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Global transcriptional responses of *Candida albicans* to histone acetyltranferases deletion

Ph.D Thesis

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Autoriza:

That the present dissertation entitled “Global transcriptional responses of *Candida albicans* to histone acetyltranferases deletion” submitted to apply for Ph.D degree has been carried out by Zahra Rashki Ghalehnoo under my direction in the Department of Microbiology and Genetics, University of Salamanca.

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All praise is due to Allah, who created the heavens and the earth and made the darkness and the light

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Acknowledgements

General characteristic of *Candida albicans*

Candida albicans, the major human fungal pathogen, undergoes a reversible morphological transition from single yeast cells to pseudohyphal and hyphal filaments (elongated cells attached end-to-end). Because typical *C. albicans* infections contain a mixture of these morphologies it has, for many years, been difficult to assess the relative contribution of each form to virulence. In addition, the regulatory mechanisms that determine growth in pseudohyphal and hyphal morphologies are largely unknown (Biswas *et al.*, 2007; Carlisle *et al.*, 2009).

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 (Hull & Johnson, 1999; Magee & Magee, 2000). Therefore, *C. albicans* has been classified as follows:

Kingdom: ..... Fungi
Phylum: .....*Ascomycota*
Subphylum: .....*Ascomycotina*
Class: .....*Ascomycetes*
Order: .....*Saccharomycetales*
Family: .....*Saccharomycetaceae*
Genus: .....*Candida*

Macroscopic and microscopic features

The colonies of *Candida* spp. are cream coloured to yellowish, grow rapidly and mature in three days (Fig. 1.1). The texture of the colony may be pasty, smooth, glistening or dry, wrinkled and dull, depending on the species (Aridogan *et al.*, 2005; Buschelman *et al.*, 1999).

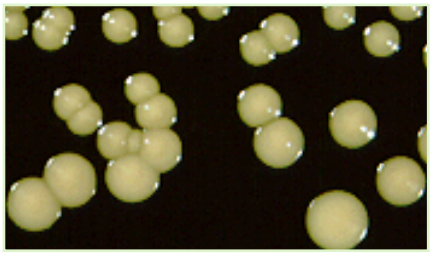

Figure 1.1 Macroscopic features of *C. albicans*

C. albicans is able to grow in two different ways; reproduction by budding, forming an ellipsoid bud, and in hyphal form, which can periodically fragment and give rise to new mycelia, or yeast-like forms (Kurzai *et al.*, 2005; Molero *et al.*, 1998; Sanchez-Martinez & Perez-Martin, 2001). Several factors have been identified that contribute, either positively or negatively for the transition from the yeast to hypha forms (Braun *et al.*, 2001). Many of these factors were identified on the basis of their homology to factors required for diploid pseudohyphal growth of *S. cerevisiae* (Khalaf & Zitomer, 2001). Transitions between the two phenotypes can be induced *in vitro* in response to several environmental cues such as pH, temperature or different compounds such as N-acetyl glucosamine or proline (Molero *et al.*, 1998).

In the laboratory, environmental conditions influence the morphological state of *C. albicans*. Serum causes blastospores to develop true hypha. High temperature (37°C), high ratio of CO_2 to O_2, neutral pH and nutrient-poor media also stimulate hyphal growth. Conversely, low temperatures, air, acidic pH, and enriched media promote blastospore growth (Cutler, 1991; Soares *et al.*, 2008).

Intermediate conditions can induce various pseudohyphal forms as well as true hypha (Fig. 1.2B and C). The transition from a commensally to pathogenic lifestyle may also involve changes in environmental conditions and dispersion within the human host. Although progress has been achieved in recent years, the molecular mechanisms governing these morphogenetic conversions are still not fully understood (Biswas *et al.*, 2007; Kurtz *et al.*, 1988). *C. albicans* can also be induced to undergo a complex morphological transition to form chlamydospores (Gow *et al.*, 2002; Odds *et al.*, 1988), which are generally defined as thick-walled asexual spores that are derived from a hyphal cell (Fig. 1.2D).

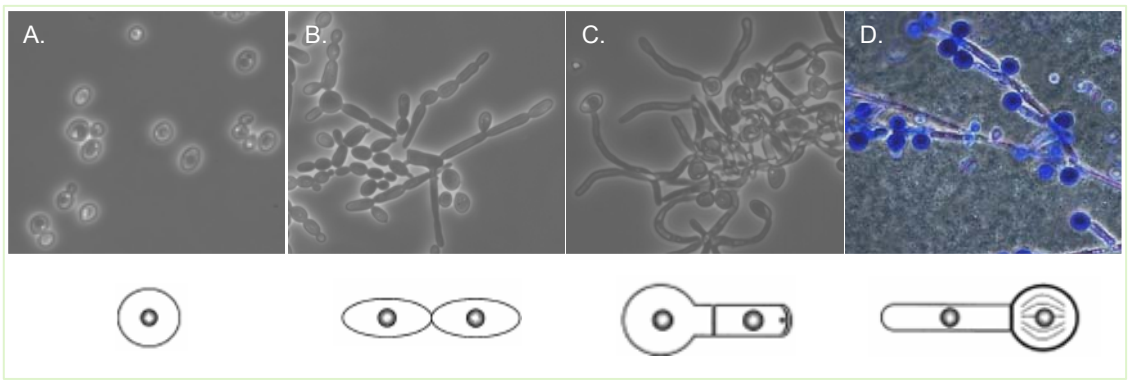

Figure 1.2 Growth of *C. albicans* yeast (A), pseudohypha (B), hypha (C) and chlamydospore (D)

The role of chlamydospores in the *C. albicans* life cycle is not understood, as they are rarely observed at sites of infection, and there is no evidence that they confer long-term

viability (Jansons & Nickerson, 1970; Martin *et al.*, 2005). However, a specific unknown role is suggested by the fact that the only *Candida* species that form chlamydospores are *C. albicans* and *Candida dubliniensis*. In fact, chlamydospore formation is an important diagnostic tool for distinguishing between different *Candida* species, since the majority of *C. albicans* clinical isolates retain the ability to form chlamydospores (Al-Hedaithy & Fotedar, 2002; Al Mosaid *et al.*, 2003; Nobile *et al.*, 2003). These observations raise the possibility that this conserved process contributes an advantage for growth as either a commensal or a pathogen in human hosts. Early studies of chlamydospores in *C. albicans* were limited to descriptions of their growth, development, and ultrastructural features (Acikgoz *et al.*, 2004; Fotedar & Al Hedaithy, 2004; Martins-Nishikawa *et al.*, 2002; Odds *et al.*, 1998; Silva *et al.*, 2003; Tekeli *et al.*, 2005; Yang *et al.*, 2003). The empirically determined conditions that favour chlamydospore production were found to include growth in the dark, microaerophilic conditions, room temperature, and nutrient-poor media containing complex carbohydrates. These conditions first trigger cells to grow in long filamentous chains; elongated suspensor cells then develop by branching off of the filaments, and then large rounded chlamydospores (8 to 12 μm in diameter) form at the end of the suspensor cells. In some cases, a chlamydospore can also form at the terminus of a filament. The mature chlamydospores contain a nucleus and other organelles but are distinguished by the presence of large lipid droplets (Kues, 2000), high RNA content (Vidotto *et al.*, 1996), and a thick outer layer of cell wall that is contiguous with the suspensor cell (Jansons & Nickerson, 1970).

The genome of *Candida albicans*

Candida albicans, one of the first eukaryotic pathogens selected for genome sequencing, is the most commonly encountered human fungal pathogen, causing skin and mucosal infections in generally healthy individuals and life-threatening infections in persons with severely compromised immune function.

The many clinical isolates of *C. albicans* used for laboratory study and genetic analysis are generally diploid and exhibit considerable natural heterozygosity and some have translocations in their genomes (Calderone *et al.*, 2000; Chibana *et al.*, 2000). Although mating governed by a mating-type-like locus can occur, a meiotic phase for the sexual cycle remains obscure and, unlike most species chosen for sequencing, a haploid or homozygous form for *C. albicans* is not available (Hull *et al.*, 2000; Tzung *et al.*, 2001). Strain SC5314 (Fonzi & Irwin, 1993), was chosen for large-scale sequencing because

of its widespread and increasing use in molecular analyses, virulence in animal models, and apparent standard diploid electrophoretic karyotype.

The Candida Genome Sequencing Project started in 1996 and in 2004, it produced a diploid assembly constructed from 10.9× coverage (Assembly 19), which provided single contigs where heterozygosity was not obvious and allelic contigs where there was significant heterozygosity (Jones *et al.*, 2004). The first was the construction of a physical map of one chromosome, chromosome 7 (Chibana *et al.*, 1994). Nucleotide sequence data for Assemblies 5, 6 and 19 of the *C. albicans* strain SC5314 genome sequence were retrieved from the Stanford Genome Technology Center (SGTC) website (http://www-sequence.stanford.edu/group/candida/) (d'Enfert *et al.*, 2005). CandidaDB (http://genodb.pasteur.fr/CandidaDB) was established in 2002 to provide the first genomic database for the human fungal pathogen *Candida albicans*.

The new version of CandidaDB houses the latest versions of the genomes of *C. albicans* strains SC5314 and WO-1 along with six genome sequences from species closely related to *C. albicans* that all belong to the CTG clade of *Saccharomycotina* - *Candida tropicalis*, *Candida* (*Clavispora*) *lusitaniae, Candida* (*Pichia*) *guillermondii*, *Lodderomyces elongisporus*, *Debaryomyces hansenii*, *Pichia stipitis* and the reference *Saccharomyces cerevisiae* genome (Rossignol *et al.*, 2008).

CandidaDB includes sequences coding for 5417 proteins with annotations collected from other databases, enriched with illustrations of structural features and functional domains and data of comparative analyses. In order to take advantage of the integration of multiple genomes in a unique database, new tools using pre-calculated or user defined comparisons have been implemented that allow rapid access to comparative analysis at the genomic scale (Rossignol *et al.*, 2008). The main facts and figures of the *C. albicans* genome sequence are as follows. Eight chromosomes (historically named 1-7 and R) constitute a haploid genome size of 14,851 kilobases (kb), containing 6,419 open reading frames (ORFs) longer than 100 codons, of which some 20% have no known counterpart in other available genome sequences. The codon CUG, which is translated abnormally by *C. albicans* as serine rather than leucine, is found at least once in approximately two-thirds of ORFs (Odds *et al.*, 2004).

Assembly 19, the diploid assembly of the genome of *C. albicans* strain SC5314 was a very important achievement. It provided a great deal of insight into many aspects of genomic organization, especially the large amount of heterozygosity (Newport & Nejentsev, 2004). The subsequent annotation of the assembly by the community demonstrated a number of important properties of the genome, including the number of genes (6,354), the number with introns (224), the frequency and characteristics of short

tandem repeats, and the characteristics of several multigene families (Braun *et al.*, 2005), also identified putative spurious genes and genes either on overlapping contigs or truncated by the end of contigs. However, they did not address chromosome location nor try to join the 266 haploid contigs of Assembly 19 into chromosome-sized assemblies. Thus, although these two projects brought the *C. albicans* genome to a very useful state, they still left it incomplete, lacking chromosome-size contigs and with some genes in an ambiguous state. Subsequently, Chibana et al (Chibana *et al.*, 2005) completed the sequence of chromosome 7, identified 404 genes, and compared the synteny to the *S. cerevisiae* genome. The completed Assembly 21 contains 15.845 Mb of DNA. The assembly does not include the complete telomeric sequences for every chromosome, and includes only one copy of the normally repeated rDNA on chromosome R (Braun *et al.*, 2005). The ORF analysis of Assembly 21 is being carried out at the Candida Genome Database (van het Hoog *et al.*, 2007).

Genetic manipulation with *C. albicans*

C. albicans has a nuclear genome of about 16 Mb, 33% larger than that of *S. cerevisiae.* Many genes are conserved between S. *cerevisiae* and *C. albicans*, and based on those similarity the mechanisms of many biological processes in *C. albicans* have been proposed. In many cases, although the specific components of relevant signalling pathways are conserved, molecular mechanisms and environmental signals have often diverged, most likely because of the coevolution of *C. albicans* and its human host. *C. albicans* poses special problems for scientists interested in studying gene function because it is diploid and because CUG is translated into a serine instead of a leucine (Kurtz *et al.*, 1988). To analyze the function of a gene, both alleles must be disrupted. Although methods of transformation (spheroplast-polyethylene glycol, lithium acetate and electroporation) are well established, the transformation efficiency is poor. For the study of gene function in *C. albicans*, a number of disruption protocols and selectable markers are commonly utilized (Tournu *et al.*, 2005).

The most widely used marker is the *URA3* gene, which encodes Orotidine-5'-phosphate decarboxylase that confers uracil prototrophy. However, this marker must be used with caution; *URA3* expression levels can affect virulence and are susceptible to chromosome position effects, thereby complicating the analysis of strains constructed with *URA3* as a selectable marker (Cheng *et al.*, 2003; Sharkey *et al.*, 2005; Staab *et al.*, 2003). To avoid the use of the *URA3* marker, various other auxotrophic markers, as well as dominant markers, have been developed and are

currently used (Morschhauser *et al.*, 2005; Noble & Johnson, 2005; Reuss *et al.*, 2004; Shen *et al.*, 2005).

To study the functions of essential genes, the *C. albicans MET3* promoter, which is regulated by the level of methionine and/or cysteine in the medium, or the tetracycline on/off system, can be used. These systems allow conditional expression so that the consequences of depletion of a gene product may be investigated (Care *et al.*, 1999; Park & Morschhauser, 2005; Saville *et al.*, 2003). In order to study the expression of certain genes at either the RNA level or the protein level, a number of *C. albicans*-optimized reporters construct, or tags, have been generated (Berman & Sudbery, 2002). The development of these molecular tools has greatly accelerated the elucidation of morphogenesis and pathogenesis in *C. albicans.*

Interest of study in the genome profile of *Candida albicans* morphogenesis

The fact that the fungi are eukaryotic organisms with simple and fast development and that are easier to manipulate than pluricellular organisms have stimulated the use of some species of these organisms as model of genetic studies in molecular biology. Morphogenesis can be defiened as the transition between unicellular yeast cells to a filamentous growth form.

With regard to morphogenesis, there is a long history of attempts to prove a relationship between the filamentous form of *C. albicans* and virulence. One of the most interesting features of the *C. albicans* genome is the occurrence of numeric and structural chromosomal rearrangements as means of generating genetic diversity, named chromosome length polymorphisms (contraction/expansion of repeats), reciprocal translocations, chromosome deletions and trisomy of individual chromosomes. These karyotypic alterations lead to changes in the phenotype, which is a strategy of adaptation the fungus.

The availability of the genome sequence has paved the way for the implementation of post-genomic approaches to the study of *C. albicans*: macroarrays and then microarrays have been developed and used to study the *C. albicans* transcriptome; proteomics has also been developed and complements transcriptional analyses; furthermore, systematic approaches are becoming available to study the contribution of each *C. albicans* gene in different contexts. The genome of *C. albicans* is highly dynamic and this variability has been used advantageously for molecular epidemiological and population studies in this species. A remarkable discovery that has arisen from the genome sequence is the presence of a parasexual cycle in *C. albicans*. This parasexual cycle is under the control of mating-type loci and switching between

white and opaque phenotypes. Investigating the role that the mating process plays in the dynamics of the *C. albicans* population or in other aspects of *C. albicans* biology and pathogenicity will undoubtedly represent an important focus for future research (Rossignol *et al.*, 2008). Additional interest in the molecular mechanisms of *C. albicans* morphopathogenic determinants originated from the necessity of identifying new drug targets due to increased drug resistance in clinical isolates.

There is hope that recently developed techniques of manipulating *C. albicans* and the sequencing of its whole genome will lead to a thorough understanding of its virulence and biology, thus offering the possibility of a knowledge-based approach to the development of novel antifungal agents. A major strategy for determining virulence genes as molecular targets for antifungal drugs and vaccines is to identify a specific biochemical or structural target unique to *C. albicans* (or to fungi in general) in an attempt to specifically and selectively disrupt them and determine their effects on virulence.

Relation between morphology and cell cycle

The cell cycle and its role in morphogenesis in *C. albicans* remain uncertain. However, some factors involved in the cell cycle have been reported to influence hyphal morphology. One of three homologues of G1 cyclin, Ccn1p, is necessary to maintain hyphal growth under certain conditions (Loeb *et al.*, 1999a; Loeb *et al.*, 1999b). Another G1 cyclin homologue, Hgc1p, is essential for hyphal formation but not required for the expression of HSGs (Zheng *et al.*, 2004). The other G1 cyclin homologue, Cln3p, is not only essential for budding, but negatively regulates yeast-hypha transition (Bachewich & Whiteway, 2005).

The two homologues of B type cyclins, Clb2p and Clb4p, affect *C. albicans* morphogenesis by negatively regulating polarized growth (Bensen *et al.*, 2005). While cell elongation of pseudohyphal growth in *S. cerevisiae* is regulated by a change in the cell cycle (Kron *et al.*, 1994), control of hyphal elongation in *C. albicans* appears to be independent of the cell cycle, as shown by several lines of evidence. The timing of cell cycle-regulated events, including the phosphorylation state of the tyrosine-18 of the *C. albicans* Cdc28p, spindle pole body duplication, spindle elongation, rearrangement of the actin cytoskeleton, actin structure, chitin ring formation and nuclear division are identical between synchronous yeast and hyphal apical cells (Hazan *et al.*, 2002) (Fig. 1.3).

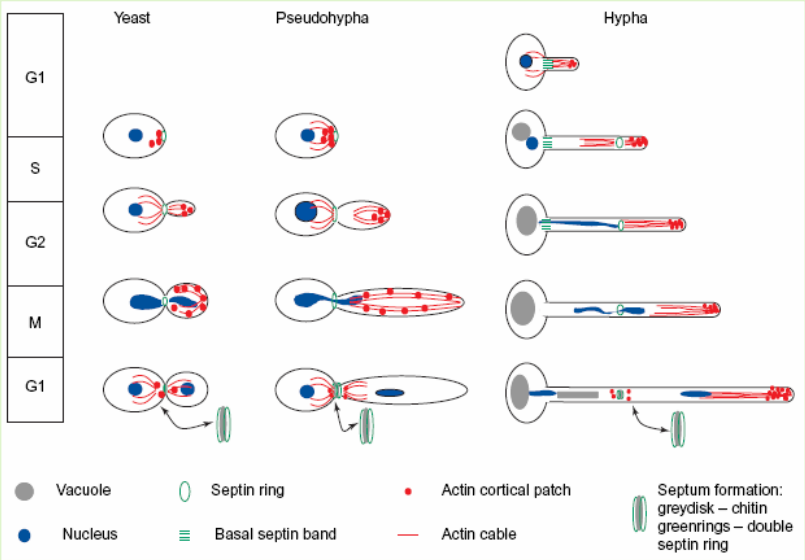

Figure 1.3 Representations of the cell cycles of yeast, pseudohypha and hypha (Sudbery *et al.*, 2004)

Several observations have reported that perturbations of the cell cycle induced a polarized growth of *C. albicans*. Deletion of *CaFKH2*, a homologue of the transcription factor *FKH2*, caused pseudohyphal growth (Bensen *et al.*, 2002). Repressing the G1 cyclin Cln3p under yeast growth conditions caused the yeast cells to arrest in G1, increase in size and then develop into hypha and pseudohypha (Bachewich & Whiteway, 2005). Cells depleted of the essential B-type cyclin Clb2p grew highly elongated filaments, while the deletion of the nonessential B-type cyclin Clb4p showed constitutive pseudohyphal growth (Bensen *et al.*, 2005). Cells lacking the cell cycle regulatory polo-like kinase *CDC5* formed hyphal-like filaments under yeast growth conditions (Bachewich *et al.*, 2003). The essential gene *MCM1* was identified as one of the *Candida* genes that were able to interfere with *FLO11* regulation, which is required for pseudohyphal development, over-expresstion of the gene in *S. cerevisiae*, and the depletion of CaMcm1p leads to constitutive induction of hypha (Rottmann *et al.*, 2003). Studies from *S. cerevisiae* indicate that cell morphogenesis may involve cell cycle regulation by cyclin-dependent kinase. *C. albicans* cells can switch from unicellular yeast growth to an alternate growth program that generates linear chains of elongated cells with no constrictions at the site of the septa (Hazan *et al.*, 2002). During hyphal growth in *C. albicans*, cell surface expansion is restricted to a small region at the hyphal tip. This apical growth zone is active during the entire hyphal growth period (Staebell & Soll, 1985). In contrast, yeast-form cells expand from a small area in a mostly apical manner only at the initial stage of budding. When the bud has reached a critical size, apical growth shuts down and general (isotropic) expansion takes place (Staebell & Soll, 1985). The localization of the actin cytoskeleton in yeast and hyphal

cells reflects these differences in morphogenesis. Polarization of the actin cytoskeleton to the hyphal tip is observed in all hyphal cells. However, in yeast-form cells, the cortical actin patches are observed at the area of apical expansion only in small budded cells but not in large budded cells (Anderson & Soll, 1986). It has been suggested that the actin cytoskeleton is essential for polarized apical growth because chloropropham, a drug affecting actin microfilament organization, has been shown to inhibit hyphal growth (Yokoyama *et al.*, 1994).

Types of pathogenesis caused by *Candida albicans*

According to Odds (Odds *et al.*, 1987), most people usually carry a single strain of *Candida* at different body sites for a long time, for instances *Candida dubliniensis* can be found in the oropharynx or in the upper respiratory tract (Sullivan *et al.*, 2005), while *C. albicans* is a normal component of the oral cavity (Barros *et al.*, 2008), the gastrointestinal tract, and the vaginal environment (Chabrier-Rosello *et al.*, 2008; Pereira-Cenci *et al.*, 2008), but this organism is also an opportunistic pathogen, commonly causing infections such as denture stomatitis, thrush, and urinary tract infections. *C. albicans* can also cause more serious systemic infections. Often these infections are transmitted in hospitals and are much more common in immunocompromised patients. *C. albicans* infection rates have increased by over a factor of twenty during the past two decades, in a large part resulting from an increase in immunocompromised persons. Mortality associated with systemic *Candida* infections is approximately 35-50% (Darwazeh *et al.*, 1990; Wey *et al.*, 1988). Approximately one half of hospital-acquired *C. albicans* infections are associated with biofilms formed on an implanted medical device, such as a central venous catheter, urinary catheter (Dominic *et al.*, 2007; Trofa *et al.*, 2008), endotracheal tube, prosthetic heart valve, pacemaker, or joint replacement. These devices provide a route through the body's barrier defences and also provide a surface for cell growth and development (Huth *et al.*, 2009; Opilla, 2008). Furthermore, oropharyngal candidiasis occurs in approximately 70% of patients with AIDS and 70% of all women (with or without AIDS) will experience at least one episode of vaginitis caused by *Candida* and 20% will experience recurrent disease.

Virulence factors of *C. albicans*

It is generally accepted now that dimorphism, i.e. the ability to grow in a yeast and a elementals growth form, not only is a virulence trait per se, but is also co-regulated with

other virulence factors, which are associated with cellular morphology (Hube, 1996; White & Agabian, 1995).

The *C. albicans* hyphal form is often found at sites of tissue invasion, and cells that do not readily form hypha often have reduced virulence (Odds *et al.*, 1988). Importantly, other *Candida* spp. that does not readily form true hypha are much less frequently isolated from the human host, indicating that they are less virulent (Braun *et al.*, 2000). It is generally thought that hyphal cells expressing cell-wall proteins that facilitate adhesion to human tissues are important for tissue invasion, as well as for escape from phagocytosis mediated by neutrophils or macrophages (Fig. 1.4). By contrast, the yeast form is thought to be important for dissemination of the pathogen through the blood stream. It is likely, therefore, that the ability to switch between the morphological forms is important for *C. albicans* virulence.

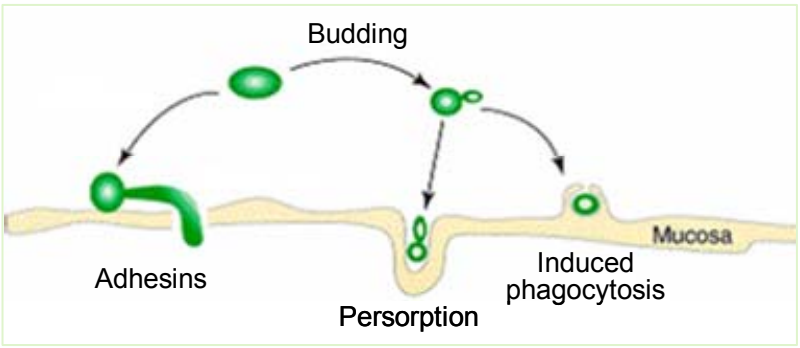

Figure 1.4 Early events in the pathogenesis of candidiasis are portrayed on a mucosal surface. A yeast cell of *Candida albicans* is shown either budding (right) or germinating (left). At the mucosal surface, germination of yeast cells (left) and penetration of the mucosa is shown.

Virulence depends on opposing reactions between host and pathogen and is intrinsically linked to the host immune status. Virulence factors rely upon microbial attributes that mediate cell damage. Activity of several *C. albicans* hydrolytic enzymes is well characterized. For example extracellular lipase released by *C. albicans* directly induced cytotoxicity and promoted the deposition of lipid droplets in the cytoplasm of macrophages and hepatocytes (Paraje *et al.*, 2008). Additionally, a membrane protein (α-1,2-mannosyltransferase) that is required for both O and N-mannosylation in fungi might be required for host recognition. Molecules involved in host cell recognition found in *Candida* are adhesions that bind to several extracellular matrix proteins of mammalian cells, such as fibronectin, laminin, fibrinogen and collagen. Adhesion function (e.g. to human buccal epithelial cells) is provided by agglutinin-like sequences of *Candida*, resembling the *S. cerevisiae* α-agglutinin protein that is required for cell-cell recognition during mating. Another potential adherence protein of *Candida* is reminiscent of the integrin family of mammalian cell receptors. Enzymes that contribute

to invasiveness are the secreted aspartyl proteinases and phospholipases which form rather large families in *C. albicans* (Haynes, 2001).

