, MNT2, MNT3* y *SUN41* fue disminuyendo a la largo de transición levadura hifa en los mutantes simples *gcn5, hda1* y en el mutante doble *gcn5, hda1*, lo que sugiere que estos genes están regulados por separado por ambos genes. El nivel de expresión de los genes *PGA4, ECM2* y *BGL2* aumenta en el mutante simple *hda1* y disminuye en los mutantes *gcn5* y doble *gcn5, hda1* que es coherente con el fenotipo dominante de *gcn5* en el mutante doble.

8. Se ha observado que el nivel de expresión de los genes relacionados con pared celular como *RHD3, RBT5, PGA10, PGA31, PGA62* y *YWP1* aumentan en ambos mutantes simple y doble durante transición levadura hifa indicando que estos genes se regulan independientemente por ambos genes. La expresión de los genes *KRE1, CRH11, PHR1* y *PGA59* aumenta en los mutantes simples *gcn5* y doble *gcn5, hda1* y disminuye en el mutante simple *hda1*. Además se ha observado que la expresión de los genes *PHR1, CHR11, KRE1* y *PGA59* aumenta en la cepa CAI4 durante la transición levadura hifa por cambio de temperatura. Estos resultados indican que *HDA1* controla la expresión de estos genes.

9. Se ha llevado a cabo el análisis del transcriptoma del mutante *odc1/odc1* durante el crecimiento en forma de levadura y durante la transición levadura hifa a concentraciones bajas de poliaminas (0,01 mM de putrescina) comparando con células crecidas en las mismas condiciones en altas concentraciones de poliaminas (10 mM de putrescina). El cambio de concentración de las poliaminas en el mutante *odc1/odc1* altera la expresión de al menos 2118 genes, considerando un factor de variación $\geq$ 1.5 veces.

10. En fase exponencial de crecimiento a 28ºC, se encuentra alterada la expresión de 137 genes, considerando un factor de variación $\geq$ 1.5 veces. Entre estos genes, aparecen subexpresados genes implicados en transporte de electrones, pared celular, transporte de drogas y azúcares, factores de transcripción y genes implicados en la ruta de glicólisis que sugerimos están relacionados con el retraso en el crecimiento.

11. Durante la transición dimórfica, aparece alterada la expresión de 1981 genes. La principal categorías asociadas a sobreexpresión son genes que codifican para proteínas ribosomicas, pared celular, factores de transcripción, RNA helicasas y

factores de inicio de transcripción.

12. Se ha observado que el nivel de expresión de los genes relacionados con el transporte de electrones y obtención de energía genes relacionados con ruta de glicólisis y ciclo de TCA disminuyen a largo de la transición levadura hifa. Además, hemos detectado subexpresion de genes relacionados con la ruta de secreción, polaridad celular, ciclo de desarrollo celular y transdución de señales que no se observó ni en los mutantes simples *gcn5, hda1* el mutante doble *gcn5*, *hda1* y ni en la cepa CAI4 durante transición levadura hifa.

13. Se ha llevado a cabo la deleción del gen *HAT1* en la cepa RDG4 (*gcn5*). La ausencia de ambas proteínas tiene un efecto intermedio en morfogénesis, ya que el doble mutante *gcn5*, *hat1* favorece la formación de pseudohifas en lugar de hifas, así como la incapacidad para formar clamidosporas como en el mutante simple *gcn5*.

14. Se llevó a cabo del análisis el transcriptoma del mutante doble *gcn5, hat1* durante el crecimiento en forma de levadura. Se observó que la deleción de los genes *GCN5* y *HAT1* altera la expresión de al menos 521 genes, considerando un factor de variación $\geq$ 1.5 veces.

15. Entre los genes sobreexpresados se identificaron genes relacionados con pared celular, estrés oxidativo, oxidas/peroxidasas y transporte de azúcares. Se detectó la subexpresión de genes relacionados con la ruta de glicólisis, metabolismo de aminoácidos y de ácidos grasos.

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