In fact, its ability to adhere to host tissues, produce secretory aspartyl protease and phospholipase enzymes and transform from yeast to hyphal phase are the major determinants of its pathogenicity.

Environmental cues regulate transcription factors

C. albicans morphology is directly related to environmental conditions. Hyphal development depends on two factors: (**i**) the nature, number and intensity of environmental signals (outside cues) and (**ii**) the activity of signalling pathways including key transcription factors (cellular-response machinery). According to current models, environmental cues trigger separate signal-transduction pathways, which regulate common targets required to initiate hyphal growth (Fig. 1.5). A threshold level of signalling may be reached either by simultaneous low-level stimulation of several pathways or by essentially a single pathway, which is fully activated (or hyperactive as in over-expression experiments).

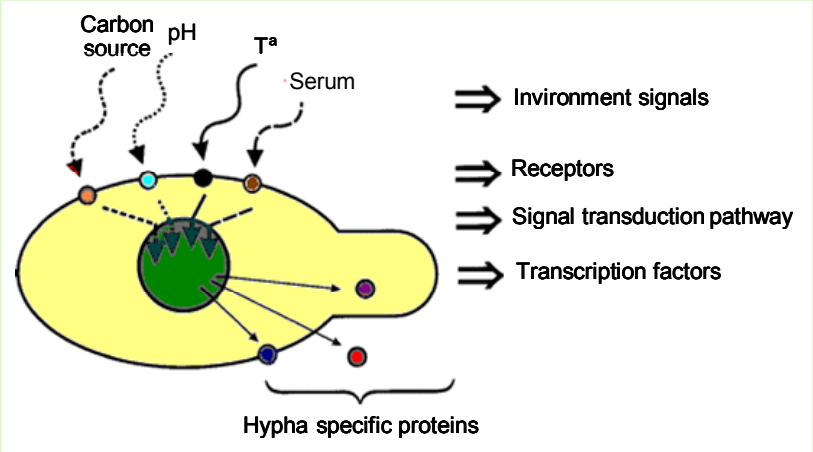

Figure 1.5 General scheme of hypha induction. Various environmental cues activate separate signalling pathways via sensor proteins. Signalling pathways may converge on separate or identical transcription factors and transcription factors may converge on common target genes to trigger expression of hypha-specific genes.

The most powerful hypha-inducing protocol involves incubation of *C. albicans* at low cell densities in liquid medium containing serum, GlcNAc or other inducers, at 37°C (Davis-Hanna *et al.*, 2008; Shepherd *et al.*, 1980). Because of strong stimulation of several pathways, minor defects in signalling pathways are not detected under this condition. Liquid induction is usually monitored for a few hours, when initial germ tubes have developed into hypha that begin to bud off yeast cells. In contrast, on solid media cells develop into colonies over several days of growth, until (pseudo-) hyphal growth

emerging from the colonies is recorded. The latter procedure is somewhat problematic, because (**i**) the molecular environment of a fully developed colony will differ greatly from the initial media composition, not allowing a clear definition of the inducing environment- in fact colonies `condition' their environment by depletion of nutrients and by secretion of fungal compounds and (**ii**) cells may element efficiently during initial growth, but may revert quickly to yeast growth and the phenotype of a grown colony will be determined by not only the yeast-hypha transition, but also the reverse process. Ernst (Ernst, 2000a) found that the use of 1% proline as a single carbon and nitrogen source in solid media strongly induced elements until microcolonies appeared, but growth later completely reverted to the yeast form, leading to a yeast-only appearance after several days.

Serum and other inducers

Serum of different sources is still the `magic potion' to rapidly induce true hypha in *C. albicans*. The responsible factor is not albumin, since albumin-free serum from a rat mutant was as efficient at promoting hypha as normal serum (Feng *et al.*, 1999). Two known inducers of hypha formation, N-acetylglucosamine (GlcNAc) and proline may contribute to the serum effect since they are generated by degradation of serum (glyco-) proteins.

Thus, several independent signalling pathways are likely to be triggered by serum. Hypha arises within minutes at 37°C at 5-20% serum in liquid; on solid media hypha development is triggered within a few hours. Numerous other compounds induce hypha *in vitro* (Odds *et al.*, 1988), but their significance for the infection process is unknown. The Efg1 protein is a strong regulator of morphogenetic processes in *C. albicans*, since it influences not only yeast-hypha inter-conversions (Lo *et al.*, 1997; Noffz *et al.*, 2008; Stoldt *et al.*, 1997), but also regulates phenotypic switching and chlamydospore formation of this pathogen (Sonneborn *et al.*, 1999a). The role of Efg1p in hyphal morphogenesis is fascinating, because under standard induction conditions, using serum or GlcNAc as inducers in liquid or on solid media, there is essentially a complete block of hyphal formation in strains lacking Efg1p (Noffz *et al.*, 2008; Stoldt *et al.*, 1997). On the other hand, under microaerophilic/embedded conditions hyphal formation is not defective at all in homozygous *efg1* mutants, but rather appears stimulated (Sonneborn *et al.*, 1999b).

Starvation

In *S. cerevisiae* nitrogen limitation stimulates pseudohyphal growth (Gimeno *et al.*, 1992). This morphogenesis is not detected in liquid medium, only on solid nitrogen-limiting medium (SLAHD); yeast colony first grows to a considerable size within several days and then lateral pseudohypha appear. *C. albicans* is also triggered on SLAHD medium to form filaments (true hypha) after several days' growth (Csank *et al.*, 1998). A similar phenotype occurs on a medium containing mannitol as a source of carbon and a complex source of nitrogen (modified Lee's medium or Spider medium) (Liu *et al.*, 1994). The late development of hypha on these media suggests that some media component has to be consumed before filamentation is triggered. It has been known for a long time that pre-growth of cells into stationary phase or a period of starvation enhances the efficiency of subsequent hyphal induction by inducers like serum or GlcNAc (Delbruck & Ernst, 1993; Shepherd *et al.*, 1980). The addition of ammonium salts to Spider medium did not block hyphal development, indicating that nitrogen limitation is not responsible for filamentation in this medium (Ernst, 2000a).

Components of the cell signalling pathways

Signal transduction networks permit cells to receive external stimuli and respond to the signals in an appropriate manner. Signal transduction describes a great number of biochemical events that transmit a signal from the cell exterior, through the cell membrane and through cytoplasm. This involves a number of molecules, including receptors, intermediate proteins and messengers. The signalling pathways are commonly used by an extensive array of biological ligands to modulate various cell processes, such as growth, differentiation and proliferation. These transduction pathways communicate information about the external environment to the inside of a cell. Signalling systems in fungi also regulate cell polarity, mating, pheromone control and hence they play a role in determining cell shape. Some of the known responses include changes in the cell cycle, polarized growth and modifications to the transcriptional profile of the cell.

Since the 50's, signalling pathways have been investigated by genetic and biochemical experimentation. In a large series of experiments, eukaryotic organisms were studied for their nutritional limitations and for their reactions to various environmental stresses, such as heat, oxidative, osmotic, or ethylic shock (Engebrecht, 2003). Pathogenic organisms sense and respond to the harsh conditions imposed by the host-activating components of signalling pathways that culminate in the expression of genes

responsible for the virulence and differentiation of the pathogen. Therefore, studies on signal transduction in various fungi may reveal common conserved mechanisms of signal transduction as well as the differences between these organisms.

- Mitogen-activated protein kinase cascades

The Mitogen-Activated Protein Kinase (MAPK) signalling pathways (Nordle *et al.*, 2007) play an important role in signal transduction in eukaryotic cells, where they modulate many cellular events including: mitogen-induced cell cycle progression through the G1 phase, regulation of embryonic development, cell movement and apoptosis, as well as cell and neuronal differentiation (Chen & Thorner, 2007; Murray *et al.*, 1998). These evolutionarily conserved pathways are organized in three-kinase modules consisting of a MAP kinase, an activator of MAP kinase (MAP Kinase Kinase or MEK) and a MAP Kinase Kinase Kinase (MEK Kinase, MEKK, or MAPK Kinase Kinase) (Arga *et al.*, 2007; Dohlman & Slessareva, 2006).

There are at least three distinct MAP kinase signal transduction pathways in mammalian cells, each named after the particular MAPK associated with it although in budding yeast *S. cerevisiae* has more than the three MAPK pathways (Levin-Salomon *et al.*, 2009). MAP kinases are proline-directed serine/threonine kinases that are activated by dual phosphorylation in response to diverse extracellular stimuli. The dual phosphorylation occurs in the activation domain of MAPKs on the threonine and tyrosine residues in the sequence pTXpY (Adam *et al.*, 2008; Alonso-Monge *et al.*, 2003; Escote *et al.*, 2004; O'Rourke *et al.*, 2002). The dual phosphorylation is facilitated by dual-specificity MAP kinase kinases (MAPKKs or MEKs), which in turn are activated by serine/threonine phosphorylation by MAP kinase kinase kinases (MEKK) (Fig. 1.6).

- The cAMP-activated protein kinase cascades

The cAMP-PKA pathway plays a very important role in filamentation in *S. cerevisiae, C. albicans* and other fungi (Lee *et al.*, 1975b; Lengeler *et al.*, 2000). Nitrogen starvation in *S. cerevisiae* results in the formation of elongated buds termed pseudohypha, which is dependent on activation of the cAMP pathway (Gimeno *et al.*, 1992; Kronstad *et al.*, 1998; Lengeler *et al.*, 2000). In *C. albicans*, an increase in cAMP levels accompanies the yeast-hypha transition, and inhibition of the cAMP phosphodiesterase induces this transition (Sabie & Gadd, 1992). Previous reports of cAMP levels during the yeast-hypha transition (Dhillon *et al.*, 2003; Maidan *et al.*, 2005; Maidan *et al.*, 2008; Roman

et al., 2007; Wolyniak & Sundstrom, 2007) are difficult to compare because of the differences in strains and in experimental conditions. Nonetheless, it is clear is that the cAMP signal is less pronounced in *C. albicans* than in *S. cerevisiae*. Our understanding of the *C. albicans* cAMP-PKA pathway is based on considerable work with budding yeast.

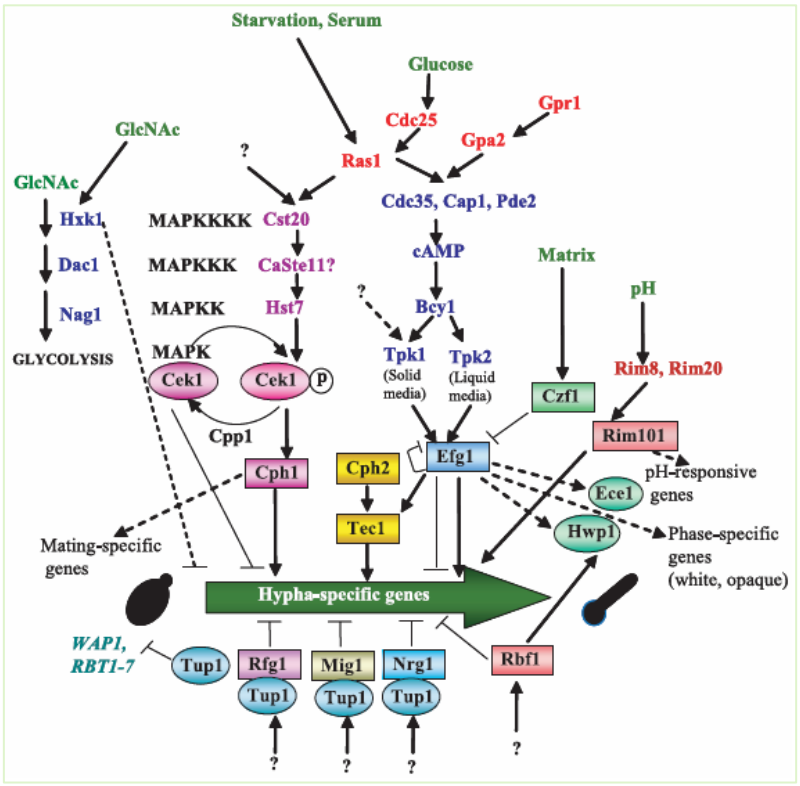

Figure 1.6 Regulation of dimorphism in *C. albicans* by multiple signalling pathways (Biswas *et al.*, 2007)

- pH induction pathway

C. albicans causes infections in a broad range of host niches, which show significant differences in ambient pH. For example, the mouse systemic pH is 7.3, whereas the pH in the rat vagina is 4.5. The ability of *Candida* to react appropriately to (among other environmental variables) rather different pH environments is crucial for its pathogenicity. The environmental pH acts as a manipulator for many physiological functions, including morphogenesis (Fonzi, 2002). Under optimal (37°C) temperature conditions, filamentation is favored by ambient pH values close to neutral and is considerably reduced at pH values lower than 6. In contrast, the yeast form predominates almost exclusively at pH 4 (Buffo *et al.*, 1984).

- Negative regulation of filamentation

Besides low pH, low temperature and high cell density, it is known that high glucose concentrations down-regulate hyphal development of *C. albicans* in liquid media. Glucose repression of morphogenesis is also observed initially during growth on solid media, although glucose consumption permits filamentation after several days of growth. On solid media, high osmolarity also inhibits hypha formation (Alex *et al.*, 1998). The presence of easily utilizable nitrogen sources, such as ammonium salts, modulates hyphal development negatively to some degree (Ernst, 2000a).

In the infected host, inhibition of filamentation by γ-interferon occurs at contact of *C. albicans* with lymphocytes (Kalo-Klein & Witkin, 1990; Levitz & North, 1996). Some chemical inhibitors of filamentation are known including diaminobutanone and some antifungals at low concentrations, such as amphotericin and azole antifungals; these compounds inhibit hyphal development by unknown mechanisms (Hawser, 1996; Hawser *et al.*, 1996; Martinez *et al.*, 1990). The Tup1 transcription factor may be involved in the hypha-repressed state in the presence of glucose and other non-inducing conditions. In *S. cerevisiae*, Tup1 protein regulates about 60 genes involved in glucose regulation, oxygen stress response and DNA damage. A *C. albicans* homologue of Tup1p was identified that is 67% identical to *S. cerevisiae* Tup1p (Braun & Johnson, 1997). Tup1p contains seven conserved WD40 repeats at the C-terminus, which could anchor Tup1p to some of its DNA-binding proteins and an N-terminal domain that could interact with a homologue of Ssn6p, as in *S. cerevisiae* (Keleher *et al.*, 1992; Komachi & Johnson, 1997). A homozygous *C. albicans tup1* mutant grew in the filamentous form in all media tested; filaments on most of the media had the characteristics of pseudohypha, although in some of them had the appearance of true hypha. Pseudohypha of a *tup1* mutant (unlike pseudohypha produced by *EFG1* over-expression) (Braun & Johnson, 1997; Stoldt *et al.*, 1997) could not be induced to form germ tubes or true hypha by the addition of serum (Braun & Johnson, 1997). Tup1p has other activities besides repression of mutants, because *tup1* mutants failed to grow at 42°C grew faster on glycerol and had misshapen cell walls compared to the wild-type.

Chromatin structure and nucleosomes

In all living cells the DNA is wrapped around proteins called histones, thus forming chromatin. The repeating subunit of the chromatin, the nucleosome, consists of 146 DNA nucleotides wrapped around the histone core, which carries one subunit of each

of the four histones: H2A, H2B, H3 and H4. The main function of the chromatin is to pack the DNA efficiently in the cell, but it was also shown to participate in crucial processes such as mitosis, replication, DNA damage and gene expression (Bakkenist & Kastan, 2004)(Fig. 1.7).

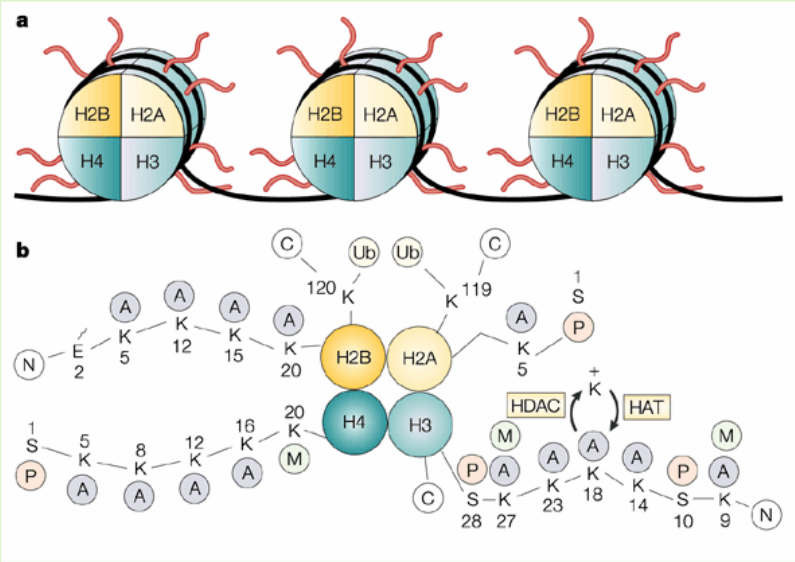

Figure 1.7 Nucleosome structure in eukaryotic cells

Much of the regulatory potential of the histones lies in their NH_2 termini. The NH_2 terminal tails, the first; 30 amino acids of each histone, are largely unstructured and contain high concentrations of lysine and arginine residues (Luger *et al.*, 1997). The physical characteristics of the NH_2-terminal tails are regulated by extensive post-translational modifications (Verreault *et al.*, 1998).

Histone acetyltransferase complexes

Changes in chromatin structure greatly influence all aspects of DNA metabolism. Remodeling of the chromatin structure at the nucleosomal level occurs by a variety of methods. One such remodeling is the direct covalent modification of histones, examples of which include acetylation, methylation, ubiquitination, and phosphorylation (Berger, 2007). A histone acetyltransferase (HAT) can be defined as an enzyme that acetylates core histones, which results in important regulatory effects on chromatin structure, assembly and gene transcription. The current understanding of HATs can be traced back over 40 years. In 1964, Allfrey and colleagues showed that histones can be modified by the addition of acetyl and methyl groups (Allfrey *et al.*, 1964). Over the course of the next 15 years, a number of studies correlated the acetylation of histones with gene activity (Brownell & Allis, 1996). Then, in 1979, Cano and Pestana isolated

protein fractions with HAT activity from the larvae of brine shrimp (Cano & Pestana, 1979). Another 15 or so years passed before the first HATs, Hat1p and Gcn5p, were isolated and cloned from *S. cerevisiae* (Brownell & Allis, 1996; Kleff *et al.*, 1995). Two years later, the first multi subunit nuclear HAT complex, SAGA (Spt–Ada–Gcn5–acetyltransferase), was isolated (Grant *et al.*, 1997). Over the past 10 years, the study of HATs has advanced significantly as they have become much more amenable to molecular and biochemical analysis. A number of HAT enzymes have been isolated from various organisms (Carrozza *et al.*, 2003). 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 (Kimura *et al.*, 2005), and the functions of the catalytic subunit depend largely on the context of the other subunits in those complexes (Utley & Cote, 2003). Furthermore, recent work on HAT complexes 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 (Yang, 2004). 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) (Ekwall, 2005; Robyr *et al.*, 2002). Histone acetylation is catalyzed by histone acetyltransferases (HATs), proteins that mediate the transfer of an acetyl moiety from acetyl CoA (acetyl-coenzyme A) to the ε-amino group of target lysine residues (Fig. 1.8).

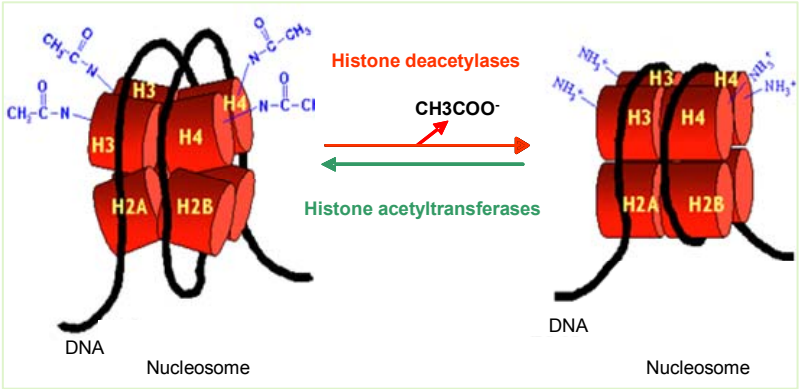

Figure 1.8 Histone acetyltransferases (HATs) remodeling. These enzymes add acetyl groups onto the basic amino acids in histone tails, which reduces the positive charge on the histone and weakens its binding to the DNA (which is negatively charged). This process can be reversed by histone deacetylases (HDACs), which remove the acetyl groups and cause the chromatin to recondense into the heterochromatin structure.

HATs that acetylate nucleosomes are usually components of large multi-protein complexes, including NuA4, SAS-C and SAGA. The enzymatic removal of these acetyl groups from lysines is performed by histone deacetylases (HDACs) (Fig. 1.8). Histones can also be post-translationally modified prior to their deposition into chromatin (Benson *et al.*, 2006).

The histone acetyltransferase superfamily

A large number of transcriptional regulators have been found to possess intrinsic HAT activity (Table 1.1) (Sterner & Berger, 2000).

Table 1.1 HAT families and their transcription-related functions **ACTR**-activator of thyroid and RA receptor; **HAT**-histone acetyltransferase; **MOF**-male absent on first; **MYST** -**MOZ**, Ybf2/Sas3, Sas2, Tip60; PCAF-p300/CBP associated factor; rOX-RNA on X; SRC-1- steroid receptor cofactor 1; not known (Lusser *et al.*, 2001).

HAT	HAT complex	Function	Organism
Gcn5-related N-acetyltransferases (GNAT)			
Gcn5	Ada, SAGA	Coactivator of transcription	Ubiquitous
Hat1	Complex with Rbap48	Cytoplasmic acetylation of H4	Ubiquitous
PCAF	PCAF complex	Coactivator of transcription	Mammals
Hpa2	-	Unknown	Yeast
Elp3	RNA polymerase II complex	Transcription (elongation)	Yeast
CBP/p300	Associates with different regulatory protein	Coactivator of transcription	Ubiquitous
Nuclear receptor coactivators			
ACTR		Coactivator of transcription	Mammals
SRC-1		Coactivator of transcription	Mammals
TIF2		Coactivator of transcription	Mammals
TAFII250	TFIID	Factor associated with TBP	Ubiquitous
MYST-family			
Sas3	Nun	Silencing	Yeast
Esa1	Nun	Cell cycle regulation	Yeast
MOF	MSL complex	Gene dosage compensation	Insects
MOZ		Malignant diseases	Human
Tip60	Tip60 complex	HIV-Tat interaction	Human
HBO1	HBO complex	Interacts with replication origin recognition complex	Human

Sequence analysis of these proteins reveals that they fall into distinct families that show high similarity within families but poor to no sequence similarity between families (Kuo *et al.*, 1998). Gcn5/PCAF family of HAT proteins (GNAT family) function as co-activators for a subset of transcriptional activators. This family depended on target site is classified as histone acetyltransferase A and histone acetyltransferase B.

Histone acetyltransferase A (*GCN5*)

GCN5 (General Control of Non-derepressed 5) was the first discovered protein capable of acetylating nuclear histones. *GCN5* was initially identified by Penn et al (Penn *et al.*, 1983) in a yeast mutation screen identifying genes involved in amino acid biosynthesis. Mutations were induced by UV-light irradiation and clones unable to grow in media deficient in preformed amino acids were considered to have disruptions in the regulation of their amino acid biosynthesis pathways. The screen lead to the discovery of 5 genes involved in the derepression (induction) and expression of proteins involved in the synthesis of histidine, arginine, tryptophan and methionine (Penn *et al.*, 1983). The genes were named *GCN1* through *GCN5*.

Eukaryotic *GCN5* acetyltransferases influence diverse biological processes by acetylating histones and non-histone proteins and by regulating chromatin and gene-specific transcription as part of multiprotein complexes. In lower eukaryotes and invertebrates, these complexes include the yeast ADA complex that is still incompletely understood; the SAGA (Spt-Ada-Gcn5 acetylase) complexes from yeast to *Drosophila* that are mostly co-activators; and the ATAC (Ada Two-A containing) complex, only known in *Drosophila* and also still poorly characterized.

In contrast, vertebrate organisms, express two paralogous *GCN5*-like acetyltransferases (*GCN5* and PCAF), which have been found so far only in SAGA-type complexes referred to here after as the STAGA (SPT3-TAF9-GCN5/PCAF acetylase) complexes (Wang *et al.*, 2008). It has been reported that in budding yeast 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 (Vernarecci *et al.*, 2008).

Histone acetyltransferase B (*HAT1* and *HAT2*)

In *S. cerevisiae* Hat1p, together with Hat2p and Hif1p, forms the histone acetiltransferase B complex. The functional significance of the association of Hif1p with the Hat1p/Hat2p complex is confirmed by the observation that *hif1* and *hat1* strains display similar defects in telomeric silencing and DNA double strand break repair. Functional analysis revealed that Hif1p is a novel histone chaperone that selectively interacts with histones H3 and H4. Hif1p is also a chromatin assembly factor,

promoting the deposition of histones in the presence of a yeast cytosolic extract. *In vivo*, the nuclear Hat1p/Hat2p/Hif1p complex is bound to acetylated histone H4, as well as histone H3. In eukaryotes Hat1p, has been linked to histone deposition, nucleosome formation and chromatin assembly during ongoing DNA replication and DNA repair, rather than to a more common function in the regulation of transcription (Ai & Parthun, 2004; Verreault *et al.*, 1998). Hat1p was originally identified as a cytoplasmic enzyme that acetylates free, but not chromatin-bound, histone H4 (Kleff *et al.*, 1995; Parthun *et al.*, 1996). Hat2p, a conserved and evolutionary protein, homolog of the human retinoblastoma binding protein Rba48p, acts as adaptor for high affinity binding of Hat1p to histone H4 (Kleff *et al.*, 1995; Parthun *et al.*, 1996), whereas Hif1p is a histone chaperone as mentioned above (Ai & Parthun, 2004).

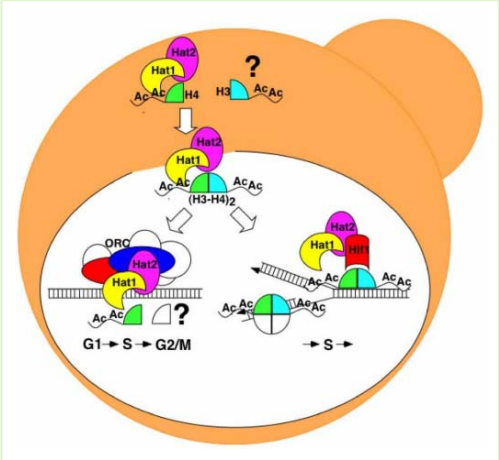

Figure 1.9 The current view on the different associations of Hat1p within the cell. In addition to the cytoplasmic Hat1p/Hat2p complex, the Hat1p/Hat2p-Hif1p chromatin assembly complex is recruited to chromatin during S-phase.

Hif1p is detected exclusively in nuclei and may accompany newly modified H3-H4 tetramers to facilitate incorporation into nascent chromatin during DNA synthesis (Suter *et al.*, 2007). The association of Hif1p with acetylated H4 requires for Hat1p and Hat2p to provide a direct link between type B histone acetyltransferases and chromatin assembly. In addition, the histone H4 associated with the nuclear Hat1p/Hat2p/Hif1p complex contains novel posttranslational modifications in the core domain. One site of core domain acetylation, lysine 91, lies at the interface between the H3/H4 tetramer and H2A/H2B dimers, and might be critical for the chromatin assembly (Ai & Parthun, 2004). In eukaryotes synthetic N-terminal histone H4 peptides found that whereas the HAT-B complex acetylates only Lys12, recombinant Hat1p is able to modify Lys12 and Lys5. Both Lys12 and Lys5 of soluble, non-chromatin-bound histone H4 are *in vivo* targets of acetylation for the yeast HAT-B enzyme. Moreover, co-immunoprecipitation

assays revealed that Lys12/Lys5-acetylated histone H4 is bound to the HAT-B complex in the soluble cell fraction. Both Hat1p and Hat2p, but not Hif1p, are required for the Lys12/Lys5-specific acetylation and for histone H4 binding. HAT-B-dependent acetylation of histone H4 was detected in the soluble fraction of cells at distinct cell cycle stages, and increased when cells accumulated excess histones. Strikingly, histone H3 was not found in any of the immunoprecipitates obtained with the different components of the HAT-B enzyme, indicating the possibility that histone H3 is not together with histone H4 in this complex (Poveda & Sendra, 2008).

Finally, the exchange of Lys for Arg at position 12 of histone H4 did not interfere with histone H4 association with the complex, but prevented acetylation on Lys5 by the HAT-B enzyme, *in vivo* as well as *in vitro* (Makowski *et al.*, 2001; Poveda & Sendra, 2008). Yeast strains deficient in either *HAT1* or *HAT2* exhibit no obvious growth deficiencies (Kleff *et al.*, 1995). However, gene silencing at telomeres is reduced when *hat1* or *hat2* mutant null alleles are combined with substitution mutations blocking acetylation of the amino terminal tail of histone H3 (Kelly *et al.*, 2000b). Moreover, *hat1* mutant in combination with N-terminal lysine substitution alleles in histone H3 confers hypersensitivity to DNA-damaging agents (Qin & Parthun, 2002). Similar phenotypes are recapitulated with deletions in the *HIF1* gene in combination with H3 tail mutants (Ai & Parthun, 2004). Collectively, these data point to the overlapping of acetylation of the N-terminal tail of histone H3 and Hat1p-mediated acetylation of histone H4 in chromosome dynamics.

In *C. albicans,* like other eukaryotes, *HAT1* and *HAT2* are conserved in other fungal species and that the most homologous regions are within the HAT and WD40 domain (Fig. 1.10 and 1.11). Many yeast genes encode histone acetytransferase proteins in *C. albicans* and their relative phenotype is totally unknown. Thus, our labotaroty have performed a phenotypic analysis of yeast strain carrying deletions of *HAT1, HAT2* and other genes encoding members of the HATs and HDACs family of *C. albicans.*

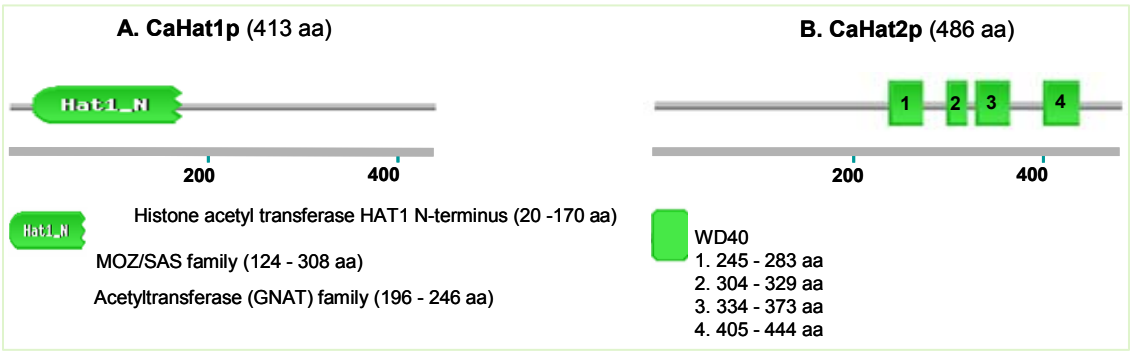

Figure 1.10 Structures of Hat1p (A) and Hat2p (B) in *C. albicans*

The *C. albicans* genome contains two ORFs that potentially encode histone acetyltransferase B complex. A search of the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/) indicates that a *Candida albicans* protein named Hat1 (orf19.705) shares the highest similarity with *Pichia guilliermondii* (62%), *Pichia stipitis* (69%), *Lodderomyces elongisporus* (69%), *Debaryomyces hansenii* (61%), *Saccharomyces cerevisiae* (57%), *Kluyveromyces lactis* (44%), *Candida glabrata* (44%), *Yarrowia lipolytica* (37%), *Schizosaccharomyces pombe* (33%) and *Homo sapiens* (26%). The GeneBank accession number for the *C. albicans HAT1* and *HAT2* nucleotide sequence are NW_139542 and NC_007436 respectively.

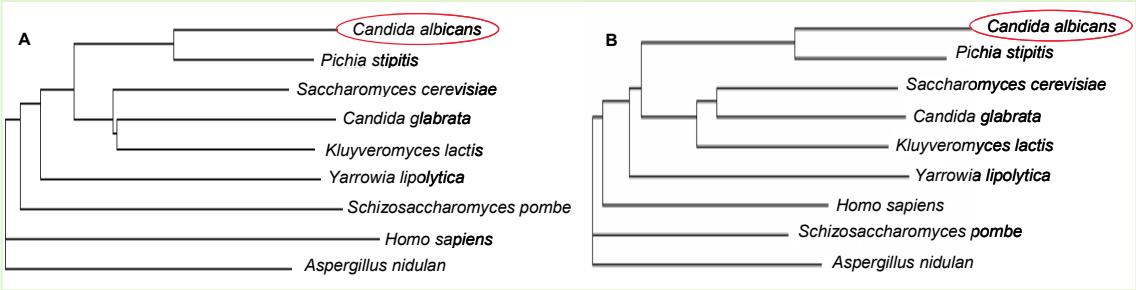

Figure 1.11 Dendograms representing the phylogenetic relationships of *C. albicans HAT1* (A) and *HAT2* (B)

Are type B histone acetyltransferases cytoplasmic or nuclear?

Cytoplasmic localization was one of the original defining characteristics of type B histone acetyltransferases. But are these enzymes really cytoplasmic? The idea that type B histone acetyltransferases are located in the cytoplasm arose from the fact that histone acetyltransferase activities, usually specific for free histone H4, are found in the soluble fraction following lysis of a variety of eukaryotic cell types. However, the fact that a protein does not pellet with nuclei, does not necessarily mean that it was not nuclear before cell lysis as many proteins have been shown to leak out of isolated nuclei (Decker *et al.*, 1987; Li & Kelly, 1984). The identification of Hat1p as a type B histone acetyltransferase provided the tools to more definitively determine the subcellular localization of this enzyme. In *S. cerevisiae*, evidence from a variety of different experiments suggests that the enzyme is actually both cytoplasmic and nuclear (Fig. 1.12).

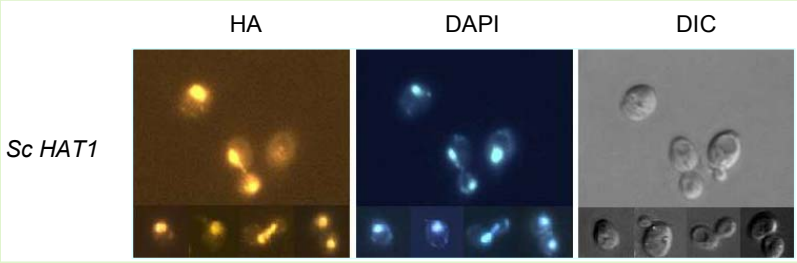

Figure 1.12 The subcellular localization of Hat1p in *S. cerevisiae* (Poveda *et al.*, 2004)

Deletion of the gene encoding *HAT1* not only eliminates the H4-specific histone acetyltransferase present in cytoplasmic extracts, but also causes the loss of an activity present in nuclear extracts as well (Parthun *et al.*, 1996; Ruiz-Garcia *et al.*, 1998). Also, distinct Hat1p/Hat2p-containing complexes can be purified from both cytoplasmic and nuclear extracts (Ai & Parthun, 2004; Poveda *et al.*, 2004). Localization of native Hat2p shows a strong concentration in the nucleus with a significant staining throughout the cytoplasm as well (Ai & Parthun, 2004) (Fig. 1.13).

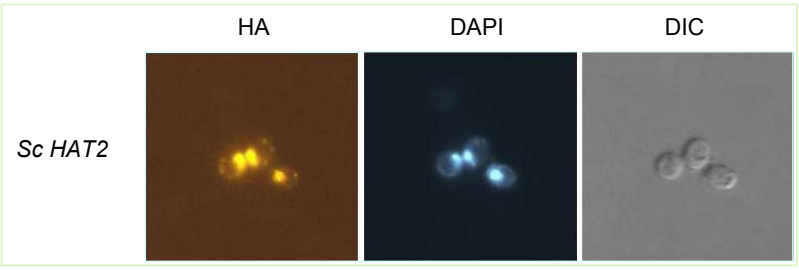

Figure 1.13 The subcellular localization of Hat2p in *S. cerevisiae* (Poveda *et al.*, 2004)

Localization of epitope-tagged Hat1p and Hat2p (expressed from their native promoters) shows a similar pattern with dense staining in nuclei and diffuse staining in the cytoplasm.

Histone deacetylase complexes

Histone deacetylases (HDACs) reverse chromatin acetylation and promote transcriptional repression and silencing. Dozens of proteins have been found to possess intrinsic HDAC activity (Cress & Seto, 2000; Grozinger & Schreiber, 2002). In *S. cerevisiae* histone deacetylases comprise a family of 10 genes, which are grouped into classes I-III based on their homology to their respective yeast orthologues. Class I, consist of 3 family members (ScRpd3p, ScHos1p and ScHos2p), Class II consist of 2 family members (ScHda1p and ScHos3p) and class III include of ScSir2p, ScHst1p, ScHst2p, ScHst3p and ScHst4p of family members. In the human genome histone

deacetylases comprise a family of 18 genes, which are grouped into classes I-IV based on their homology to their respective yeast orthologues. Classes I, II and IV consist of 11 family members, which are referred to as ‘‘classical” HDACs, whereas the 7 class III members are called sirtuins. The phylogenetic grouping of HDACs clearly shows that, in general yeast have fewer HDACs than humans. Because redundancy is often a problem in genetic analysis, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are excellent model systems for the study of HDACs.

The Sin3-histone deacetylase (HDAC) corepressor

Mammals possess two highly related Sin3 proteins, mSin3A and mSin3B, which serve as scaffolds tethering HDAC enzymatic activity, and numerous sequence-specific transcription factors to enable local chromatin regulation at specific gene targets (David *et al.*, 2008). *SIN3* was first identified genetically as a global regulator of transcription. Sin3p is a large protein composed mainly of protein-interaction domains, whose function is to provide structural support for a heterogeneous Sin3/histone deacetylase (HDAC) complex. In addition to HDACs, Sin3p can sequester other enzymatic functions, including nucleosome remodeling, DNA methylation, N-acetylglucosamine transferase activity and histone methylation. Since the Sin3/HDAC complex lacks any DNA-binding activity, it must be targeted to gene promoters by interacting with DNA-binding proteins. Although most research on Sin3p has focused on its role as a corepressor, mounting evidence suggests that Sin3p can also positively regulate transcription. Furthermore, Sin3p is a key to the propagation of epigenetically silenced domains and is required for centromere function. Thus, it provides a platform to deliver multiple combinations modifications to the chromatin, using both sequence-specific and sequence-independent mechanisms (Silverstein & Ekwall, 2005). In *C. albicans* Sin3p, a component of a specific histone deacetylase complex, is shown to bind to the *EFG1* promoter. The *sin3* mutants grew as budding pseudohypha and are unable to form true hypha, similar to strains constitutively expressing *EFG1*. It has been reported that the PKA signalling pathway, in addition to its importance in the initial steps of filament formation, is part of a feedback loop that controls *EFG1* expression allowing continued hypha formation in inducing conditions. This auto-regulation of *EFG1* expression is probably mediated through the Sin3p-containing histone deacetylation complex (Tebarth *et al.*, 2003). The Sin3p is optimized for multiple protein interactions with its four-paired amphipathic helices and a histone interaction domain (HID) (Fig. 1.14).

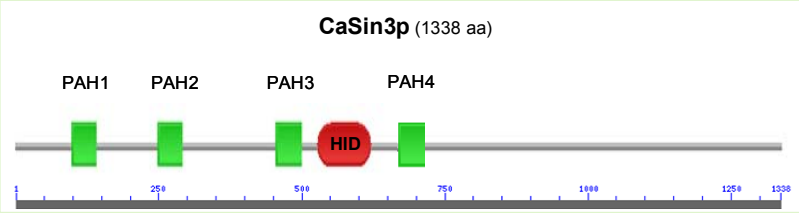

Figure1.14 Estructure of Sin3p in *C. albicans* contains four paired amphipathic helix (PAH) and HID domain.

The core of this complex, which is highly conserved from yeast to man, consists of eight components in mammals: SIN3, HDAC1, HDAC2, RbAp46, RbAp48, SAP30, SAP18 and SDS3. The yeast complexes contain only one HDAC each and seem to lack the SAP proteins. The resulting complexes provide specialized combinations of enzymatic specificities to the target chromatin. Sin3p has no intrinsic DNA-binding activity. Yet it must localize to a gene promoter if it is to exert any control over its transcriptional regulation. The targeting of the Sin3/HDAC complex is accomplished by flexibly interacting with DNA-binding proteins.

Sin3p is able to directly interact with transcription factors, although it sometimes requires an adaptor molecule. Co-repressors such as N-CoR and SMRT provide such a link between Sin3p and the nuclear hormone receptors. By regulating the interaction between Sin3p and its targeting proteins, the cell is able to tightly regulate gene expression. It also clearly has a role in silencing at both centromeres and the rDNA, as well as DNA-methylation-linked silencing. Other transcription-independent roles for Sin3p include influencing retrotransposon transposition frequency, replication timing and DNA repair. By providing a flexible scaffold for various chromatin modifying enzymes and DNA-binding proteins, Sin3 is able to globally regulate gene expression and to help maintain overall genome stability.

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

Microarray technology evolved from Southern blotting, where fragmented DNA is attached to a substrate and then probed with a known gene or fragment. The use of a collection of distinct DNAs in arrays for expression profiling was first described in 1987, and the arrayed DNAs were used to identify genes whose expression is modulated by interferon (Kulesh *et al.*, 1987). These early gene arrays were made by spotting cDNAs onto filter paper with a pin-spotting device. The use of miniaturized microarrays for gene expression profiling was first reported in 1995 (Schena *et al.*, 1995), and a complete eukaryotic genome (*S. cerevisiae*) on a microarray were published in 1997

(Lashkari *et al.*, 1997). Genomic research tools such as microarrays have provied to be important resources to study the complex regulation of genes that respond to environmental perturbations (Shaw *et al.*, 2007). It consists of an arrayed series of thousands of microscopic spots of DNA oliogonucleotides, called features, each containing picomoles of a specific DNA sequence. This can be a short section of a gene or other DNA element that are used as probes to hybridize a cDNA or cRNA sample (called target) under high-stringency conditions. Probe-target hybridization is usually detected and quantified by fluorescence-based detection of fluorophore-labeled targets to determine relative abundance of nucleic acid sequences in the target. The recently developed DNA microarray technology provides a powerful and efficient tool to rapidly compare the differential expression of a large number of genes. 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 (Liu *et al.*, 2005). Microarray analysis gives researchers the ability to identify genes involved in processes such as biofilm formation and 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. In human by cDNA microarray technology and methodology more focuses on their application in molecular classification of tumors, drug sensitivity and resistance studies and identification of biological markers of cancer. 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 (Carroll, 2000; Gompel *et al.*, 2005).

Table 1.2 Some of example about cDNA microarray analysis of differential gene expression in *C. albicans*

Analysis/publication	*Abstract of result*
DNA microarray based on arrayed-primer extension technique for identification of pathogenic fungi responsible for invasive and superficial mycoses (Campa *et al.*, 2008)	The microarray was tested for its specificity with a panel of reference and blinded clinical isolates. The APEX technique was proven to be highly discriminative, leading to unequivocal identification of each species, including the highly related ones *C. parapsilosis*, *C. orthopsilosis* and *C. metapsilosis*. Because of the satisfactory basic performance traits obtained, such as reproducibility, specificity, and unambiguous interpretation of the results, this new system represents a reliable method of potential use in clinical laboratories for parallel one-shot detection and identification of the most common pathogenic fungi.
Genome-wide expression profiling of the response to terbinafine in *Candida albicans* using a cDNA microarray analysis (Zeng *et al.*, 2007)	In this work A total of 222 genes were found to be responsive to terbinafine, including 121 up-regulated genes and 101 down-regulated genes. These included genes encoding membrane transport proteins belonging to the members of the ATP-binding cassette (ABC) or major facilitator superfamily (*MFS; CDR1, AGP2, GAP6, PHO84, HOL3*, *FCY23, VCX1*), genes involved in stress response and detoxification (*CDR1, AGP2, HOL3*), and gene involved in the ergosterol biosynthesis pathway (*ERG12*). The up-regulation of the gene encoding the multidrug resistance efflux pump *CDR1* may contribute to the terbinafine resistance in *Candida albicans.*
cDNA microarray analysis of differential gene expression and regulation in clinically drug-resistant isolates of *Candida albicans* from bone marrow transplanted patients (Xu *et al.*, 2006)	In this work more than 198 differentially expressed genes were identified and they were confirmed and validated by RT-PCR independently. Not surprisingly, the resistant phenotype is associated with increased expression of CDR mRNA, as well as some common genes involved in drug resistance such as *CaIFU5, CaRTA2* and *CaIFD6*. Meanwhile, some special functional groups of genes, including ATP binding cassette (ABC) transporter genes (*IPF7530, CaYOR1* and *CaPXA1*), oxidative stress response genes (*CaALD5, CaGRP1, CaSOD2* and IPF10565), copper transport and iron mobilization-related genes (*CaCRD1/2, CaCTR1/2, CaCCC2* and *CaFET3*) were found to be differentially expressed in the resistant isolates. Furthermore, among these differentially expressed genes, some co-regulated with *CaCDR1*, *CaCDR2* and *CaIFU5*, such as *CaPDR16* and *CaIFD6*, have a DRE-like element and may interact with *TAC1* in the promoter region. These findings may shed light on mechanisms of azole resistance in *C. albicans* and clinical antifungal therapy.
cDNA microarray analysis of differential gene expression in *Candida albicans* biofilm exposed to farnesol (Cao *et al.*, 2005)	A total of 274 genes were identified as responsive, with 104 genes up-regulated and 170 genes down-regulated. Independent reverse transcription-PCR analysis was used to confirm the important changes detected by microarray analysis. In addition to hyphal formation-associated genes (e.g., *TUP1, CRK1* and *PDE2*), a number of other genes with roles related to drug resistance (e.g., *FCR1* and *PDR16*), cell wall maintenance (e.g., *CHT2* and *CHT3*), and iron transport (e.g., *FTR2*) were responsive, as were several genes encoding heat shock proteins (e.g., *HSP70, HSP90, HSP104, CaMSI3* and *SSA2*). Further study of these differentially regulated genes is warranted to evaluate how they may be involved in *C. albicans* biofilm formation. Consistent with the down-regulation of the cell surface hydrophobicity-associated gene (*CSH1*), the water-hydrocarbon two-phase assay showed a decrease in cell surface hydrophobicity in the farnesol-treated group compared to that in the control group. Our data provide new insight into the molecular mechanism of farnesol against *C. albicans* biofilm formation.
Transcriptional and physiological adaptation to defective protein-O-mannosylation in *Candida albicans* (Cantero *et al.*, 2007)	Comparisons of genome-wide transcript patterns of each pmt mutant revealed commonly down-regulated genes involved in glycolysis and glycerol production. Increased phosphorylation of the Cek1p- but not the Mkc1p-MAP kinase, as well as increased transcript levels for some stress-related genes was detected in the *pmt1* strain but not in the other pmt mutants. The transcriptomal pattern after short-term inhibition of Pmt1p activity confirmed stress responses, but did not indicate an alteration of glycolytic flow. Short- but not long-term adaptation to Pmt1p inhibition required signalling components Cek1p, Mkc1p, Efg1p and Tpk1p. Cna1p (calcineurin) but not its downstream effectors Crz1p and Crz2p was generally essential to allow growth during Pmt1p inhibition; accordingly, cyclosporin A strongly inhibited growth of the *pmt1* mutant. The lack of Pmt isoforms influenced transcript levels for the remaining isoforms both positively and negatively, suggesting complex cross-regulation among PMT genes. These results confirm individual functions of Pmt isoforms but suggest a common biphasic adaptation response to Pmt deficiency. While known signalling pathways modulate adaptation for a short-term, long-term adaptation requires calcineurin, adjustments of remaining Pmt activities and of glycolytic flow.

Global transcriptional responses of *Candida albicans* to histone acetyltransferases deletion

Abstract

We have studied the behavior of a *C. albicans* which encode two histone acetyltransferases. We have deleted the *C. albicans HAT1* gene in a homozygous *hat2*Δ mutant genetic background and generated a *hat1*Δ*, hat2*Δ double mutant strain. The double mutant behaves as the *hat1*Δ simple mutant, respect to growth rate, hyphal outgrowth, chlamydospore formation and sensitivity to Calcofluor white, SDS, Congo red, Amphotericin B, Fluconazole, Itraconazole and Caffeine. In a location analysis by GFP-fusion to Hat1p and Hat2p, we have found that Hat1p and Hat2p are mainly localized in the nucleus and that location of Hat2p is dependent on the Hat1p.

We were also observed that the double mutant like *hat1Δ* simple mutant showed attenuated virulence in a mice model. We have carried out the transcriptional profile of the *hat2*Δ and *hat1Δ, hat2Δ* during both yeast mode of growth and during the yeast-hypha transition. We have also compared our results with those obtained with the *C. albicans hat1Δ* simple mutant. All the three mutants show a different transcriptional profile supporting the idea that *HAT1* and *HAT2* control different set of genes and that both acetyltransferases interact to regulate the expression of another set of *C. albicans* genes.

Introduction

In the eukaryotic organisms, acetylation of histone is a reversible process that depends on two different sets of enzymatic activities, histone acetyltransferases (HAT) and histone deacetylases (HDAC). This post-translational modification is involved in processes such as activation or repression of gene transcription, nucleosome assembly during replication, DNA repair and recombination or cell cycle and growth control (Carrozza *et al.*, 2003; Kurdistani & Grunstein, 2003; Rosaleny *et al.*, 2005). The yeast *HAT1* was the first HAT gene described (Kleff *et al.*, 1995) that specifically modifies Lys 12 of free histone H4 and has been implicated in the acetylation of cytoplasmic histone molecules required for post-replicative nucleosome assembly (Poveda & Sendra, 2008; Rosaleny *et al.*, 2005). In *Saccharomyces cerevisiae,* it has been demonstrated that HAT-B complex is mainly localized in the nucleus and that is composed of three proteins, Hat1p, Hat2p and Hif1p (Ai & Parthun, 2004; Parthun, 2007; Poveda *et al.*, 2004). Deletion of *HAT1*, in combination with specific histone H3 amino terminal tail mutations, results in a significant defect in telomeric silencing (Kelly

et al., 2000b). Hat2p acts as a bridge between Hat1 and Hif1 proteins (Poveda *et al.*, 2004) and is required for high affinity binding of Hat1p to histone H4 (Parthun *et al.*, 1996). It seems to be essential for all the functions carried out by Hat1p, because deletion of *HAT2* always produces similar defects to those of *HAT1* (Kelly *et al.*, 2000b; Parthun *et al.*, 1996; Ruiz-Garcia *et al.*, 1998). Deletion of *HIF1* displays similar defects to those described in telomeric silencing (Ai & Parthun, 2004; Poveda *et al.*, 2004) and in DNA double-strand break repair to those described for the deletion of *HAT1* (Ai & Parthun, 2004). At present, in *C. albicans* no unique phenotype has been specifically and directly associated to a particular HAT-B subunit. We wanted to investigate whether deletion of *HAT1* and *HAT2* play any role in the yeast-hypha transition or yeast mode of growth and virulence. We have observed that *hat1*Δ and *hat2*Δ mutants display a different phenotype and transcriptional profile. The *hat1*Δ*, hat2*Δ double mutant also display a different transcriptional profile of both simple mutants. All these results indicate that Hat1p and Hat2p have different functions and that very few overlaps exits between them.

Experimental procedures

Strains, media and culture conditions

The *S. cerevisiae* strains used were pJ69-4A and pJ69-4α, bearing the yeast two-hybrid system. The relevant genotypes of the strains used are listed in Table 2.1. For general purposes, yeasts were grown in 250-ml flasks containing 50 ml yeast peptone dextrose (1% Yeast extract, 2% Bactopeptone, 1% Glucose) or YNB (0.67% Yeast Nitrogen Base without aminoacids (YNB w/o aa, Difco), 1% Glucose) plus the required amino acids for plasmid maintenance. For the induction of hypha in *C. albicans*, 4% (v/v) bovine calf serum (GIBCO) was added to the culture media. The Lee, Spider and SLAHD media were prepared as described (Buffo *et al.*, 1984; Castilla *et al.*, 1998; Lee *et al.*, 1975a; Liu *et al.*, 1994). Yeast growth temperature was 28°C for general purposes and 37°C for expression of the phenotype in the mutant strains or induction of the filamentous morphology.

Table 2.1 Strains and plasmids used in this study

	Relevant genotype or characteristics	Parental strain	Reference
Strains			
SC5314	Clinical isolated	-	(Gillum, 1984)
CAI4	*ura3Δ::imm434/ura3Δ::imm434*	CAF2-1	(Fonzi and Irwin, 1993)
CAMR	*ura3Δ::imm434/URA3*	CAI4	(Rodrigues and Dominguez, 2005)
RDH4	*hat2Δ::hisG /hat2Δ::hisG ura3Δ:: imm434/ura3Δ:: imm434*	CAI4	(Degano and Dominguez, 2005)
RGL4	*hat1Δ::hisG /hat1Δ::hisG ura3Δ:: imm434/ura3Δ:: imm434*	CAI4	(Degano and Dominguez, 2005)
MAH1	*hat1Δ::hisGURA3hisG/HAT1 ura3Δ:: imm434/ura3Δ:: imm434 hat2Δ:: hisG /hat2Δ::hisG*	RDH4	present work
MAH2	*hat1Δ::hisG/HAT1*	MAH1	present work
MAH3	*hat1Δ::hisG/hat1Δ::hisGURA3hisG ura3Δ:: imm434/ura3Δ:: imm434 hat2Δ:: hisG /hat2Δ::hisG*	MAH2	present work
MAH4	*hat1Δ::hisG/hat1Δ::hisG ura3Δ:: imm434/ura3Δ:: imm434 hat2Δ:: hisG /hat2Δ::hisG*	MAH3	present work
MAHR1	*hat1Δ::hisG /hat1Δ::hisG hat2Δ::hisG /hat2Δ::hisG ura3Δ:: imm434/URA3*	MAH4	present work
RDHR9	*hat2Δ::hisG /hat2Δ::hisG ura3Δ:: imm434/URA3*	RDH4	present work
RGLR6	*ura3Δ::imm434/URA3 hat1Δ::hisG /hat1Δ::hisG*	RGL4	present work
PJ69-4A	*MATa trp1-901ura3-52 his3-200 leu2-3,112 gal4Δgal80Δ GAL2-ADE2 LYS2::GAL1-HIS·met2::GAL7-lacZ*		(James *et al*., 1996)
Plasmids			
pAG1	Plasmid containing the ampicillin resistance gene, the *C. albicans ACT1* promotor fused to the GFP protein (with *Sma*I/*BamH*I places), the *LEU2* and *URA3* genes and the *ARS2* sequence.	pIR4	(Novo *et al*., 2004)
pGEM-T	Plasmid containing ampicillin resistance gene, T7 and SP6 RNA Polymerase transcription initiation site, T7 and SP6 RNA Polymerase promoter, multiple cloning site, *lacZ* start codon, lac operon sequences, lac operator, β-lactamase coding region, phage f1 region, binding site of pUC/M13 Forward Sequencing Primer, binding site of pUC/M13 Reverse Sequencing Primer.		Promega (Robles, 1994)
pMR1	Plasmid containing the reinsertion cassette of the *URA3* gene.		(Rodriguez and Dominguez, 2005)
pZRG1	Plasmid containing the *HAT2* gene under control of the *ACT1* promotor fused in frame to the GFP protein (in the *Sma*I site).		present work
pAHR1	Plasmid containing the *HAT1* gene under control of the *ACT1* promotor fused in frame to the GFP protein (in the *Sma*I site).		present work
pGBD-C	Plasmid containing the *S. cerevisiae ADH1* promotor, *GAL4* DNA-binding domain polypeptide, *TRP1* coding sequence, Ampicillin resistance gene.		(James *et al*., 1996)
pROS1	Plasmid containing the *S. cerevisiae ADH1* promotor, *GAL4* DNA-binding domain polypeptide, *TRP1* coding sequence, Ampicillin resistance gene, *HAT2* gene.		present work
pGAD-C	Plasmid containing the promoter fragment carrying the truncated *S. cerevisiae ADH1* promotor, *GAL4* activation domain polypeptide, *LEU2* coding sequence, Ampicillin resistance gene.		(James *et al.,* 1996)
pMAH1	Plasmid containing the promoter fragment carrying the truncated *S. cerevisiae ADH1* promotor, *GAL4* activation domain polypeptide, *LEU2* coding sequence, ampicillin resistance gene, *HAT2* gene.		present work
pJBR3	Hat1 promoter and terminator flanked in *URA3*-marked pSNC1 vector		(Degano and Dominguez, 2000)

Strain construction

Plasmid pJBR3 was constructed in our laboratory (Degano, Ph.D thesis in process). Gene disruption was performed by the "Ura-blaster" protocol (Fonzi and Irwin, 1993). *C. albicans* CAI4 genomic DNA was used as template for PCR using the oligonucleotides (Table 2.2). A 5.1-kb *Not*I-*Xho*I fragment was isolated and used to transform strain RDH4 (*hat2*Δ). Correct integration of the cassette into the *HAT1* locus of the *URA3* ⁻ transformants was verified by PCR and Southern blot analysis. Spontaneous *URA3* ⁻ derivatives of one of the heterozygous disruptants were selected on medium containing 5-fluoroorotic acid (Sigma). These clones were screened by PCR and Southern blot hybridization to identify those which had lost the *URA3* gene *via* intrachromosomal recombination mediated by the *hisG* repeats. The procedure was then repeated to delete the remaining functional allele of *HAT1*.

Southern hybridization

Samples of genomic DNA (10 μg) were digested with restriction enzymes and the resulting fragments were separated by electrophoresis in 1% agarose gel in TAE buffer (2 M Tris-HCl/NaOAc, 50 mM EDTA pH 8.0). The agarose gel was then submerged in 0.25 N HCl for 15 min (twice), in 0.5 M NaOH/1.5 M NaCl for 30 min, and finally in 0.5 M Tris-HCl, pH 7.5/1.5 M NaCl for a further 30 min. The DNA was then transferred onto a Hybond-N^{TM} membrane (Amersham Pharmacia Biotech) by capillarity, and the membrane was blocked by UV cross-linked 120 mJoules cm^{-2} for 40 s (Statagene UV crosslinker) to ensure DNA immobilization. Prehybridization was performed in 1% NaCl, 1% Dextran sodium sulphate salt (Amersham) and 1% SDS with denatured salmon sperm DNA (Clontech) for 4 h at 65°C. The blot was then hybridized with ^{32}P-labelled DNA probe (α^{32}P, dCTP with specific activity > 3000 Ci/mmol), with the same solution for at least 16 h at 65°C. The membrane was then washed in SSC 2× (twice) for 5 min at room temperature and in SSC1×, 0.1% SDS for 45 minutes at 65°C. Detection of the hybridized probe was carried out according to the manufacturer's instructions for the ^{32}P labelling protocol and detected with a BAS-1500 Storage Phosphor Imageing System (Fujifilm).

DNA manipulations

Except where specified, standard procedures were used for DNA manipulation (Sambrook, 2001). *C. albicans* and *S. cerevisiae* transformations were carried out using the protoplast and lithium acetate methods respectively (Kohler *et al.*, 1997;

Rose, 1990). Genomic DNA from CAI4 *C. albicans* and mutant strains were obtained as described elsewhere (Sherman, 1986). The plasmid bearing *GFP3* and optimized for the genetic code of *C. albicans* has been described in (Barelle *et al.*, 2004; Cormack *et al.*, 1997) and plasmids bearing the cassette for *GFP3* tagging directly to the chromosome have been described in (Gerami-Nejad *et al.*, 2001).

The yeast two-hybrid system

The yeast two-hybrid system was carried out as have been described by James et al (James *et al.*, 1996). To construct the plasmids for the two-hybrid system, the complete *HAT1* and *HAT2* genes were isolated from *C. albicans* genomic DNA using two primer sets: "CaHAT1F and CaHAT1R" and "CaHAT2F and CaHAT2R," respectively (Table 2.2). The corresponding fragment was digested and cloned into the pGAD-C and pGBD-C expression vector at the *Sma*I/*Sal*I site. Both plasmids were used to transform the *S. cerevisiae* pJ69-4A and pJ69-4α strains. Transformants were selected on SD minus Leu and Trip media in the presence of plasmid. The transformants that display the expected phenotypes was conjugated. The resultant transformants contain pGAD-C and pGBD-C was tested for *GAL4* activity and survival on selective media: SD/-Leu/-Trip/-His/-Ade.

Table 2.2 Oligonucleotides used in this study

Primer	Sequence 5´-3´	Primer	Sequence 5´-3´
URA1	GGATACTATCAAACAAGAGG	**HAT2GFPR**	CCCGGGTGGTGGATCTTTCATTTTTGTATCCTC
URA2	AATGCTGGTTGGAATGCTTA	**HAT1GFPF**	CCCGGGATGTCATCCGCAAAAGAA
AMH1	CCGAACATCAACCAATCGT	**HAT1GFPR**	CCCGGGTGGTGGCACTTTTTGCTTTTTGGAA
IP2	TTACAATCAAAGGTGGTCC	**CaHAT2F**	CCCGGGATGTTTTCGAGACCATTAGAG
IP3	GGTACAGTTGTTCCTCACA	**CaHAT2R**	GTCGACATCTTTCATTTTTGTATCCTC
MAH38	GGTTGTTAATGCACCAAGAGA	**HAT1GFPCO**	CCCGGGTGGTGGAATGTTCAACTTCAAGTCT
MAH39	CTCTTGTTCTCCAAACTGC	**CaHAT1F**	CCCGGGATGTCATCCGCAAAAGAACAA
MAH40	ACCAATCCTGAAGCAGCAC	**CaHAT1R**	GTCGACTTACACTTTTTGCTTTTTGGA
MAH41	CAAAGCTTGATAAGCAGTCTG	**HAT1 inner**	TTCCTTTATTGAATCGACCCA
MAH32	GGCAATCACATCAAGACT	**HAT1 outer**	TCCAGGTATTTTATACCCATT
MAH33	TGTTCAACTTCAAGTCTC	**HAT2 iner**	ACTTCCATTAGGTGAAACTTT
RD48	GTATAGTACTGCAGTTGGCT	**HAT2 outer**	ATAACTACGAATAACACCATC
RD49	CCAACTCGAGCAACCCTC		

RLM-RACE

The *C. albicans* transcript was mapped using the FirstChoice RLM-RACE kit (Ambion). RNA was isolated from CAI4 exponential cultures by breaking cells in a micro-dismembrator (Braun, Melsungen), following by 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´GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA3´) 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 2.2). The 5´ RACE inner PCR products were cloned into pGEM-T (Promega) and sequenced.

DNA sequencing and sequence analysis

Both strands of the PCR product described above were sequenced using the DNA sequenciation service from University of Salamanca with an Applied Biosystems ABI PRISM 3100 Genetic Analyzer. For sequence analysis DNAstar program was used.

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

Methods for testing the *C. albicans* strains were similar for all the effectors. Cultures were grown in 50 ml of YEPD medium until the exponential phase and diluted to an O.D 600_{nm} of 0.4. Five microliters of pure and 1/10 serial dilutions of each cell culture were spotted onto YEPD plates containing NaCl (1 M), Calcofluor white (1 mg/ml), Caffeine (15 mM), SDS (0.05%), Fluconazole (2 μg/ml), Itranconazole (4 μg/ml), Amphptericin B (100 μg/ml), Hygromycin (300 μg/ml) and Congo red (0.5 mg/ml). Differences in growth were recorded after incubation of the plates at 28°C for 4 days.

Zymolyase sensitivity phenotypic test

Cultures of the CAI4, *hat1Δ*, *hat2Δ* simples and double mutant, were grown in YEPD medium until 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 Zymolyase 20T 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 decreased in optical density reflected the portion of cells that have been lysed.

Alcian blue binding assay

The Alcian blue binding assay was carried out using the method of Herrero et al (Herrero *et al.*, 2002). A series of solutions containing different amounts of Alcian blue was 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* amounts of Alcian blue. To quantify Alcian blue binding to the cell surface, 1000 μl exponential-phase yeast cells (O.D 600_{nm}, 2.5) 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. The O.D 600_{nm} 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.

Mouse strain and infection model

Pathogen-free 4-week-old CD1 mice were purchased from Animal Science Center of Salamanca University. For inoculation, fresh cultures of *C. albicans* were washed, resuspended in ice-cold PBS and counted with a hemocytometer. Cell concentration was adjusted with PBS to 5×10^8 yeast cells per 200 μl. Microscopic examination showed the cell suspension to be predominantly composed of single cells, with minimal clumping. Yeast suspensions were placed on ice until ready for injection. Immediately prior to injection, the yeast suspensions were agitated for 10 to 15 seconds and loaded into 1-ml syringes fitted with a 30G needle. Two hundred microliters of suspension was introduced into peritoneum, delivering a total of 5×10^8 yeast cells. Mice were returned to their cages and monitored for 30 days.

Green fluorescent protein fusions to *CaHAT1* and *CaHAT2*

GFP3 was placed at the carboxyl terminus of *CaHAT1* and *CaHAT2*. Two prolines were inserted between both proteins in order to preserve their correct folding. The pAG1 plasmid (a kind gift of Gonzalez-Novo) (Gonzalez-Novo *et al.*, 2004) is based on the pIR4 plasmid, which carries the *ACT1* promoter and a *C. albicans* ARS sequence. The *CaHAT1* and *CaHAT2* ORF were amplified by PCR and cloned in the pAG1 plasmid bearing the GFP and releasing the stop codon. These plasmids thus bore the *CaHAT1*-GFP and *CaHAT2*-GFP fusion under control of the *ACT1* promoter. This construction was introduced into *C. albicans* CAI4 and *hat1Δ* and *hat2Δ* mutant strains.

Microscopy

The observation of GFP-fusion proteins was performed. Images were captured with a Leica DMRXA (Leica, Germany) fluorescence microscope equipped with a 63× objective and a Leica DFC 350 FX monochrome digital camera, using the Qfish 2.3 program. Images were processed with Adobe Photoshop.

The cell and colony morphology on liquid medium and plates were inspected microscopically using a Leica and Zeiss Stemi SV 6 stereoscopic microscope, respectively.

RNA isolation, cDNA preparation and microarray hybridization

C. albicans CAI4 (*HAT1, HAT2*), RDH4 (*hat2Δ)* and MAH4 (*hat1Δ, hat2Δ*) strains were grown in YNB and Lee cultures for yeast mode of growth and during the yeast-hypha transition, respectively. Cells were harvested at room temperature, resuspended in a small volume of the supernatant and immediately frozen by releasing small drops of cell samples into liquid nitrogen. Cells were then stored at -80°C till RNA extraction. Total RNA was isolated by breaking cells in a microdismembrator (Braun, Melsungen, Germany), followed by trizol extraction method as described in Galar Fungal 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 (Hitachi, U-2001) analysis at 260_{nm}. The *C. albicans* microarrays used in this study were manufactured by Eurogentec SA (Ivoz-Ramet, Belgium) in collaboration with the European Galar Fungail Consortium. Two independent sets of RNA from CAI4 (control) and mutant cells (biological replicates) were used in these studies to prepare two independent cDNA probe sets. Fifteen micrograms of total RNA sample were added to a mixture of 1 μl of oligo (dT12-18)(Sigma), 1 μl primer mix (0.1 pmole/μl + 1 μg/μl AncT) (*C. albicans*-specific primer mix Plus) (Eurogentec); 3.6 mM of each dATP, dGTP, dTTP; 1 mM dCTP (Sigma); 1 mM Cy3- or Cy5-dCTP (Amersham); and 0.1 M DTT (Invitrogen). The reaction mixture was denatured at 65°C for 5 min, incubated at 42°C for 5 min, after which 1 μl of RNAsin (Promega, Madison, WI, USA) and 200U of Superscript II reverse transcriptase (Invitrogen) were added to the mixture. The reaction proceeded at 42°C for 1 h after which an additional 200U of Superscript II reverse transcriptase was added, and the reaction mixture was incubated at 42°C for an additional hour. To stop the reaction, EDTA (pH 8.0) and sodium hydroxide were added to a final concentration of 5 mM and 0.4 M, respectively, and the mixture was incubated at 65°C for 20 min. Finally, acetic acid was added to achieve a final concentration of 0.4 M. The labelled cDNA probes

were purified using Qia-Quick columns (Qiagen, Valencia, CA, and USA) following the manufacturer's instructions. The cDNA probes were then fluorescently labelled. One set of cDNA probes was labelled using Cy5 for those representing RNA from mutant's cells and Cy3 for those representing RNA from control's cells. The second set was labelled using Cy3 for those representing RNA from mutant's cells and Cy5 for those representing RNA from control's cells. Five microlitres each of the Cy3- and Cy5-labelled probes were mixed with 50 mg of heat denatured salmon sperm DNA (Sigma, Aldrich), incubated at 95°C for 2 min and snap-cooled on ice. The mixture was added to 60 μl of hybridization buffer (DIG easy hyb; Roche, Basel, Switzerland) and applied to the array slides under cover slips (Sigma). Hybridization was performed at 42°C overnight in a humidified chamber (Corning Life Sciences, Acton, MA, USA). After that microarrays were washed fallowing Eurogentec recommendations and dried by centrifugation at 1100 rpm at room temperature. Slides were scanned using a GenePix 4000B scanner.

Data analysis

GenePix 4.0 software (Axon Instruments) was used for image analysis and data visualization. Preliminary normalization, logarithmic transformation and determination of correlation coefficients were performed with Excel 2003 (Microsoft). Data were loaded into GeneSpring version 5.0.3 (Silicon Genetics, CA, and USA). Signals were log-transformed and per chip normalized by an intensity-dependent method (Lowess) applied to the print-tip region. Significance analysis of the results was conducted using Student's *t* test (Gene-Spring). Data with p values of ≤ 0.02 were considered to be significant. In the present study, only spots with a mean balanced differential expression ratio ≥ 1.5 or ≤ 0.66 (1/1.5) were considered to be differentially expressed for both spots representing a given cDNA on the array in two independent experiments.

Results

Determination of transcription initiation site using RNA ligase-mediated Rapid Amplification of 5' cDNA Ends (5' RLM-RACE) of *HAT1* and *HAT2* genes

To identify transcription initiation sites, RLM-RACE was performed. The results are shown in Fig. 2.1. We have amplified one band of ~450 bp and ~500 bp for *HAT1* and *HAT2* respectively (Fig 2.1A) that was cloned in pGEM-T. After sequencing four different clones, three putative transcription start sites (two identical and two different) were identified for *HAT1*. These products may have originated from different

transcription start sites. The three positions 21, 24 and 48 bp can be taken as the transcription start site (Fig. 2.1B). In any case the *HAT1* transcript is relatively short only a few bp upstream of the ATG codon. We must point out that very few data about the length of transcripts exist in *C. albicans*.

We have detected three putative transcription start sites for *HAT2,* all of them inside of coding sequence (ORF) that has been predicted by "in silico" analysis (CandidaDB) (Fig. 2.1C). Our result open the possibility that the real place of start of the *CaHAT2* gene is the one marked in red in Fig. 2.1C and purification of the protein is necessary to assess this point.

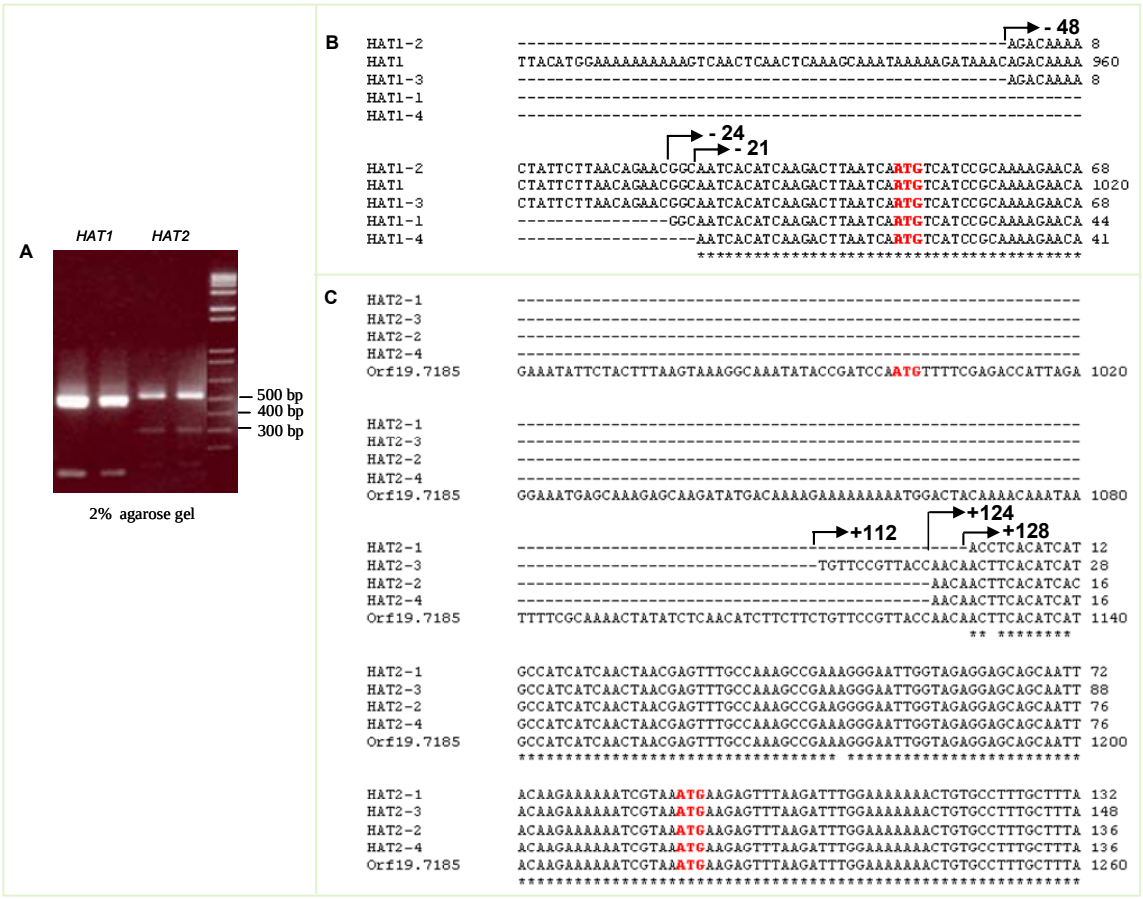

Figure 2.1 Mapping of *HAT1* and *HAT2* transcrirtion initiation sites by RLM-RACE. Predicted of the transcription start site of *HAT1* and *HAT2* genes

```
C. dubliniensis   ---------------------------------------------MPSSTNEFAKAERE 14
C. albicans       MFSRPLEEMSKEQDMTKEKKMDYKTNNFRKTISQHLLSFRYQQLHIMPSSTNEFAKAERE 60
C. tropicales     ---------------------------------------------MPSTTNEYAKAERE 14
P. stipitis       ---------------------------------------------MTITQKDLAVAERE 14
                                                                *. : :: * ****

C. dubliniensis   LVEEQQLQEKIVNEEFKIWKKTVPLLYDFIHTFALDSPSLVFQWLPTTNVS--QSDLELK 72
C. albicans       LVEEQQLQEKIVNEEFKIWKKTVPLLYDFIHTFALDNPSLVFQWLPTTSVS--QSDLELK 118
C. tropicales     LANEQQLQEKIVNEEFKIWKKTVPLLYDFVHTFALDSPSLVFQWLPDYNVS--QSDLEVK 72
P. stipitis       IVEEHQLKEKVVNEEFKIWKKTVPLLYDTIHTYVLDYPSLAIKWLPDYTYSDNKNSVNVK 74
                  :.:*:**:**:***************** :**:.** ***.::***  . *  :.:::*

C. dubliniensis   FLIGTNTINKADNYLKLASINLPSTLVG-------ATESIPVPSDDID-TSNFKVITQWK 124
C. albicans       FLIGTNAINKSENYLKLTSISLPSTLVG-------ATDSIPVPSDGID-TSNFKVVTQWK 170
C. tropicales     FLIGTNTINKSENYLKLGSVTLPSTLVENS----TNPQGIPIPTEDLD-TSNFRIINQWK 127
P. stipitis       FLIGTNTSHNSSNYLKLGSVNIPSTLAPDFSTVNPDVDSITVPSSVIEDTSDFRILSKWK 134
                  ******: :::.***** *:.:****.          :.*.:*:. :: **:*::::.:**

C. dubliniensis   QSQEVNKLKVSPNGSLAVGFNADGVLRSYNLDNFDSVDYKYHKQGGIALDWVDNNGFLSG 184
C. albicans       QTQEINKLKVSPNGSLAVGFSADGVIRSYNLDNFDSVDYKYHKQGGIALDWVDNNGFLSG 230
C. tropicales     QSCEINKLKVSSNGGLAVGFGADGIIRGFNLKNYDIVDYKYHKQEGSALNWINENSFISG 187
P. stipitis       QTSEINKLDISPNGKKVLSFNSDGVVHSYDLENNDVIDYKYHKSEGYALTWFGNDSFISG 194
                  *: *:***.:*.**  .:.*.:**:::.::*.* * :******. * ** *.::.*:**

c. dubliniensis   SNDSQIALWQVDKPSTPLQLFKGHHGAINDISY-VKEKHLFGSVSDDSTTQFHDSRVNSA 243
C. albicans       SNDAQIALWQVDKSSTPLQLFKGHHGAINDISS-IKEKHLFGSVSDDSTTQFHDTRVNAT 289
C. tropicales     AKDSQIALWQVDKPSTPIQLFKGHRGAINDLSS-IKGKTLFGSVSDDSTTQFYDGRIGSI 246
P. stipitis       SNDSQIALWSLDKPSTPIQLFKSHNGAVNDISYNPNFVSIFGSVSDDSSTQFHDSRASGD 254
                  ::*:*****.:**.***:****.*.**:**:*   :   :********:***:* * ..

C. dubliniensis   DINPVITVENSHIQKCIQFHPDIPTLYATGGKDNVVSLYDMRNYSTPFRKFYGHNDSVRQ 303
C. albicans       DINPVITVENSHIQNCIQFHPDIQTLYATGGKDNVVSLYDIRNYSTPFRKFYGHNDSVRQ 349
C. tropicales     DANPVISVENNHIQNCIQFHPDIHTMYATAGKDNIVSLYDMRNYKTPFRKFYGHNDTIRQ 306
P. stipitis       --NPVIKQENQHIQMAISVHPEIETLYATGGKDNVVSLYDIRNYKIPLRKFFGHNDSVAG 312
                    ****. **.*** .*..**:* *:***.****:*****:***. *:***:****::

C. dubliniensis   LQWDWNNPDILVSCGLDKRIIFWDLKNLDEDFTYPDAMSNGKDTNSKKKQAVKTDPCLKY 363
C. albicans       LQWDWNNPDILVSCGLDKRIIFWDLKNLDEDFTYPDATSNGKDTNSKRKQAVKTDPCLKY 409
C. tropicales     LQWDSYNPNLLVSCGLDKRVLFWNLESLDEDYTYPDQTSNGKDSNSKKKQVNKVDPCLKY 366
P. stipitis       IKWDVEDPRTLISWSLDKRIITWDLKDLEEEYAYPDGNEN-----SRRRAAVKIDPCLRF 367
                  ::**  :*  *:* .****:: *:*:.*:*:::***  .*     *::: . * ****::

C. dubliniensis   VHGGHTRRTNDFDIHPKIKNIFGSVGDDKLLEIWKPKTLPSDEPEQESADVQEDEEMKDA 423
C. albicans       VHGGHTRRTNDFDIHPKVKNIFGSVGDDKLLEIWKPKSLPGDEPEQESTEVQEDEEMKDA 469
C. tropicales     IHGGHTNRINDFDIHTKVRNLFGSVGNDRLLEIWKPKTLPGDEPEEEEEEEVEEEALPEE 426
P. stipitis       IHGGHTNRVNDFDVHPKIRSLYASVGDDNLLEVWK------------------------ 402
                  :*****.* ****:*.*::.::.***:*.***:**

C. dubliniensis   D--AKGDKEGEEDSKMKD 439
C. albicans       E--TKGVKDGEEDTKMKD 485
C. tropicales     KKVSEETKESEQDVEMKD 444
P. stipitis            ------------------
```

Figure 2.2 Comparison of *Candida albicans* Hat2p protein with following species: *C. dubliniensis, C. tropicales* and *P. stipitis*

The comparison of the Hat2p protein length between *C. albicans* and other *Candid* spp. support our data. Fig. 2.2 shows the shorter length of the other *Candida* Hat2p proteins.

Disruption of *HAT1* in a *hat2Δ* mutant background strain

Many yeast genes encode histone acetytransferase proteins in *C. albicans* but phenotypes of the putative mutants were unknown. We performed a phenotypic analysis of a yeast strain by deletion of *HAT1* and *HAT2* genes which encode members

of the HAT families in *C. albicans*. We have deleted the ORF of the *HAT1* gene by the "Ura-blaster" procedure (Fonzi & Irwin, 1993) using protoplast method (Rose, 1990) in a *hat2Δ* background strain.

Deletion was verified by using two pairs of primers for PCR analysis (data not shown) and by Southern blot analysis (Fig. 2.3B). The oligonucleotides used for this study are listed in Table 2.2. With the double mutant, we performed a whole set of experiments and below we describe the results obtained.

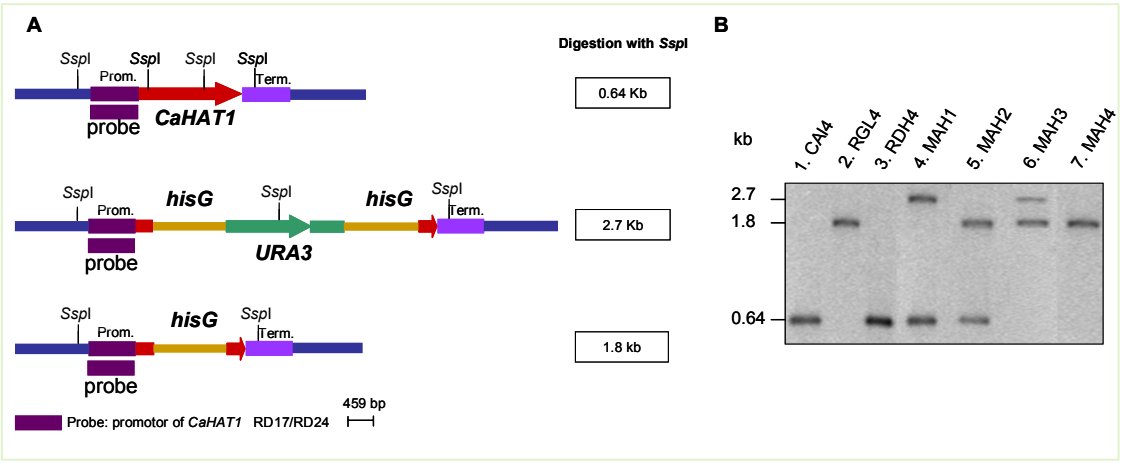

Figure 2.3 Deletion of *CaHAT1* alleles. (A) Structure of different alleles. The wild-type *HAT1* gene and the alleles disrupted by the *hisG-URA3-hisG* cassette or by *hisG* alone are shown. (B) Southern blot analysis of genomic DNA was performed with the following strains digested with *Ssp*I.
Lane 1, CAI4 (*HAT1/HAT1; HAT2/HAT2*)
Lane 2, RGL4 (*hat1Δ::hisG/hat1Δ::hisG; HAT2/HAT2)*
Lane 3, RDH4 (*HAT1/HAT1; hat2Δ::hisG/hat2Δ::hisG*)
Lane 4, MAH1 (*hat2Δ::hisG/hat2Δ::hisG; HAT2/hat2Δ::hisGURA3hisG*)
Lane 5, MAH2 (*hat2Δ::hisG/hat2Δ::hisG; HAT1/hat1Δ::hisG*)
Lane 6, MAH3 *(hat2Δ::hisG/hat2Δ::hisG; hat1Δ::hisGURA3hisG/hat1Δ::hisG)*
Lane 7, MAH4 (*hat2Δ::hisG/hat2Δ::hisG; hat1Δ::hisG/hat1Δ::hisG*)
A 459 bp fragment of the *CaHAT1* promoter was used as probe

The double mutant grew like *hat1Δ* simple mutant, on solid and liquid media compared with the *hat2Δ* and CAI4. To further analyze the growth rate of the double mutant, we performed a comparison of growth between all four strains: CAI4 (*HAT1, HAT2*) and RGL4 (*hat1Δ*), RDH4 (*hat2Δ*) and the MAH4 (*hat1Δ, hat2Δ*) double mutant.

The beginning of the exponential growth phase in the double mutant, as in the case of the *hat1Δ* simple mutant, was delayed by up to 16 h (Fig. 2.4). These results suggest that *HAT1* would be involved in vegetative growth in *C. albicans*. In our laboratory, we have been unable to detect any significant difference in growth rate, cell size and morphology between the *hat2Δ* and CAI4 strains (Degano, Ph.D thesis in process).

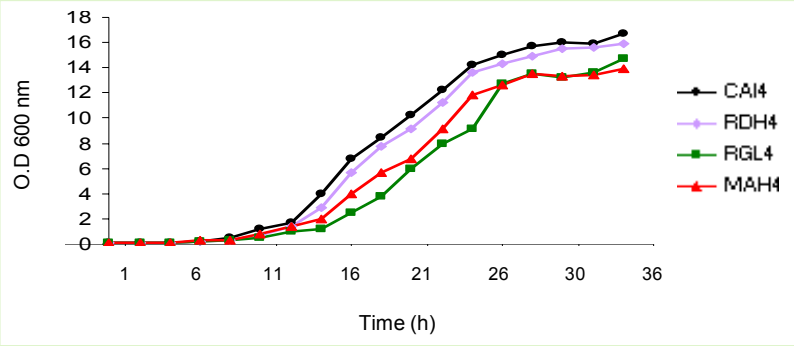

Figure 2.4 Growth curves of CAI4, RGL4 *(hat1Δ)*, RDH4 *(hat2Δ)* and MAH4 *(hat1Δ, hat2Δ).* Strains were pre-grown on rich medium containing 1% glucose and inoculated at an O.D at 600nm of 0.1 YEPD. Growth was monitored over a period of 34 h by measuring the optical density of the cultures.

Morphologically, the double mutant, like the *hat1Δ* simple mutant, is very different to the CAI4 strain as shown in Fig. 2.5. The culture of the *hat1Δ, hat2Δ* double mutant in YEPD liquid medium was composed of a mixture of large cells and large chains of elongated, aberrantly shaped cells attached to one another suggesting that Hat1p activate or suppress transcription of genes that directly or indirectly control cell morphology.

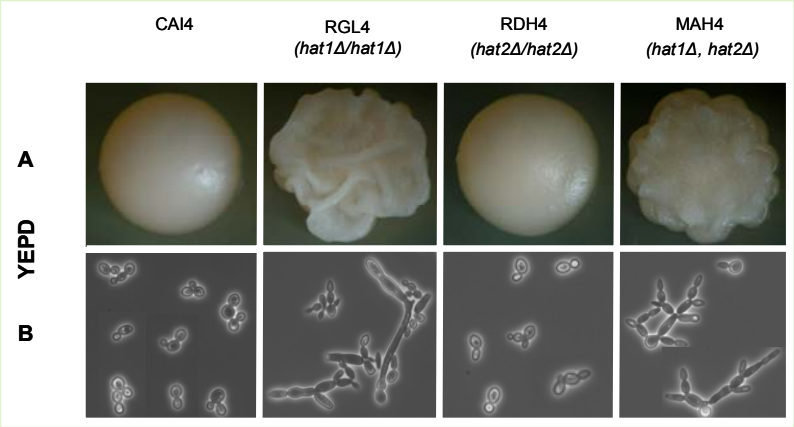

Figure 2.5 (A) Colony growth and (B) morphological characteristics of *C. albicans* homozygous strain CAI4, RGL4, RDH4 and MAH4 in YEPD media

Behavior of the *hat1Δ, hat2Δ* double mutant in solid media

For the observation of the filamentous growth on solid media, cells of the simples and double mutant as well as the CAI4 strain were plated at the 50 ufc on plates. Solid media normally induces filamentous growth after 3 days. The double and the *hat1Δ* simple mutants displayed hyperfilamentation, whereas *hat2Δ* cells formed hypha like the wild-type parent under these conditions (Fig. 2.6). These results indicated that the alternative pathway was almost fully induced in a *hat1*Δ simple and *hat1*Δ, *hat2*Δ double mutant. A similar morphological phenotype has been reported in the *efh1*Δ,

*efg1*Δ double mutant (Doedt *et al.*, 2004). We also observed that, like in both simple mutants, the double mutant was also able to form hypha in Embedded medium (Fig. 2.6).

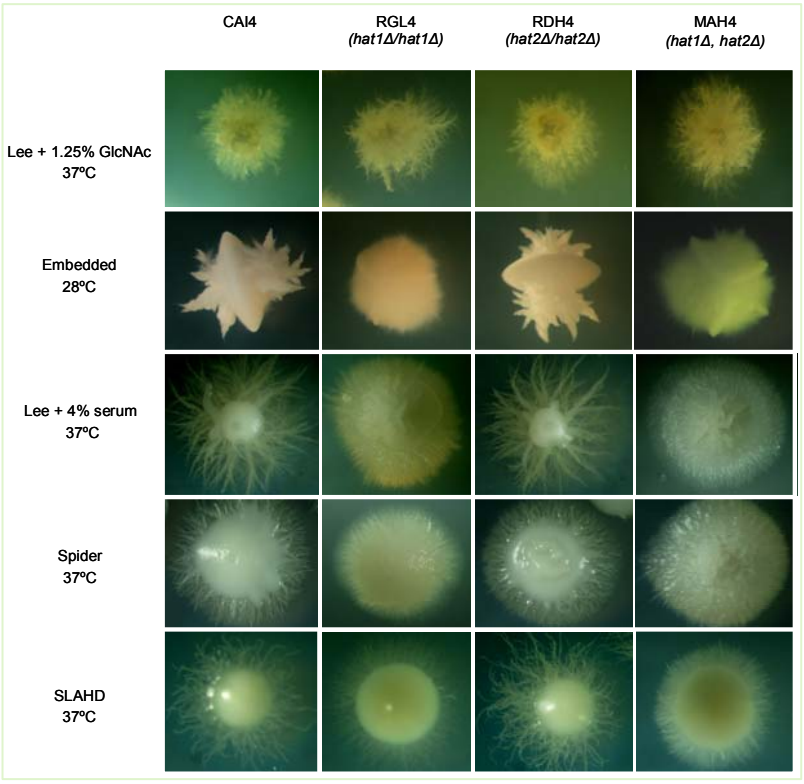

Figure 2.6 Growth of the CAI4, RGL4, RDH4 and MAH4 on solid media. All strains were grown on solid medium which induce hyphal development. The plates were incubated for 4 days at 37°C.

Behavior of the *hat1Δ, hat2Δ* double mutant in liquid media

The behavior of the double mutant strain was next investigated by assessing hyphal formation in Lee (pH 6.8, 37°C) (data not shown) or in serum inducing media.

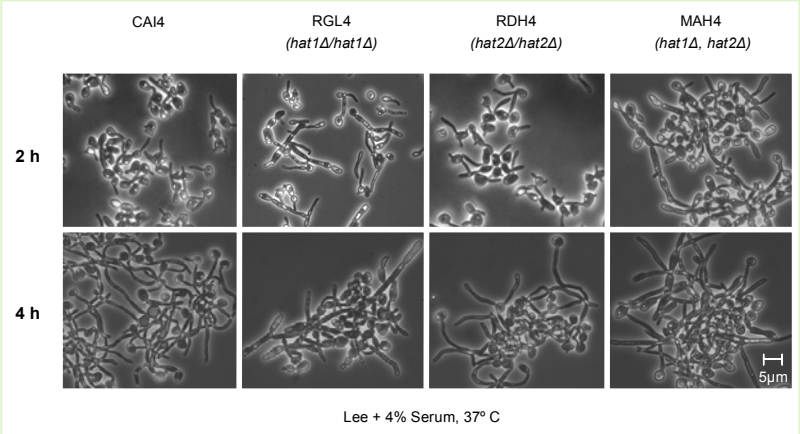

Figure 2.7 Germinative behavior of CAI4, RGL4, RDH4 and MAH4 strains during the yeast-hypha transition in Lee+ 4% serum at 37ºC

Fig. 2.7 shows that whereas CAI4 and *hat2Δ* mutant formed normal hypha, *hat1Δ, hat2Δ* double mutant like *hat1Δ* simple mutant have a block of hypha formation in both medium and an aberrant morphology with long, thick, stubby protrusions resembling pseudohypha were observed. A similar phenotype has been reported previously in our laboratory in the *hat1*Δ*, hda1*Δ double mutant.

Chlamydospore formation

We try to determine whether the deletion of both genes might influence chlamydospore formation. We analyzed the growth of the double mutant in the presence of the cornmeal agar. The results are shown in Fig. 2.8. The *hat1*Δ*, hat2*Δ double mutant like both simple mutants were able to formed chlamydospore. These results suggesting that deletion of the *HAT1* and *HAT2* genes did not lead to an apparent defect in chlamydospore formation. It has been reported that other histone acetyltransferase like *GCN5* failed to form chlamydospore (Degano, Ph.D thesis in process). Collectively, our data indicate that a *hat1*Δ and *hat1*Δ*, hat2*Δ mutation does not influence the various morphogenetic processes that are strongly affected by a *gcn5*Δ mutation (morphogenesis and phenotypic switching).

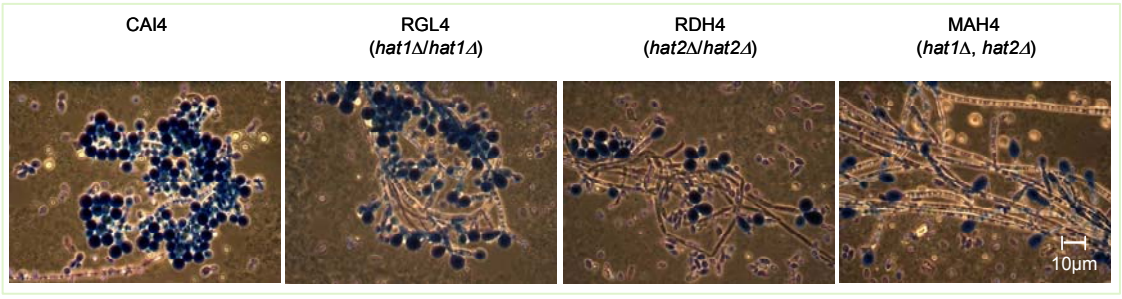

Figure 2.8 Hat1p and Hat2p are not required for chlamydospore formation. The strains were streaked out lightly on chlamydospore induction medium (cornmeal agar (Difco)–0.33% Tween 80.

Phenotype analysis of the *hat1Δ, hat2Δ* double mutant

To gain further characterize and better define of both simples and double mutant, we carried out further phenotypic tests including sensitivity to SDS, NaCl, CFW, Caffeine, Amphotericin B, Hygromycin, Fluconazole, Itraconazole and Congo red. To do so, the sensitivity of both simple and the *hat1Δ, hat2Δ* double mutant was compared with that of the CAI4. We found that the *hat1Δ* and double mutant were more sensitive than the *hat2Δ* and the CAI4 strain when they were incubated in the presence of 1 M of NaCl, 1 mg/ml of Calcofluor white (CFW), 0.5 mg/ml of Congo red and 15 mM of Caffeine (Fig. 2.9). The sensitivity of a *hat1Δ, hat2Δ* double mutant was similar to that of the single

mutants and the CAI4 strain in the medium supplemented with SDS (0.05%). In the present of Amphotericin B, Fluconazole and Itraconazole, the *hat1Δ* and double mutant were showed a moderate sensitivity when compared to that of *hat2Δ* and CAI4. These results suggest that Hat1p maybe control expression some of cell wall genes.

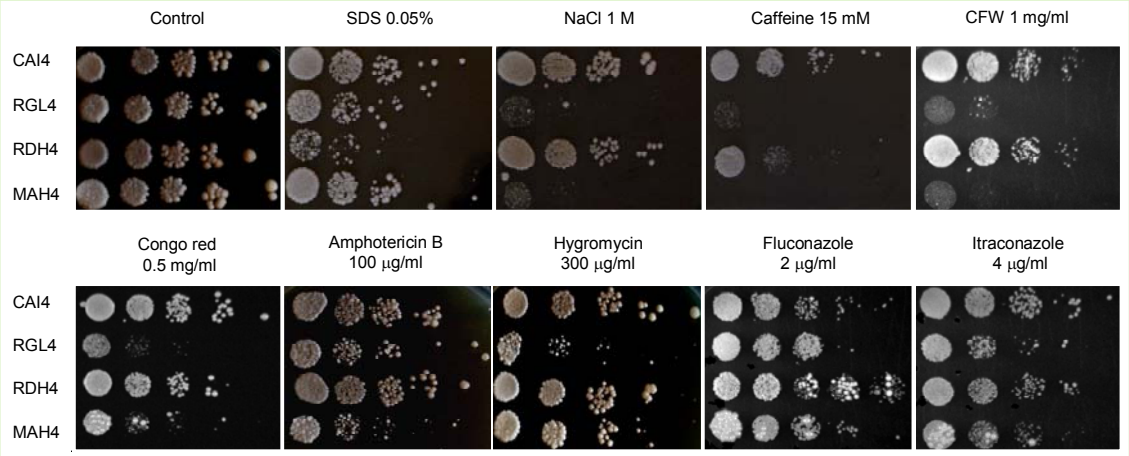

Figure 2.9 Sensibility assays of CAI4, RGL4 (*hat1Δ*), RDH4 (*hat2Δ*) and MAH4 (*hat1Δ, hat2Δ*) to different agents. All strains were grown in liquid YEPD. The optical density at 600nm of the cultures were adjusted to 0.4 with the same medium and 5 µl aliquots from the cultures and from 10-fold serial dilutions were spotted onto YEPD plates supplemented with different agents. Plates were incubated at 28°C for 4 days.

The sensitivity of yeast cells to Zymolyase has been used to detect changes in cell wall composition and arrangement (Ram *et al.*, 1994). To address this issue, we monitored the response of all strains to the presence of the Zymolyase 20T for 70 minutes at 30°C. We found that like the *hat1Δ,* the double mutant, are slightly more sensible to Zymolyase than the *hat2Δ* and CAI4 strain (Fig. 2.10).

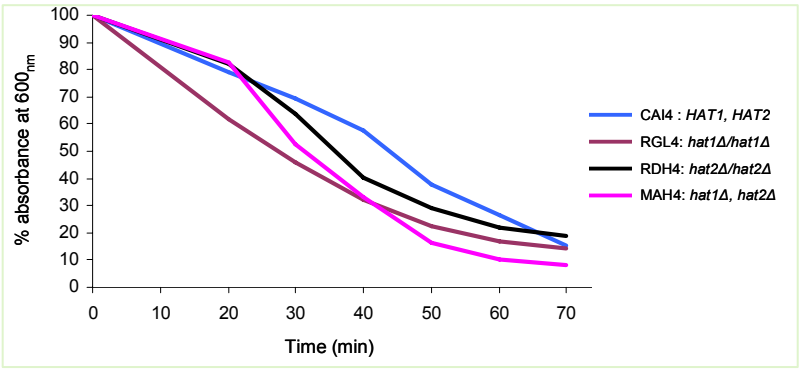

Figure 2.10 Resistance to a cell wall-degrading enzymatic complex of *C. albicans*. All strains were grown until the exponential phase in YEPD, at 28°C. Aliquots (2×10^7 cells) were resuspended in 10 mM Tris-Hcl PH 7.5 and treated with 10 µg of Zymolyase per ml. The decrease in optical density (O.D) (percentage of resistant cells) is monitored as described in Methods.

Alcian blue binding assay

Next we determined the level of Alcian blue binding to CAI4 and to the three mutant strains of *C. albicans,* using the method previously described (Herrero *et al.*, 2002). As shown in Fig. 2.11, the level of Alcian blue binding was reduced to 48.14% in the *hat1Δ* mutant. In the *hat2Δ* mutant a small increase was detected and the double mutant present a similar level of Alcian blue binding to the CAI4 strain suggesting a compensatory effect of the *HAT2* deletion on the *hat1Δ* mutation.

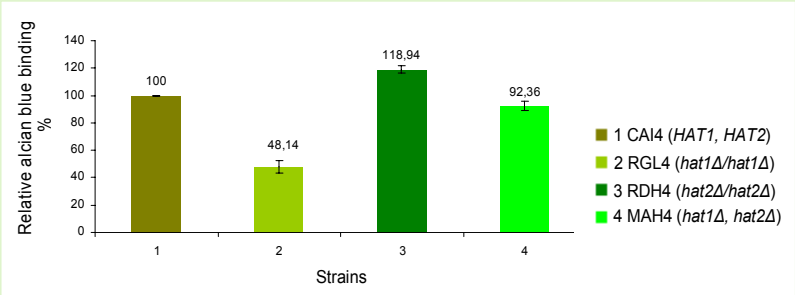

Figure 2.11 Comparison of Alcian blue binding of double mutant with parental and simple mutants. Relative dye binding of MAH4 was calculated as the percentage of dye bound compared with results for the parental strains (RGL4, RDH4 and CAI4). Results are average of three independent determinations; bars indicate standard deviations.

Virulenece assay

We decided to determine whether the absence of the Hat1p and Hat2p might lead to a defect in virulence in a mouse model. We tested the virulence of the *C. albicans* hat mutant strains in a mouse model infection because hyphal growth is hypothesized to be important for the pathogenicity of *C. albicans*. Isogenic *URA3* strains were used because *ura3* strains have reduced virulence (Cole *et al.*, 1995; Leberer *et al.*, 1996). All of the mice injected with the CAI4 and *hat2Δ* mutant died by day 3 (Fig. 2.12).

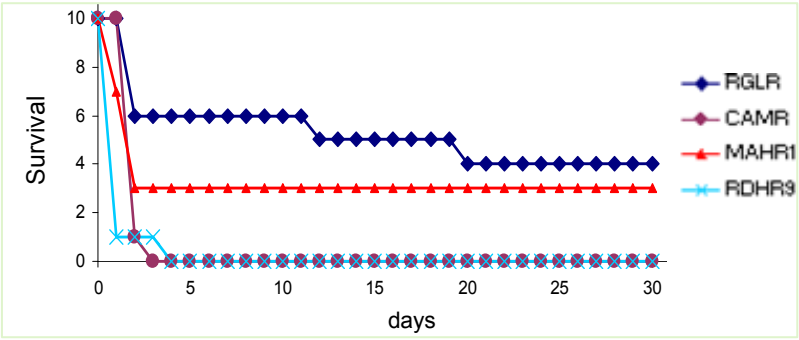

Figure 2.12 *C. albicans* double and *hat1Δ* simple mutants are hypovirulent in a mouse model. Survival curves for mice. (n = 10 for each *C. albicans* strain at each inoculation dose) infected with 5×10^8 cells of *C. albicans*. • CAMR (CAI4-*URA*$^+$), ♦ RGLR6 (*hat1Δ-URA*$^+$), RDHR9 (*hat2Δ-URA*$^+$) and ▲MAHR1 (*hat1Δ, hat2Δ-URA*$^+$).

In contrast, the *hat1Δ* and *hat1Δ, hat2Δ* homozygotes were less virulent; 40% and 30% of the mice were alive at the end of the experiment respectively. Similar to the results was reported for the *gcn5Δ, hat1Δ* double mutant (Rashki, Ph.D thesis 2009).

Cellular location of the Hat1p and Hat2p proteins

In order to determine the cellular location of the Hat1p and Hat2p in the *C. albicans*, we constructed strains that carried Hat1-GFP and Hat2-GFP fusion proteins and observed it under the fluorescence microscope. We found that the two GFP fused proteins were mainly located in the nucleus (Fig. 2.13 and Fig. 2.14). A transformed with a construct of the GFP not in phase wild-type strain was used as a negative control for both proteins.

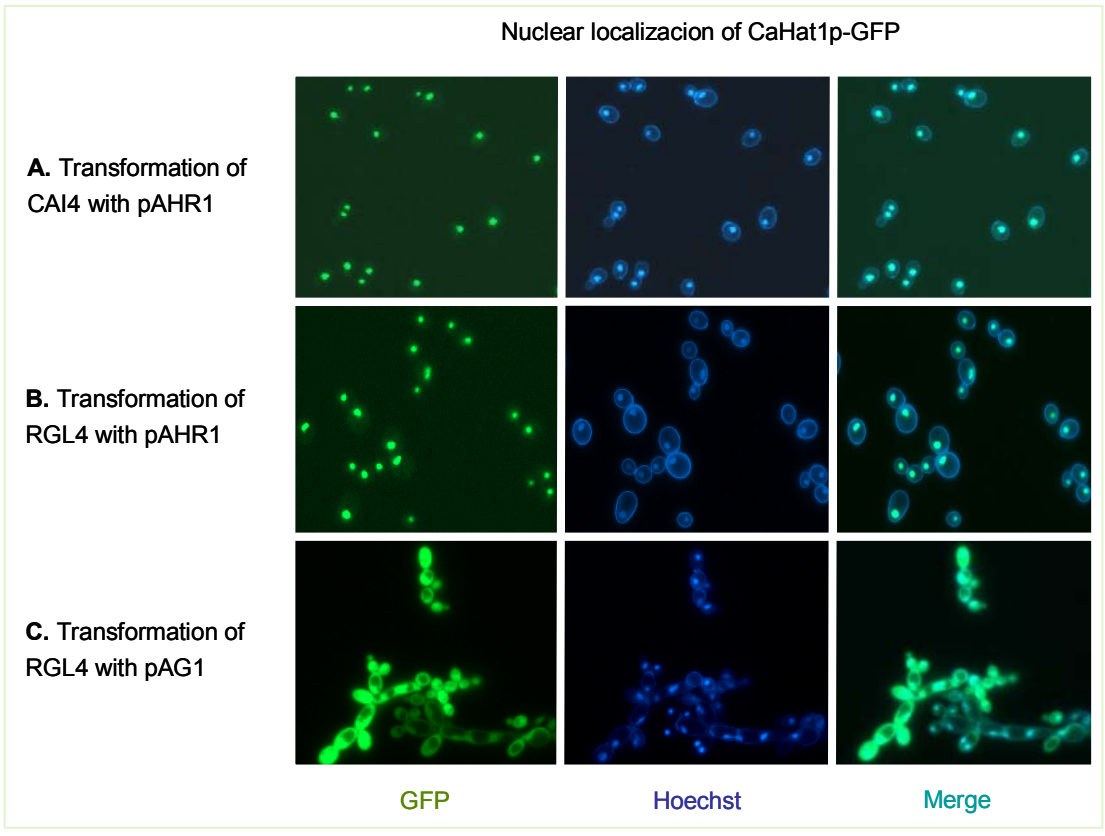

Figure 2.13 Subcellular localization of Hat1-GFP in wild-type and mutant strains. Localization of the fusion protein was visualized by fluorescence microscopy. Nuclei were visualized by Hoechst staining.

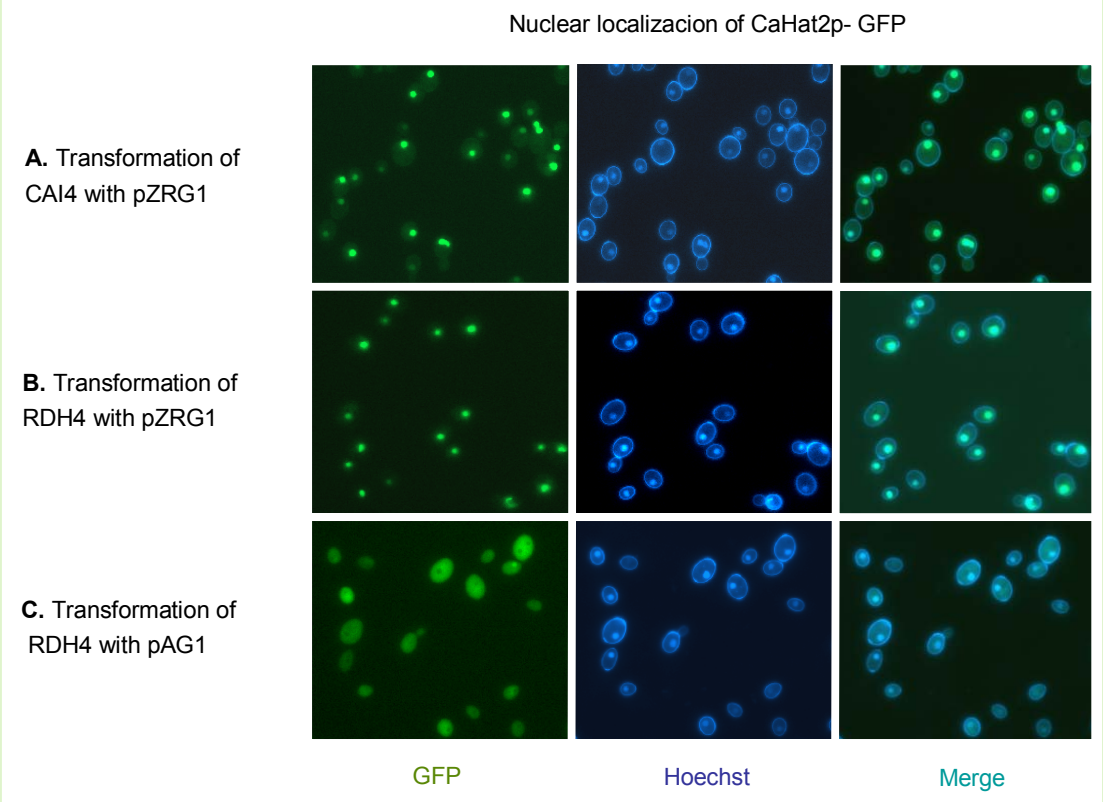

Figure 2.14 Subcellular localization of Hat2-GFP in wild-type and mutan strains. Localization of the fusion protein was visualized by fluorescence microscopy. Nuclei were visualized by Hoechst staining.

Next, we asked whether their nuclear residence is interdependent. To address this question, Hat1-GFP and Hat2-GFP were visualized in the *Candida* cells defective in any of the other companion protein. The nuclear residence of Hat1-GFP exhibited no difference between cells with a wild-type and *hat2*Δ genetic background, suggesting that Hat2p is not necessary for localization of that Hat1p in the nucleus (Fig. 2.15A).

We also analyzed location of Hat2p in a *hat1Δ* mutant. In this case, we transformed a plasmid that carrying GFP-fused Hat2p. As shown in Fig. 2.15B, we detected that the GFP-Hat2p signal localized in the cytoplasm suggesting that Hat1p is necessary for localization of the Hat2p in the nucleus.

All these results strongly suggested that Hat1p would interact directly o indirectly with the Hat2p in a histone acetyltransferase complex.

In order to see whether Hat1p was directly interacting with Hat2p, we performed the standard two-hybrid assays in *S. cerevisiae*. For this purpose, we tested fusions of Hat1p or Hat2p to the Gal4p DNA-binding (BD) or activating domains (AD). We found that Hat1p didn't interact directly with Hat2p (result not shown) suggesting that maybe other protein o proteins are involved in a bridge between Hat1p and Hat2p.

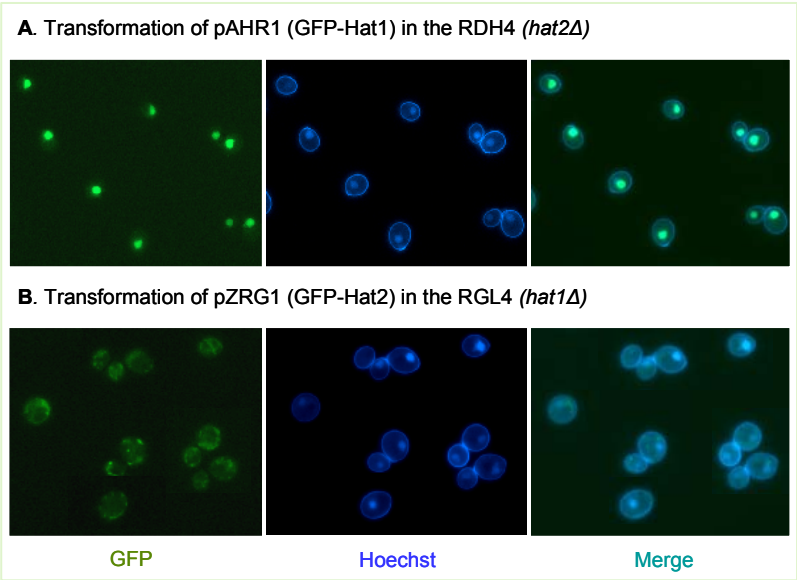

Figure 2.15 Subcellular localization of Hat1-GFP in the *hat2Δ* (A) and Hat2-GFP in the *hat1Δ* mutant (B). Localization of the fusion protein was visualized by fluorescence microscopy. Nuclei were visualized by Hoechst staining.

Transcriptional profile of the *hat2*Δ mutant during the yeast mode of growth

We have analysed the transcriptional profile of the *hat2*Δ mutant while the transcription profile of the *hat1*Δ mutant has been previously performed in our laboratory. The experiments were done in duplicate by using two different exponentially growing YNB culture for each mutant. In the *hat2Δ* mutant, we detected an altered expression of 109 genes: 26 genes being up-regulated and 83 being down-regulated. It has been reported that 62 genes were affected in the *hat1Δ* mutant (45 genes up- and 17 down-regulated). Only two genes (*HHF1* and *STF2*) were commonly up-regulated in both single mutants suggested that transcription profile of the *hat1*Δ and *hat2*Δ are different during the yeast mode of growth and thus demonstrating that both acetyltranferases regulate a different set of genes.

The major gene categories of HAT2-dependent genes down-regulated during the yeast mode of growth included cell wall proteins (5 genes), genes encode for elongation factor (6 genes), ribosomal proteins (10 genes), stress response (4 genes) and transcription factors (4 genes) (Table 2.3). Seven genes involved in respiration (*ATP6, COX3A, COX3B, NAD2, NAD5, NAD6* and *STF2*), three genes encoding known histone assembly proteins and two genes encoding for ribosomal proteins were found to be up-regulated in the yeast mode of growth (Table 2.3). Eleven genes involved in glycolysis and TCA cycle (*CDC19*, *PDB1, FBA1, PFK2, PYC2, ACO1, ACO2, CIT1, GDH3*, *MDH1-1* and *MIS11*) (McAlister & Holland, 1985; Overkamp *et al.*, 2002;

Reifenberger *et al.*, 1995; Sonneborn *et al.*, 2000; Walsh *et al.*, 1983) were found to be down-regulated in the *hat2Δ* mutant. Expression of *ACO1, ACO2, CIT1, MIS1* and *PYC2* genes has been reported to be decreased in the *hat1Δ* mutant suggesting that this group of genes were regulated separately by Hat1p and Hat2p. Six genes encoding for translation elongation factors (*CAM1-1, CEF3, EFB1, EFT2, TEF1* and *TIF1*) (Lorenz *et al.*, 2004; Maneu *et al.*, 1996; Mirbod *et al.*, 1996; Myers *et al.*, 1992) also were down-regulated in the *hat2Δ* mutant. Although their roles in the yeast mode of growth in *C. albicans* are unknown, it has been reported that transcription of translation-related genes are down-regulated upon phagocytosis (Liu *et al.*, 2005; Lorenz *et al.*, 2004).

Table 2.3 Differentially expressed genes in *hat2Δ* mutant during the yeast mode of growth

Gene name	ORF	Function	Fold regulation
Up-regulated genes			
Respiration			
ATP6	-	Subunit6 of the F0 sector of mitochondrial F1F0 ATP synthesis	1.77
COX3A	-	Subunit III of cytochrome c oxidase	3.31
COX3B	-	Subunit III of cytochrome c oxidase	2.49
NAD2	-	Subunit 2 of NADH: ubiquinone oxidoreductase	2.02
NAD5	-	A multisubunit enzyme complex (complex I)	1.64
NAD6	-	Subunit 6 of NADH: ubiquinone oxidoreductase	1.91
STF2	orf19.2107.1	Protein involved in ATP biosynthesis	2.19
Histone assembly			
HHF1	orf19.1059	Putative histone H4	2.04
HTA1	orf19.6924	Putative histone H2A	1.75
NHP6A	orf19.4623.3	Protein described as nonhistone chromatin component	2.36
Ribosomal proteins			
RPL26A	orf19.3690.2	Ribosomal protein	1.71
RPL40B	orf19.4684.2	Protein with similarity to the ribosomal protein of *S. cerevisiae* Rpl40Bp	1.72
Down-regulated genes			
Glycolisis			
CDC19	orf19.3575	Putative pyruvate kinase	0.45
PDB1	orf19.5294	Similar to pyruvate dehydrogenase	0.63
FBA1	orf19.4618	Putative fructose-bisphosphate aldolase	0.63
PFK2	orf19.6540	Beta subunit of phosphofructokinase	0.59
PYC2	orf19.789	Putative pyruvate carboxylase	0.43
TCA cycle			
ACO1	orf19.6385	Protein described as aconitase	0.56
ACO2	orf19.6632	Protein described as aconitate hydratase 2	0.60
CIT1	orf19.4393	Protein described as citrate synthase	0.60
GDH3	orf19.4716	Similar to NADp-glutamate dehydrogenase	0.52
MDH1-1	orf19.4602	Predicted malate dehydrogenase	0.51
MIS11	orf19.2364	Putative protein of glycine catabolism	0.57
Ribosomal proteins			
RPL8B	orf19.2311	Predicted ribosomal protein	0.55
RPL13	orf19.2994	Protein described as ribosomal subunit	0.59
RPL16A	orf19.6085	Similar to *S. cerevisiae* ribosomal protein Rpl16ap	0.64
RPL81	orf19.6002	60s ribosomal protein l7a.e.b	0.52
RPP0	orf19.7015	Putative ribosomal protein	0.27
RPS4A	orf19.5341	Predicted ribosomal protein	0.51
RPS15	orf19.5927	Putative ribosomal protein	0.42
RPS23A	orf19.13632	Putative ribosomal protein	0.63
RPS8A	orf19.6873	Putative ribosomal protein	0.34
YST1	orf19.6975	Ribosome-associated protein	0.57
Elongation factor			
CAM1-1	orf19.2651	Putative translation elongation factor	0.53
CEF3	orf19.11629	Translation elongation factor 3	0.47
EFB1	orf19.3838	Translation elongation factor ef-1 beta	0.56

EFT2	orf19.5788	Elongation factor 2 (eEF2)	0.61
TEF1	orf19.9009	Translation elongation factor 1-alpha	0.55
TIF1	orf19.3324	Similar to *S. cerevisiae* translation initiation factor	0.53
Cell wall			
PGA45	orf19.2451	Putative GPI-anchor	0.62
PGA59	orf19.2767	Putative GPI-anchored protein of unknown function	0.29
PGA62	orf19.10281	Putative GPI-anchored protein	0.24
TOS1	orf19.1690	Similar to alpha agglutinin anchor subunit	0.55
YWP1	orf19.3618	Putative GPI-anchor; cell wall and secreted	0.17
Stress response			
SOD1	orf19.2770.1	Cytosolic copper- and zinc-containing superoxide dismutase	0.62
SOD2	orf19.3340	Mitochondrial manganese-containing superoxide dismutase	0.57
SSB1	orf19.6367	Putative heat shock protein	0.32
HSP90	orf19.6515	Chaperon of Hsp90p family	0.57
Transcription factors			
RGT1	orf19.2747	Transcriptional repressor	0.56
SKO1	orf19.1032	Putative transcription factor	0.61
EFG1	orf19.8243	Transcriptional repressor	0.31
NRG1	orf19.7150	Transcriptional repressor	0.59

Transcriptional profile of the *hat2*Δ during the yeast-hypha transition

Next, we focused on the identification of genes whose expression was altered in the *hat2*Δ mutant strain during the yeast-hypha transition. We generated the transcriptional profile for the *hat2Δ* mutant in Lee medium for 15, 60 and 180 min at 37°C using the CAI4 strain as reference. In a previous study in our laboratory, the transcriptional profile of *hat1*Δ has been performed after growth in hypha-inducing condition at the same times. A total of 429 genes were found to change at the consensus level of ≥ 1.5 fold, including 196 up- and 233 down-regulated genes. The distribution of genes altered at the three points is represented in the Venn diagram, and the clustering of genes regulated by deletion of *HAT2* is represented in Fig. 2.16. We found that 2 genes (up-regulated) were common to the three times assayed, 54 genes at 15 and 60 min (35 genes up- and 19 down-) and 11 genes at 15 and 180 min (9 up- and 2 down-). 156 genes (85 up- and 71 down-), 167 genes (40 up- and 127down-) and 39 genes (25 up- and 14 down-) varied only at one time of induction (at 15, 60 and 180 min respectively). Only 2 genes *BUD7* encoding a protein with similarity to the *BCH1* gene of *S. cerevisiae* that has a role in cellular bud site selection (Braun *et al.*, 2005) and *MEF2* a mitochondrial elongation factor, similar to Mef2p of *S. cerevisiae* involved in translational elongation (Rasmussen, 1995) were up-regulated commonly during three times of incubation.

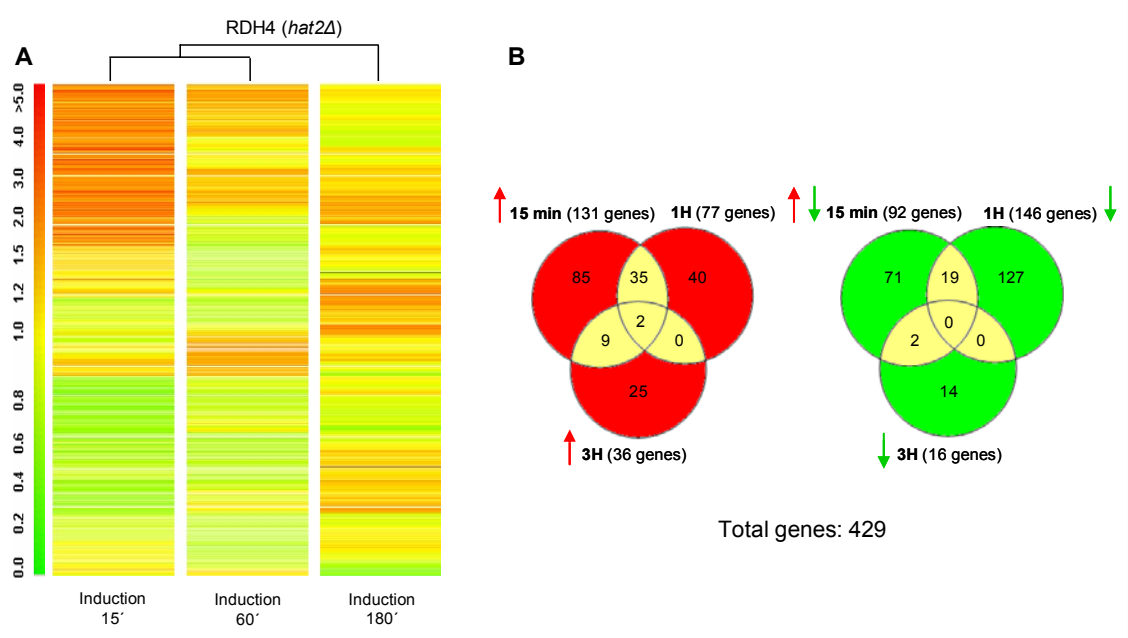

Figure 2.16 Transcript profiles of *C. albicans hat2Δ* mutant during the yeast-hypha transition (A). Venn diagrams of genes regulated by Hat2p in the RDH4 (B). The CAI4 strain was used as reference, which all were grown in identical conditions: Lee/37°C (inducing).

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 by the *hat2*Δ mutant. Many genes can not be readily categorized into functional groups and represent a wide range of cellular metabolic processes (Supplementary tables 2.3 and 2.4). The microarray results demonstrate that the lack of Hat2p affect a large number of cells functions and most likely serve to globally reprogram in *C. albicans* physiology to allow the *hat2*Δ mutant to adapt to this environment.

Expression of transcription factors

Only one transcription factor was down-regulated at the 15 and 60 min of incubation; namely, *ZPR1*, which has been described as a putative zinc finger transcription factor with unspecified role in morphogenesis, regulated by Gcn4p and repressed in response to amino acid starvation (Rogers & Barker, 2003; Yin *et al.*, 2004). Six genes including IPF19804 (Karababa *et al.*, 2004) (at 15 min), *MRR1* (Morschhauser *et al.*, 2007) (at 60 min), three transcription factors member of a family of telomere-proximal genes *CTA2*, *CTA24, CTA29* (Doedt *et al.*, 2004; Kaiser *et al.*, 1999; van het Hoog *et al.*, 2007; Zakikhany *et al.*, 2007), *GAL4* (Martchenko *et al.*, 2007) (at 180 min) and *HAC1* (Nobile

& Mitchell, 2005), *TAF145* (Davis *et al.*, 2002), *SPT20* (Marchais *et al.*, 2005), *ZCF20* (Yan *et al.*, 2008) (at 15 min), *CAP1* (Alarco *et al.*, 1997), *CPH1* (Chen *et al.*, 2002; Magee *et al.*, 2002), *CTA4* (Kaiser *et al.*, 1999), *CTA8* (Kaiser *et al.*, 1999), *MIG1* (Garcia-Sanchez *et al.*, 2004), *TBP1* (Leng *et al.*, 1998), *TUP1* (Murad *et al.*, 2001a) (at 60 min) were up- and down-regulated respectively.

Only *HAC1* has been reported to be down-regulated in the *hat1Δ* mutant at 15 min of incubation suggesting that the *hat2*Δ mutant has a distinct transcriptome of *hat1Δ* mutant implying that Hat2p has additional functions in the cell. Among the uncharacterized ORFs without significant homologies to *S. cerevisiae*, two ORF (*CTA2* and *CTA24*) which were named *TLO3* and *TLO12* are putative members of a family of telomeric proximal genes In the Candida Data Base.

Cell wall genes: Our results indicate that the transcript levels of seven encoding for cell wall proteins including *BMT5* (at 15 min), *IRS4, FGR41* (at 60 min), *HWP1, PGA54* (at 180 min), *BMT9, CHS2 and GSL1* (at 15 and 60 min) and *PGA45* (at 15 and 180 min) were up-regulated and expression of *PHR2, TOS1* (at 15 min), *ALS4, ECM31, HYR3, PGA6, PGA63* (at 60 min), *HYR1* (at 180 min) and *GSC1* (at 15 and 60 min) were down-regulated (Table 2.4).

Only *HWP1, TOS1* (at 60 min) and *ALS4* (at 60 min) have been reported to be down- and up-regulated in the *hat1Δ* mutant suggesting again that the *hat2*Δ mutant has a distinct transcriptome of *hat1Δ* mutant. Expression of *HWP1, PGA54* (at 15 min), *HYR1* (at 15 and 60 min) and *TOS1* (at 60 min) were down-regulated in the *hat1Δ, hat2Δ* double mutant. *HWP1, PGA54, PHR1, TOS1, PGA6* and *HYR1* have been found to be up-regulated in previous studies in our laboratory on transcriptional response of *C. albicans* to temperature (Rashki, Ph.D thesis 2009).

Table 2.4 Differentially expressed genes in *hat2Δ* mutant at the three times of incubation (at 15, 60 and 180 min)

Gene name	ORF	Function	Fold regulation		
Up-regulated genes					
Glycolysis			**15 min**	**60 min**	**180 min**
ADH1	orf19.3997	Alcohol dehydrogenase	5.91	1.37	0.96
ADH5	orf19.2608	Putative alcohol dehydrogenase	1.76	0.79	0.72
ALD5	orf19.13228	An aldehyde dehydrogenase	0.81	0.94	1.79
CDC19	orf19.3575	Putative pyruvate kinase	3.66	0.81	1.93
ENO1	orf19.395	Enolase (2-phospho-D-glycerate-hydrolyase)	4.77	0.81	1.26
FAB1	orf19.9088	Phosphatidylinositol 3-phosphate 5-kinase	1.15	1.24	1.70
FBA1	orf19.4618	Putative fructose-bisphosphate aldolase	2.97	0.64	1.01
GPH1	orf19.7021	Putative glycogen phosphorylase	2.25	0.74	0.93
GPM1	orf19.903	Described as phosphoglycerate mutase	2.54	0.55	1.08
GLG2	orf19.7434	Protein described as self-glucosylating	1.75	1.28	1.42
HXK2	orf19.8176	Protein described as hexokinase II	2.66	0.93	0.79
PCK1	orf19.7514	Phosphoenolpyruvate carboxykinase	3.68	0.90	1.04
PDC11	orf19.2877	Protein similar to pyruvate decarboxylase	2.62	0.92	1.19

PFK2	orf19.6540	Beta subunit of phosphofructokinase	1.88	0.61	1.07
PGK1	orf19.3651	Phosphoglycerate kinase	3.93	1.03	1.07
TPI1	orf19.6745	Putative ortholog of *S. cerevisiae* Tpi1p	1.58	0.93	0.85
TKL4	orf19.5112	Putative transketolase	1.08	0.39	1.78
Cell wall					
BMT5	orf19.1464	Putative beta-mannosyltransferase	3.30	1.48	1.15
BMT9	orf19.4673	Putative beta-mannosyltransferase	1.64	2.02	0.98
CHS2	orf19.7298	Chitin synthase	1.99	2.55	0.84
GSL1	orf19.2495	Subunit of beta-1.3-glucan synthase	1.77	2.58	1.08
IRS4	orf19.6953	Protein with roles in cell wall integrity	1.14	1.67	0.99
FGR41	orf19.4910	Putative GPI-anchored protein	1.35	2.18	1.20
HWP1	orf19.1321	Hyphal cell wall protein	0.76	1.05	1.78
PGA45	orf19.2451	Cell wall protein; putative GPI-anchor	2.08	1.01	2.24
PGA54	orf19.2685	Putative GPI-anchored protein	0.59	0.61	1.89
Glucose transport					
HGT1	orf19.4527	High-affinity glucose transporter	1.81	0.54	1.69
HGT7	orf19.2023	Putative glucose transporter	2.29	1.47	1.97
HGT8	orf19.2021	Putative glucose transporter	2.14	1.33	1.44
HGT9	orf19.644	Putative glucose transporter	1.43	2.75	0.98
SHA3	orf19.3669	Protein similar to S. cerevisiae Sha3p, which is a serine/threonine kinase involved in glucose transporter	1.92	2.09	1.42
Transcription factors					
CTA29	orf19.7127.1	Transcription is upregulated in an RHE mode	1.29	1.38	1.98
IPF19804	orf19.3835	Putative DNA-binding transcription factor	1.93	1.40	1.06
GAL4	orf19.5338	Putative transcription factor with zinc cluster DNA-binding motif	1.14	0.90	2.48
MRR1	orf19.7372	Regulator of *MDR1* transcription	1.31	8.69	1.00
TYE7	orf19.4941	Putative bHLH transcription factor	2.22	1.46	1.73
Telomere-proximal genes family					
TLO3 (CTA2)	orf19.6112	Transcriptional activator family of telomere-proximal genes	1.17	1.18	1.72
TLO4	orf19.7276.1	Member of a family of telomere-proximal genes	1.25	1.38	1.68
TLO12 (CTA24)	orf19.4054	Transcriptional activator family of telomere-proximal genes	1.11	0.85	1.75
Ribosomal protein					
RPL4B	orf19.7217	Putative ribosomal protein	1.72	1.18	1.11
RPL5	orf19.6541	Predicted ribosomal protein	1.75	1.44	1.26
RPL33	orf19.6882.1	Ribosomal protein L35a	0.98	1.78	1.12
RPL42	orf19.4909.1	Ribosomal protein L36a	1.77	1.20	1.07
RPS6A	orf19.4660	Predicted ribosomal protein	1.11	2.15	1.16
Down-regulated genes					
Respiration					
COB		Cytochrome b	0.41	0.88	1.12
COX1		Subunit I of cytochrome c oxidase	0.59	0.73	1.43
COX3A		Subunit III of cytochrome c oxidase	0.41	0.70	1.28
COX3B		Subunit III of cytochrome c oxidase	0.48	0.74	1.49
COX6	orf19.873.1	Flucytosine induced	0.88	0.47	1.33
COX15	orf19.3656	Transcription is regulated by Nrg1p and Tup1p	0.79	0.64	1.11
NAD2		Subunit 2 of NADH: ubiquinone oxidoreductase	0.41	0.69	1.74
NAD6		Subunit 6 of NADH: ubiquinone oxidoreductase	0.48	0.81	1.20
Cell wall					
ALS4	orf19.4556	ALS family protein	0.95	0.55	0.78
ECM31	orf19.6057	Involved in cell wall biogenesis and architecture	0.93	0.52	0.74
GSC1	orf19.2929	Subunit of beta-1.3-glucan synthase	0.50	0.32	0.97
HYR1	orf19.12440	Nonessential, GPI anchored	1.06	0.71	0.51
HYR3	orf19.575	Putative GPI-anchored protein of unknown function	0.94	0.48	1.02
PHR2	orf19.3829	Glycosidase of cell surface	0.50	0.67	1.44
PGA6	orf19.12229	Putative GPI-anchored cell-wall protein of unknown function	0.85	0.51	1.25
PGA63	orf19.6217	Putative protein of unknown function	1.05	0.34	No data
TOS1	orf19.1690	Similar to alpha agglutinin anchor subunit	0.59	0.81	1.24

Ribosomal protein					
RPL11	orf19.2232	Predicted ribosomal protein	0.49	1.01	0.86
RPL20B	orf19.4632	Predicted ribosomal protein	0.58	0.77	0.84
RPL8B	orf19.6002	Predicted ribosomal protein	0.51	0.84	1.00
RPS4A	orf19.5341	Predicted ribosomal protein	0.57	0.67	0.78
RPS15	orf19.5927	Putative ribosomal protein	0.49	0.69	1.10
MRPL7	orf19.2214	Ribosomal protein of the large subunit	0.96	0.47	1.11
MRPL19	orf19.13611	Protein described as a ribosomal protein	1.00	0.47	0.98
MRPL38	orf19.5684	Ribosomal protein of the large subunit(L14)	0.69	0.54	1.23
RPL10	orf19.10452	Putative ribosomal protein	0.68	0.54	1.29
RPL14B	orf19.4931.1	Ribosomal protein L14B	0.89	0.39	0.98
RPL16A	orf19.6085	Protein similar to *S. cerevisiae* ribosomal protein Rpl16Ap	0.70	0.38	0.90
RPS16	orf19.2994.1	Ribosomal protein	0.101	0.49	0.89
RPL82	orf19.2311	Predicted ribosomal protein	0.46	0.51	0.97
Transcription factors					
CPH1	orf19.4433	Transcription factor required for mating and hyphal growth	0.71	0.40	1.26
CAP1	orf19.9191	Transcription factor	0.85	0.47	1.15
CTA4	orf19.7374	Predicted transcription factor	0.85	0.56	0.85
CTA8	orf19.4775	Protein that activates transcription	0.81	0.60	1.19
HAC1	orf19.2432	Putative transcription factor	0.56	0.88	1.26
MIG1	orf19.4318	Transcriptional repressor	0.85	0.38	0.95
SPT20	orf19.422	Protein described as a transcription factor	0.62	1.36	1.49
TAF145	orf19.735	Putative DNA-binding transcription factor; putative TFIID subunit; flucytosine repressed	0.65	0.89	1.33
TBP1	orf19.1837	Transcription initiation factor	0.79	0.38	1.15
TUP1	orf19.6109	Transcriptional corepressor	1.25	0.55	1.43
ZCF20	orf19.11621	Predicted zinc-finger protein of unknown function	0.56	1.28	0.69
ZPR1	orf19.3300	Protein with putative zinc finger	0.57	0.59	1.07
Ergoestrol biosynthesis					
ERG4	orf19.5379	As similar to sterol C-24 reductase	1.13	0.43	1.02
ERG7	orf19.9143	2,3-epoxysqualene-lanosterol cyclise	0.89	0.42	1.09
ERG9	orf19.3616	Putative farnesyl-diphosphate farnesyl transferase	1.23	0.42	0.96
ERG26	orf19.2909	C-3 sterol dehydrogenase	1.03	0.58	0.77

Genes involved in protein synthesis and ribosomal proteins

A second important category includes up-regulation of *RPL42, RPL4B, RPL5* (at 15 min), *RPS6A, RPL33* (at 60 min) and down-regulation of *RPL11, RPL20B, RPL8B, RPS15, RPS4A* (at 15 min), *MRPL7, MRPL19, MRPL38, RPL10, RPL14B, RPL16A, RPS16* (at 60 min) and *RPL82* (at 15 and 60 min) in the *hat2Δ* mutant. Two genes, *RPL33* and *RPS4A*, that have been found to be up-regulated in previous studies on transcriptional response of *C. albicans* to temperature (Rashki, Ph.D thesis 2009), were up- and down-regulated in our experiment respectively. No genes of this category have been found to be up- and down-regulated in the *hat1Δ* mutant.

Central carbon metabolism

The regulation of 17 genes (13 up-regulated after 15 min) involved in the glycolytic pathway was detected. The expression all of them decreased with time, suggesting a

variation in regulation of this group of genes in *hat2Δ* mutant during the yeast-hypha transition (Table 2.4). Only expression three genes, *ADH1, ENO1* and *PDC11* have been found to be up-regulated in the *hat1Δ* mutant after 15 min of incubation. The expression all of them have been reported to be down-regulated after 15 min and increased with time in the transcriptional response of *C. albicans* to temperature (Rashki, Ph.D thesis 2009) suggesting that *HAT2* play a role in regulation of this set of genes.

Expression all of this group of genes were down-regulated in the *hat1Δ, hat2Δ* double mutant and have been reported to be decreased in the *gcn5Δ* and *gcn5Δ, hda1Δ* and *odc1Δ* mutant strains during the yeast-hypha transition (Rashki, Ph.D thesis 2009). These changes reflect differences in the modulation of metabolic genes during the hypha transition in different mutant strains. We have found up-regulation of five genes belonging to the glucose transporter *HGT1, HGT7* (at 15 and 180 min)*, HGT8* (at 15 min)*, HGT9* (at 60 min) and *SHA3* (at 15 and 60 min) (Brown *et al.*, 2006; Davis *et al.*, 2002; Fan *et al.*, 2002; Uhl *et al.*, 2003; Varma *et al.*, 2000) in the *hat2Δ* mutant.

Set of genes regulated only at one incubation time

After 15 and 60 minutes of incubation at 37°C, several sets of genes were either up- or down-regulated. The main groups of up-regulated genes at one time point included five genes contributing to stress response, *HSP12* (at 15 min), *HSP70, HSP90, SSA2* and *SSC1* (at 180 min) (Supplemenary table 2.3). Among the up-regulated genes after 15 min of incubation were *HAT1* (histone acetyltransferase), *PLB3* (putative secreted phospholipase B), two genes encode for *DRAP* deaminase (*RIB2* and *RIB21*), *RIM21* (protein involved in the pH response pathway), *SMF11* (manganese transporter) and two genes involved in ubiquitin (*UBI4* and *UBP1*) (Supplemenary table 2.3).

The genes up-regulated at 60 min were *CDC23* (similar to anaphase-promoting complex component), *CDC73* (protein similar to *S. cerevisiae* Cdc73p), *HHT21* (putative histone H3), *HOG1* (MAP kinase of osmotic and core stress response) (Supplemenary table 2.3). Among the down-regulated genes after 15 min, we noted six genes corresponding to respiration (*COB, COX1, COX3A, COX3B, NAD2* and *NAD6*), two genes involved in RNA helicases (*DBP2* and *DBP9*) and four genes involved in transport (*DAL9, DUR32, TPO4* and *FET34*). Among the down-regulated genes after 60 min, we noted four genes corresponding to ergosterol biosynthesis (*ERG4, ERG7, ERG9* and *ERG26*), five genes involved in respiration (*ABC1, COX15, COX6, MCI4* and *MCR1*) and five genes encoding for translation elongation factor (*EFB1, EFT2, SUP35, TIF35* and *TUF1*) (Supplemenary table 2.4).

Transcriptional profile of *hat1Δ, hat2Δ* double mutant

A transcriptional profiling analysis was performed to further examine the roles of Hat1p and Hat2p proteins in *C. albicans*. The transcriptome of the *hat1Δ, hat2Δ* double mutant was compared with the CAI4 strain and 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 2.5-2.8). To explore the functions of the *hat1Δ, hat2Δ* 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 28°C. In the *hat1Δ, hat2Δ* double mutant we detected an altered expression of 219 genes by ≥ 1.5-fold: however in the transcriptome of cells growing in Lee medium at 37°C during the hypha transition, 793 genes modified their expression level, including 376 up-regulated genes and 417 down-regulated genes. Approximately 16% of the *C. albicans* genes showed significant changes in their expression levels (ρ -value ≤ 0.02), indicating a global transcriptional response.

Differential gene expression in exponential growth phase

During the yeast mode of growth more genes were up-regulated (160 genes) than down-regulated (59 genes). For up-regulated genes, the transcript ratios had values ≥1.5-2.0 for 113 genes, 2–3 for 37 genes (e. g. *SMF11, PTR2*) and ≥ 3 for ten genes (e.g. IPF8762, IPF525, *HSP12* and *CCL1*). For down-regulated genes, the transcript ratios had values between 0.33−0.66 for 51 genes and < 0.33 for eight genes.
Distinct groups of genes were either up- or down-regulated during the yeast mode of growth in the *hat1*Δ*, hat2*Δ double mutant (Table 2.5).

Table 2.5 Differentially expressed genes in *hat1Δ, hat2Δ* mutant during the yeast mode of growth

Gene name	ORF	Function	Fold regulation
Up-regulated genes			
Cell wall			
BMT6	orf19.5602	Putative beta-mannosyltransferase	1.61
CHS1	orf19.4477	Member of aldo-ketonreductase family	2.22
CPS37	orf19.2531	Plasma membrane	3.14
IFF11	orf19.5399	Secreted protein required for normal cell wall structure	2.67
IPF885	orf19.7214	Protein described as similar to glucan 1.3-beta-glucosidase	1.99
PGA10	orf19.5674	Plasma membrane protein involved in heme-iron utilization	1.92
PGA14	orf19.968	Putative GPI-anchored protein of unknown function	1.69
PIR1	orf19.220	Structural protein of cell wall	1.57
RBT5	orf19.5636	GPI-anchored cell wall protein	2.12
RHD3	orf19.5305	Putative GPI-anchored protein that localizes to the cell wall	1.61

Transcription factors			
CBF1	orf19.2876	Transcription factor	2.28
TCA cycle			
FDH2	orf19.1117	Formate dehydrogenase	2.51
FDH3	orf19.1774	Formate dehydrogenase	1.80
FDH4	18026	Similar to formate dehydrogenase	4.19
Glycolysis			
GPM2	orf19.1067	Protein described as phosphoglycerate mutase	1.61
PCK1	orf19.7514	Phosphoenolpyruvate carboxykinase	2.65
Stress response			
HSP104	orf19.13747	Functional homolog of *S. cerevisiae* Hsp104p	2.60
HSP12	orf19.3160	Heat-shock protein	5.06
HSP31	orf19.11148	Protein repressed during the mating process	1.53
HSP70	orf19.4980	Putative chaperone of Hsp70 family	1.94
HSP78	orf19.884	Protein described as a heat-shock protein	1.61
Proteasome			
PRE3	orf19.6991	Protein described as beta-1 subunit of proteasome	2.05
PRE4	orf19.11705	20S proteasome subunit	1.78
PRE6	orf19.544.1	Protein described as alpha-4 subunit of proteasome	1.83
PRE7	orf19.2755	Putative subunit of 20S proteasome	1.66
PRE9	orf19.350	Alpha3 (C9) subunit of the 20S proteasome	1.81
PUP2	orf19.709	Alpha5 subunit of the 20S proteasome	1.60
RPN12	orf19.213	26S proteasome regulatory subunit	1.97
RPN4	orf19.1069	26S proteasome subunit	1.77
Telomere-proximal genes family			
TLO1	orf19.7544	Member of a family of telomere-proximal genes	1.68
TLO3	orf19.6112	Putative transcriptional activator	2.11
TLO11	orf19.5700	Member of a family of telomere-proximal genes	1.57
TLO12	orf19.4054	Putative transcriptional activator	1.74
TLO16	orf19.7127	Member of a family of telomere-proximal genes	1.78
Down regulated genes			
TCA cycle			
ACO2	orf19.6632	Protein described as aconitate hydratase 2	0.62
CIT1	orf19.4393	Protein described as citrate synthase	0.32
FUM12	orf19.6724	Protein described as similar to fumarate hydratase	0.52
GDH3	orf19.4716	Similar to NADP-glutamate dehydrogenase	0.61
Glycolysis			
PDC11	orf19.2877	Protein similar to pyruvate decarboxylase	0.64
PYC2	orf19.789	Putative pyruvate carboxylase	0.48
SDH12	orf19.10389	Protein with similarity to *S. cerevisiae SDH1*	0.53
Cell wall			
ECM17	orf19.4099	Predicted enzyme of sulfur amino acid biosynthesis	0.51
IFF5	orf19.10397	Putative GPI-anchored protein of unknown function	0.28
PGA49	orf19.4404	Putative GPI-anchored protein of unknown function	0.47
Transporter			
DAL9	orf19.6956	Putative allantoate permease	0.45
DUR1,2	orf19.780	Transcription is regulated by Nrg1p	0.43
DUR31	orf19.781	Putative urea transporter	0.52
IFC1	orf19.3746	Oligopeptide transporter	0.38
IFC3	orf19.3749	Oligopeptide transporter	0.38
IPF1471	orf19.304	Putative transporter similar to MDR proteins	0.20
MEP2	orf19.5672	Ammonium permease and regulator of nitrogen	0.30
NUP	orf19.6570	Nucleoside permease	0.56
PMA1	orf19.5383	Plasma membrane H(+)-ATPase	0.51
Transcription factors			
SPT20	orf19.422	Protein described as a transcription factor	0.54

We have found up-regulated in the *hat1Δ* (*TLO2, TLO3, TLO4, TLO9, TLO10, TLO11, TLO12* and *TLO34*) and in the *hat1Δ, hat2Δ* mutants a family of genes involved in telomeric silencing although those genes have not been described in other organisms (*TLO1, TLO3, TLO11, TLO12, TLO16*) (Harcus *et al.*, 2004; Maglott *et al.*, 2007; van het Hoog *et al.*, 2007).

We proposed that in *C. albicans* the *HAT1* gene plays a role in telomeric silencing. In *Saccharomyces cerevisiae* has been reported that deletion of the *HAT1* and/or *HAT2* gene doesn't changes in the level of telomeric silencing (Poveda *et al.*, 2004). This result is a difference between the putative Hat-B complex of *C. albicans* and *S. cerevisiae.*

However, in the double mutant, we detected the up-regulation of ten genes encoding for cell wall proteins (*IFF11*) (Bates *et al.*, 2007)*, BMT6, CHS1, CSP37, RBT5, RHD3,* IPF885*, PGA10, PGA14* and *PIR1*) and down-regulation of three cell wall genes (*ECM17, IFF5* and *PGA49*), of five genes involved in stress response (*HSP104, HSP12, HSP31, HSP70* and *HSP78*), of eight genes encoding for proteasome (*PRE3, PRE4, PRE6, PRE7, PRE9, PUP2, RPN12* and *RPN4*).

Our results were in contrast which those obtained with the *hat1Δ* simple mutant, in which genes encoding for proteasome and cell wall proteins and the majority of genes involved in the glycolytic pathway and sugar metabolism did not appear regulated during the yeast mode of growth. We can conclude that the transcriptional response in *C. albicans hat1Δ, hat2Δ* double mutant differs significantly from those of *hat1Δ* and *hat2Δ* simple mutants.

Transcriptional profile of the *hat1*△, *hat2*△ during the yeast-hypha transition

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 *hat1*△, *hat2Δ* mutant in Lee medium for 15, 60 and 180 min at 37°C using the CAI4 strain as reference. A total of 793 genes were found to change at the consensus level of ≥ 1.5 fold, including 376 up- and 417 down-regulated genes. The distribution of genes altered at the three points is represented in the Venn diagram, and the clustering of genes regulated by deletion of *HAT1* and *HAT2* is represented in Fig. 2.17 (A and B).

We found that 16 genes were common to the three times assayed (6 genes up- and 10 down-regulated), 193 genes at 15 and 60 min (62 up- and 131 down-) and 16 genes at 60 and 180 min (10 up- and 6 down-). 272 genes (189 up- and 83 down-), 265 genes (97 up- and 168 down-) and 29 genes (10 up- and 19 down-) varied only at one time of induction (at 15, 60 and 180 min respectively). However, only 2 genes were common between 15 and 180 min validating our experimental approach. Sixteen genes common at the three times are listed in Table 2.6.

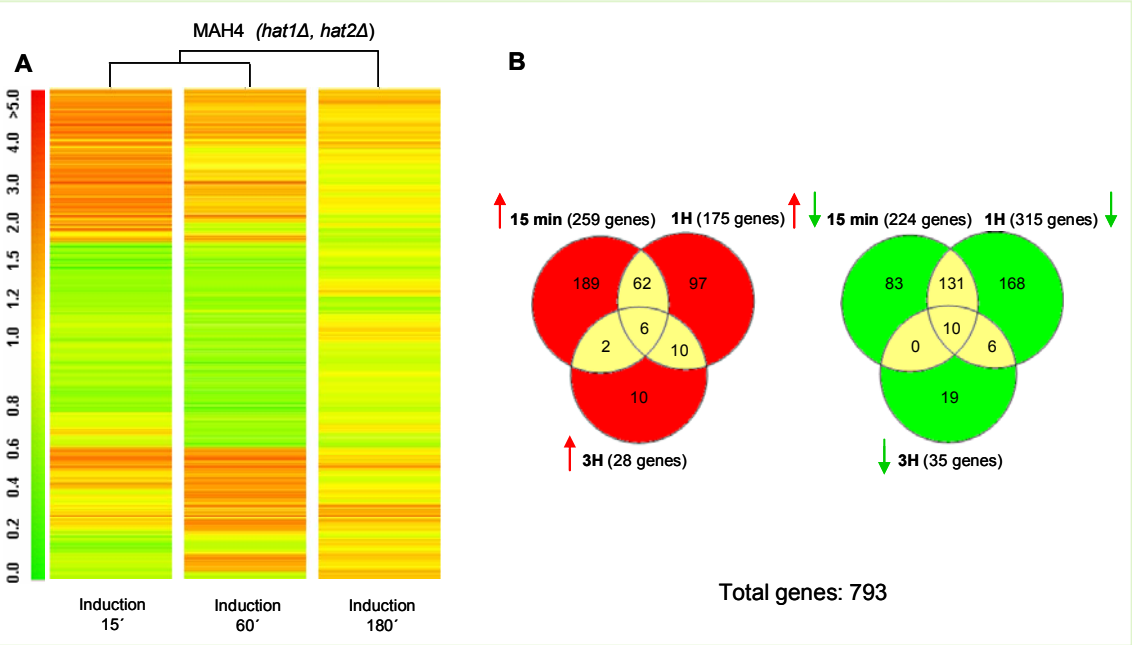

Figure 2.17 Transcript profiles of *C. albicans hat1Δ, hat2Δ* double mutant during the yeast-hypha transition (A). Venn diagrams of genes regulated by Hat1p and Hat2p in the double mutant (B). The CAI4 strain was used as reference, which all were grown in identical conditions: Lee/37°C (inducing).

Table 2.6 Common differentially expressed genes in *hat1Δ, hat2Δ* mutant at the three times of incubation (at 15, 60 and 180 min)

Gene name	ORF	Description	Fold regulation 15 min	Fold regulation 60 min	Fold regulation 180 min
Up-regulated genes					
COX3A		Subunit III of cytochrome c oxidase	1.85	2.38	1.97
COX3B		Subunit III of cytochrome c oxidase,	1.63	1.88	1.78
CTR1	orf19.3646	Copper transporter	2.01	3.86	2.09
HHT1	orf19.6791	Putative histone H3	2.37	2.21	2.36
RPL2	orf19.2309.2	Ribosomal protein L8	2.78	3.32	2.34
RPL27A	orf19.5225.2	Ribosomal protein L27	1.76	1.79	1.68
Down-regulated genes					
ADH1	orf19.3997	Alcohol dehydrogenase	0.39	0.38	0.59
ATP14	orf19.5491.1	Macrophage/pseudohyphal-induced	0.59	0.43	0.49
ATP4	orf19.3579	Macrophage/pseudohyphal-induced	0.40	0.36	0.58
CDC19	orf19.3575	Putative pyruvate kinase	0.20	0.34	0.49
ERG251	orf19.12101	Ketoconazole-induced; amphotericin B, caspofungin repressed	0.44	0.36	0.51
FCY24	orf19.7331	Putative transporter	0.49	0.39	0.57
PDC11	orf19.2877	Protein similar to pyruvate decarboxylase;	0.29	0.37	0.63
PHM7	orf19.2170	Putative transporter	0.60	0.41	0.64
YHB1	orf19.3707	Nitric oxide dioxygenase	0.46	0.59	0.45
YTH1	orf19.6881	Protein described as an mRNA cleavage and polyadenylation	0.26	0.29	0.42

Six up-ORFs can be classified in respiration (i.e *COX3A* and *COX3B*), one gene encodes for copper transporter (*CTR1*), two genes encode for ribosomal proteins (*RPL2* and *RPL27A*) and one gene is involved in histone assembly (*HHT1*). The ten commonly down-regulated genes belong to the glycolytic pathway were *ADH1, CDC19* and *PDC11*, two genes involved in ATP biosynthesis (*ATP14* and *ATP4*), two genes encoding for putative transporter proteins (*FCY24* and *PHM7*) and three genes with different function included *ERG251* (putative C-4 methyl sterol oxidase) (Marichal *et al.*, 1999), *YHB1* (nitric oxide dioxygenase) and *YTH1* (essential RNA-binding component of cleavage and polyadenylation factor) (Barabino *et al.*, 2000).

Common up- and down-regulated genes after two time points of induction

The analysis of the microarray data of cells obtained during the hyphal transition showed a total of 193 genes to be differentially expressed commonly between 15 and 60 min of induction: 62 genes were up-regulated and 131 were down-regulated (Supplementary tables 2.7 and 2.8).

Among the up-regulated genes, we highlight the induction of genes encoding for rRNA processing protein (*RPL11, RPL14B, RPL23A, RPS24, RRP1, RSA2, SIK1* and *SNU23*), genes encoding for translation initiation factors (*SUI2, TEF1* and *TIF11*), genes encoding for stress-response proteins (*HSP70* and *HSP90*) and histone assembly (*HHF1* and *HHF22*). Among the down-regulated genes, we observed genes encoding for cell wall proteins (*ALS1, ALS3, ALS4, ECM21, FGR51, HYR1,* IPF8796*, PGA63, PMT1, PMT2, PMT4* and *RBT1)*, glycolytic pathway (*ADH2, PFK1, PFK2, GPM1, GSY1, PGK1* and *TPS2*), glucose transport (*HGT6, HGT7* and *HGT8*), cell polarity and signal transduction (*ARP3, ARP8, BEM1, BEM2, BUD2, CLC1, MYO2, RAS1, RGS2* and *TPM2*), secretory pathway (*ERV46, SEC11, SEC16, SEC24, SEC61, SEC9*) and transcription factors (*ECM22, HAC1, SSN6, TEC1* and *UME6*). The down-regulation of genes involved in the secretory pathway and in o-glycosylation has been reported to be down-regulated in *odc1Δ* mutant after 15 and 60 min of incubation (Rashki, Ph.D thesis 2009). Differentially expression of the PMT genes family were also reported in pmt mutant strains (Cantero *et al.*, 2007).

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

Of the ten ORFs, we detected three genes involved in respiration (*COB, NAD2* and *NAD6*), two genes encoding for ferric reductases (*FRE7* and *FRE30*), one gene encoding for a predicted GPI anchor (*PGA10*), one gene encoding for a predicted

ribosomal protein (*RPS4A*), and two genes of unknown function. The down-regulated genes, were *CRH11* (putative GPI-anchor), *CYT1* (component of the mitochondrial respiratory chain), IPF11725 (described as a Gag-related protein), IPF3912 (similar to *S. cerevisiae TOS8* a putative transcription factor found associated with chromatin), *QCR8* (Subunit 8 of ubiquinol cytochrome-c reductase complex) and *ZRT2* (a protein described as predicted zinc transporter).

Sets of genes regulated only at one incubation time

A total of 272 genes were expressed differentially at 15 min of hypha transition, among which 189 and 83 genes were up- and down-regulated respectively.

Expression of some genes include those encoding for rRNA processing protein (*MPP10, MRP1, MRP7, MRPL16, MRPL17, MRPL31, MRPL36, RPF1, RPL10, RPL21A, RPL25, RPL37B, RPL39, RPL40B, RPL6, RPL9B, RPS1, RPS17B, RPS19A, RPS25B, RPS28B, RPS3, RPS7A*), genes encoding for nucleolar proteins (*MAK16, MAK21, MAK5, NOP1, NOP14, NOP15, NOP2, NOP4, NOP5, RRP15, RRP6, RRP8*), genes involved in DNA-direcred RNA polymerase (*RPA190, RPA43, RPA49, RPC10, RPC25, RPC40*), stress response (*ASR1, ASR3, DDR48, HSP12, SOD5, SSA2*), elongation factors (*EFT2, SUI3, TFS1, TIF1, TIF3*) and one transcription factor (*ZPR1*) (Rogers & Barker, 2003) were up-regulated. The main categories of down-regulated genes were correspond to those encoding for cell wall proteins (*ALS2, EAP1, ECE1, ECM17, ECM33, HWP1, MNT1, PGA54, PGA58, PGA62*), secretory pathway (*CHC1, ERV29, SEC23, SEC26*), glycolytic pathway and glyoxylate cycle (*ENO1, GLK1, ICL1, MIS11, MLS1, TDH3, TPI1*) and transcription factors (*GAL4,* IPF29*, SKO1*) (Table 2.7).

Table 2.7 Differentially expressed genes in *hat1Δ, hat2Δ* mutant during the yeast-hypha transition (at 15, 60 and 180 min)

Gene name	ORF	Function	Fold regulation		
Up-regulated genes					
Amino acid biosynthesis			15 min	60 min	180 min
ARG1	orf19.7469	Similar to argininosuccinate synthase	1.37	1.89	0.81
ARG3	orf19.5610	Alkaline downregulated	1.36	2.63	0.82
GAP4	orf19.4456	Putative amino acid permease	1.21	1.72	1.21
MET14	orf19.946	Predicted role in sulfur metabolism	1.14	1.53	1.43
MET3	orf19.5025	Putative ATP sulfurlyase of sulfate assimilation	1.22	1.61	1.32
SAM2	orf19.657	S-adenosylmethionine synthetase	1.04	1.98	1.15
Stress response					
ASR1	orf19.2344	Protein described as similar to heat shock proteins	1.72	1.15	1.07
ASR3	orf19.8462	Gene regulated by cAMP and by osmotic stress	1.65	0.78	0.87
HSP60	orf19.717	Protein described as mitochondrial heat shock protein	1.29	2.14	0.73
HSP70	orf19.4980	Putative chaperone of Hsp70 family	2.63	2.53	1.04
HSP90	orf19.6515	Chaperone of Hsp90 family	3.20	2.68	1.41
MSI3	orf19.2435	Protein of the HSP70 family	2.54	2.01	1.01

SSA2	orf19.1065	Hsp70 family chaperone	2.19	1.49	0.76
SSZ1	orf19.3812	Protein described as an *HSP70* chaperone	1.60	1.06	1.01
TSA1	orf19.7417	Protein of TSA/alkyl hydroperoxide peroxidase C	1.80	1.32	0.91
Protein synthesis and ribosomal proteins					
MPP10	orf19.1915	Downregulated in core stress response	1.96	1.19	1.10
MRP1	orf19.1661.1	Mitochondrial ribosomal protein of the small subunit	1.71	1.24	0.98
MRP7	orf19.7203	Mitochondrial ribosomal protein YmL2 precursor	1.78	1.35	1.02
MRPL16	orf19.9569	Ribosomal protein	1.56	1.44	1.08
MRPL17	orf19.585	Ribosomal protein of the large subunit	1.74	1.14	0.91
MRPL31	orf19.1485	Mitochondrial ribosomal protein	1.60	1.26	1.13
MRPL36	orf19.3205	Ribosomal protein YmL36 precursor	1.89	1.48	0.84
NMD3	orf19.706	Downregulated in core stress response	1.66	1.17	1.06
NMD5	orf19.4188	Involved in protein-nucleus import	1.91	1.24	1.07
PWP1	orf19.12110	Downregulated in core stress response	1.98	1.48	1.17
RPL10	orf19.10452	Ribosomal protein L10	1.83	1.49	1.09
RPL21A	orf19.840	Putative ribosomal protein	1.70	1.40	1.11
RPL25	orf19.687.1	Ribosomal protein L23a (by homology)	1.99	1.25	1.23
RPL37B	orf19.667.1	Ribosomal protein	1.61	1.37	1.12
RPL39	orf19.827.1	Ribosomal protein L39	1.73	1.16	1.24
RPL40B	orf19.4684.2	Protein with similarity to the ribosomal protein portion	1.57	1.17	1.09
RPL6	orf19.3003.1	Protein similar to *S. cerevisiae* Rpl6p	1.59	1.49	1.28
RPL9B	orf19.236	Predicted ribosomal protein	1.82	1.34	1.26
RPS1	orf19.10520	Probable ortholog of *S. cerevisiae* ribosomal protein Rp10	1.78	1.28	1.42
RPS17B	orf19.2329.1	Putative ribosomal protein	1.70	1.37	1.19
RPS19A	orf19.5996.1	Putative ribosomal protein	1.65	1.45	1.18
RPS25B	orf19.6663	Predicted ribosomal protein	1.57	1.35	1.18
RPS28B	orf19.7048.1	Ribosomal protein S28B	1.62	1.13	1.25
RPS3	orf19.6312	Putative ribosomal protein	1.68	1.42	1.40
RPS7A	orf19.9267	Predicted ribosomal protein	1.71	1.49	1.26
RRS1	orf19.6014	M utation confers hypersensitivity to 5-fluorocytosine	2.43	1.34	1.14
SOF1	orf19.5407	Involved in 18S pre-rRNA production	1.61	1.08	1.03
PRP31	orf19.1296	Pre-mRNA splicing protein (by homology)	0.84	1.70	0.83
RRP43	orf19.6259	rRNA processing protein (by homology)	1.15	1.62	1.16
RPL20B	orf19.4632	Predicted ribosomal protein	1.24	1.22	1.74
RPL11	orf19.2232	Predicted ribosomal protein	2.47	2.06	1.29
RPL14B	orf19.4931.1	Ribosomal protein L14B (by homology)	2.39	2.09	1.26
RPL23A	orf19.3504	Putative ribosomal protein	3.74	1.96	1.18
RPS24	orf19.5466	Predicted ribosomal protein	2.14	1.80	1.08
RRP1	orf19.6828	Involved in processing rRNA precursor	3.30	1.81	1.12
RSA2	orf19.6355	Involved in ribosome biogenesis (by homology)	1.77	1.60	0.97
RPS4A	orf19.5341	Predicted ribosomal protein	1.43	1.78	1.73
RPL2	orf19.2309.2	Ribosomal protein L8	2.78	3.32	2.34
RPL27A	orf19.5225.2	Ribosomal protein L27	1.76	1.79	1.68
Cell wall					
CSP37	orf19.2531	Plasma membrane, hyphal cell wall protein	2.17	1.27	0.93
PGA14	orf19.968	Putative GPI-anchored protein of unknown function	2.16	1.29	1.09
PGA52	orf19.9467	Putative GPI-anchored protein of unknown function	1.74	1.05	1.06
XOG1	orf19.2990	Exo-1,3-beta-glucanase	1.80	0.96	0.98
ALG8	orf19.1659	Putative glucosyltransferase	0.86	1.67	1.14
CSE1	orf19.1231	Importin-beta-like protein	1.26	1.55	0.83
IFF5	orf19.10397	Putative GPI-anchored protein of unknown function	1.25	1.79	1.07
PGA26	orf19.2475	Putative GPI-anchored protein of unknown function	0.84	2.58	1.01
PGA59	orf19.2767	Putative GPI-anchored protein of unknown function	0.86	2.04	0.94
RBT5	orf19.5636	GPI-anchored cell wall protein	1.26	1.45	2.16
YWP1	orf19.3618	Putative GPI-anchor; cell wall and secreted	1.02	1.38	2.23
RBR1	orf19.535	Glycosylphosphatidylinositol anchored cell wall protein	1.62	1.54	1.07
PGA10	orf19.5674	Predicted GPI anchor	1.23	1.60	2.42
Nucleolar protein					
MAK16	orf19.5500	Decreased expression in response to prostaglandins	1.87	1.16	0.74

MAK21	orf19.5912	Mutation confers hypersensitivity to tubercidin	1.71	1.45	0.97
NOP1	orf19.3138	Nucleolar protein; flucytosine induced	2.32	1.13	1.11
NOP2	orf19.501	Protein described as; hyphal-induced expression	2.03	1.48	1.02
NOP4	orf19.5198	Nucleolar protein	2.27	1.36	1.27
NOP5	orf19.1199	Protein similar to *S. cerevisiae* Nop5p protein	2.13	1.28	0.88
NOP14	orf19.5959	Mutation confers resistance to 5-fluorocytosine	1.78	1.20	0.99
NOP15	orf19.7050	A nucleolar ribosome biogenesis factor	2.23	1.40	1.14
RRP15	orf19.563	Decreased expression in response to prostaglandins	1.87	1.29	1.03
RRP6	orf19.58	Mutation confers hypersensitivity to 5-fluorocytosine	1.55	1.27	1.02
RRP8	orf19.3630	Induced upon biofilm formation	2.10	1.44	1.30
DNA directed RNA polymerase					
RPA190	orf19.1839	lucytosine induced	2.05	1.16	1.02
RPA43	orf19.2594	DNA-directed RNA polymerase I	1.83	1.44	1.07
RPA49	orf19.9567	DNA-directed RNA polymerase A	2.36	1.46	1.09
RPC10	orf19.7255	Flucytosine induced	2.05	1.46	0.91
RPC25	orf19.443	DNA-direcred RNA polymerase III	1.78	1.34	1.06
RPC40	orf19.3564	RNA polymerase	1.73	1.00	0.89
Elongation factors					
CAM1	orf19.7382	Putative translation elongation factor eEF1 gamma	1.32	1.62	1.22
EFT2	orf19.5788	Elongation Factor 2 (eEF2)	1.70	1.37	1.35
SUI2	orf19.6213	Putative translation initiation factor	1.90	1.60	1.33
SUI3	orf19.7161	Putative translation initiation factor	1.64	1.45	1.29
TFS1	orf19.1974	Transcription is regulated upon yeast-hyphal switch	1.69	1.10	1.33
TEF1	orf19.9009	Translation elongation factor 1-alpha	1.74	1.67	1.12
TIF11	orf19.5351	Predicted translation initiation factor eIF1a	2.15	1.81	1.44
TIF1	orf19.3324	Protein similar to *S. cerevisiae* translation initiation factor	1.67	1.00	0.99
TIF3	orf19.3423	Putative translation initiation factor	1.63	1.24	1.09
Feric reductase and Feroxidase					
CFL1	orf19.1263	Protein similar to ferric reductase Fre10p	1.28	2.01	1.24
CFL2	orf19.1264	Iron utilization protein; similar to an *S. cerevisiae* oxidoreductase	1.11	1.57	1.19
FET34	orf19.4215	Protein similar to multicopper ferroxidase	1.05	1.71	1.16
Transcription factors					
IPF29	orf19.5975	Putative transcription factor with zinc finger DNA-binding motif	0.59	1.98	1.23
NUT2	orf19.5268	Negative transcription regulator from artifical reporters	0.81	1.69	1.19
ZCF20	orf19.11621	Predicted zinc-finger protein of unknown function	1.28	1.84	0.72
ZCF4	orf19.1227	Putative transcription factor with zinc cluster DNA-binding motif	1.32	1.80	1.07
Down-regulated genes					
Cell wall					
ALS2	orf19.2122	ALS family protein includes cell-surface glycoproteins	0.52	0.67	0.88
BMH1	orf19.3014	14-3-3 protein, role in hyphal growth	0.64	0.89	1.07
EAP1	orf19.1401	Cell wall adhesin required for cell-cell adhesion and biofilm formation	0.32	0.99	1.18
ECE1	orf19.3374	Hyphal-specific expression	0.65	0.86	0.84
ECM33	orf19.3010.1	Cell wall protein; putative GPI-ancho	0.64	0.67	0.94
ECE17					
HWP1	orf19.1321	Hyphal cell wall protein	0.38	0.90	0.76
MNT1	orf19.1665	Alpha-1,2-mannosyl transferase	0.58	0.80	0.96
PGA54	orf19.2685	Putative GPI-anchored protein	0.32	0.69	1.07
PGA58	orf19.4334	Putative GPI-anchored protein of unknown function	0.46	0.90	1.02
PGA62	orf19.10281	Putative GPI-anchored protein	0.56	1.00	0.93
ALG7	orf19.2187	Protein involved in cell wall mannan biosynthesis	0.77	0.65	0.98
ALS5	orf19.5736	Adhesin; ALS family protein	0.85	0.55	0.92
CHS4	orf19.7349	Activator of Chs3p chitin synthase	0.89	0.54	1.12
FGR41	orf19.4910	Putative GPI-anchored protein	0.87	0.31	0.91
GLC3	orf19.13067	Similar to 1,4-glucan branching enzyme	0.71	0.58	1.07
RBT4	orf19.13583	Similar to plant pathogenesis-related	0.83	0.54	0.69

		proteins			
ALS1	orf19.5741	Adhesin; ALS family of cell-surface glycoproteins	0.63	0.63	0.89
ALS3	orf19.2355	ALS family protein	0.37	0.48	0.70
ALS4	orf19.4556	ALS family protein	0.52	0.55	1.00
FGR51	orf19.156	Protein lacking an ortholog in *S. cerevisiae*	0.55	0.62	1.02
HYR1	orf19.12440	Nonessential, GPI anchored	0.31	0.37	0.71
PGA63	orf19.6217	Putative protein of unknown function	0.48	0.44	1.03
PMT1	orf19.5171	Protein mannosyltransferase	0.52	0.52	0.92
PMT2	orf19.6812	Essential protein mannosyltransferase	0.57	0.56	1.03
PMT4	orf19.4109	Protein mannosyltransferase	0.35	0.47	0.91
RBT1	orf19.1327	Putative cell wall protein with similarity to Hwp1p	0.29	0.42	0.72
CRH11	orf19.2706	Probable membrane protein	0.87	0.52	0.58
IPF3964	orf19.675	Similar to cell wall proteins	0.91	0.64	0.84
RBT4	orf19.13583	Similar to plant pathogenesis-related proteins	0.83	0.54	0.68
TOS1	orf19.1690	Protein described as similar to alpha agglutinin anchor subunit	0.67	0.65	0.91
WAL1	orf19.6598	Protein required for hyphal growth and for wild-type cell morphology, polarized budding	0.73	0.47	1.09
Glycolysis					
ENO1	orf19.395	Enolase (2-phospho-D-glycerate-hydrolyase)	0.54	1.02	0.97
FBA1	orf19.4618	Putative fructose-bisphosphate aldolase	0.32	0.69	1.31
GLK1	orf19.1408	Aldohexose specific glucokinase (by homology)	0.57	0.77	1.14
PGI1	orf19.3888	Protein described as glucose-6-phosphate isomerise	0.61	0.73	0.91
ICL1	orf19.6844	Isocitrate lyase	0.51	1.18	1.08
MLS1	orf19.4833	Malate synthase; enzyme of the glyoxylate cycle	0.59	1.05	1.19
TDH3	orf19.6814	Glyceraldehyde-3-phosphate dehydrogenase	0.49	1.04	1.34
TPI1	orf19.6745	Putative ortholog of *S. cerevisiae* Tpi1p	0.47	0.72	0.91
ADH5	orf19.2608	Putative alcohol dehydrogenase	0.96	0.62	0.71
GPM2	orf19.1067	Described as phosphoglycerate mutase	0.71	0.44	0.93
ADH2	orf19.5113	Putative alcohol dehydrogenase	0.47	0.54	0.95
GSY1	orf19.3278	Protein described as glycogen synthase	0.37	0.26	0.69
PFK1	orf19.3967	Alpha subunit of phosphofructokinase (PFK)	0.35	0.32	0.98
PFK2	orf19.6540	Beta subunit of phosphofructokinase (PFK)	0.19	0.31	0.75
PGK1	orf19.3651	Phosphoglycerate kinase	0.36	0.65	0.83
TPS2	orf19.3038	Trehalose-6-phosphate (Tre6P) phosphatise	0.49	0.49	0.98
ADH1	orf19.3997	Alcohol dehydrogenase	0.39	0.38	0.59
CDC19	orf19.3575	Putative pyruvate kinase	0.20	0.34	0.49
PDC11	orf19.2877	Protein similar to pyruvate decarboxylase	0.29	0.37	0.63
Secretory pathway					
CHC1	orf19.3496	Clathrin heavy chain	0.47	0.90	1.20
ERV29	orf19.4579	Similar to *Saccharomyces cerevisiae* Erv29p ER-Golgi transport vesicle protein	0.60	0.69	1.06
SEC26	orf19.528	Beta chain of secretory vesicles coatomer complex	0.57	0.67	1.18
SEC9	orf19.7764	Transport protein	0.54	0.59	1.06
SEC11	orf19.3259	Signal peptidase subunit	0.56	0.52	0.94
SEC16	orf19.11823	Multidomain vesicle coat protein	0.59	0.64	1.01
SEC23	orf19.1254	Transcription is regulated upon yeast-hyphal switch	0.63	0.71	1.05
SEC24	orf19.12194	Possible role in ER to Golgi transport	0.51	0.65	1.06
SEC61	orf19.6176	Essential protein	0.51	0.46	0.93
Transport					
CAN2	orf19.111	Putative amino acid permease	0.55	0.67	0.84
CRP1	orf19.4784	Copper transporter of the plasma membrane	1.21	0.61	0.77
GAP1	orf19.4304	General amino acid permease	1.02	0.52	1.18
GAP6	orf19.6659	General amino acid permease	1.11	0.65	0.91
HAK1	orf19.6249	Putative potassium transporter	0.99	0.60	1.11
IFC1	orf19.3746	Oligopeptide transporter	0.78	0.56	0.88
IFC4	orf19.2292	Oligopeptide transporter	0.80	0.66	0.86
GNP1	orf19.8784	Asparagine and glutamine permease	0.60	0.35	0.92
HGT6	orf19.2020	Putative glucose transporter	0.56	0.59	0.70
HGT7	orf19.2023	Putative glucose transporter	0.61	0.61	0.75
HGT8	orf19.2021	Putative glucose transporter	0.56	0.55	0.69

Multi Drug and other transporter					
CDR1	orf19.6000	Multidrug transporter of ATP-binding cassette	1.24	0.52	1.02
CDR2	orf19.5958	Multidrug transporter, ATP-binding cassette	1.14	0.51	1.08
CDR4	orf19.5079	Putative transporter of ATP-binding cassette	0.81	0.66	0.89
QDR1	orf19.8138	Putative transporter of antibiotic resistance	0.52	0.72	0.87
CRP1	orf19.4784	Copper transporter of the plasma membrane	1.21	0.61	0.77
IFC1	orf19.3746	Oligopeptide transporter	0.78	0.56	0.88
IFC4	orf19.2292	Oligopeptide transporter	0.95	0.66	0.97
HAK1	orf19.6249	Putative potassium transporter	0.73	0.60	0.86
Transcription factors					
GAL4	orf19.5338	Putative transcription factor with zinc cluster	0.52	0.77	1.10
IPF29	orf19.5975	Putative transcription factor with zinc finger	0.44	1.59	1.23
SKO1	orf19.1032	Putative transcription factor	0.61	0.77	1.08
CPH2	orf19.1187	Transcriptional activator of hyphal growth	0.72	0.58	0.90
GAT2	orf19.405	Putative DNA-binding transcription factor	1.19	0.44	0.96
IPF16124	orf19.173	Putative transcription factor with zinc finger	0.73	0.63	0.87
MOT1	orf19.4502	Transcriptional accessory protein	0.75	0.64	0.99
MSN4	orf19.4752	Putative zinc finger transcription factor	0.68	0.63	0.92
ZCF1	orf19.255	Predicted zinc-cluster protein of unknown function	0.82	0.53	1.10
ECM22	orf19.2623	Putative protein involved in cell wall biogenesis	0.61	0.63	0.91
HAC1	orf19.2432	Putative transcription factor	0.43	0.54	0.91
SSN6	orf19.6798	Functional homolog of *S. cerevisiae* Cyc8p/Ssn6p	0.56	0.60	1.01
TEC1	orf19.5908	TEA/ATTS transcription factor	0.64	0.44	1.01
UME6	orf19.9381	Transcription factor; required for wild-type hyphal extension	0.36	0.36	0.74
Cell polarity and signal transduction					
ARP3	orf19.2289	Shows Myo5p-dependent localization to cortical actin patches at hyphal tip	0.28	0.52	1.01
ARP8	orf19.10867	Mutation confers hypersensitivity to toxic ergosterol analog, and to amphotericin B	0.64	0.35	1.06
BEM1	orf19.4645	Protein required for wild-type budding, hyphal growth, and virulence in a mouse systemic infection	0.52	0.62	1.03
BEM2	orf19.6573	Putative Rho1p GTPase activating protein (GAP); serum-induced transcription	0.36	0.36	0.86
BUD2	orf19.940	GTPase activating protein (GAP) for Rsr1p	0.50	0.44	0.91
CLC1	orf19.4594	Clathrin light chain (by homology)	0.63	0.58	0.88
MYO2	orf19.5015	Class V myosin	0.44	0.58	1.13
RAS1	orf19.1760	RAS signal transduction GTPase	0.57	0.59	0.89
RGS2	orf19.695	Protein of RGS superfamily	0.41	0.53	1.03
TPM2	orf19.6414.3	Protein described as tropomyosin isoform 2	0.61	0.60	0.89

The expression of 265 genes was affected by a factor of ≥1.5 in the *hat1Δ, hat2Δ* double mutant after 60 min; among these, 97 and 168 genes were up- and down-regulated respectively.

The main categories of up-regulated genes were genes belong to amino acid biosynthesis (*ARG1, ARG3, GAP4, MET14, MET3, SAM2*), ferric reductases and ferroxidases (*CFL1, CFL2, FET34*), cell wall *ALG8, CSE1, IFF5, PGA26, PGA59*) and transcription factors (IPF29, *NUT2, ZCF20, ZCF4*). Genes involved in multidrug and other transporter (*CDR1, CDR2, CDR4, CRP1, QDR1, IFC1, IFC4, HAK1*), genes encoding for cell wall proteins (*ALG7, ALS5, CHS4, FGR41, GLC3*, IPF3964, *RBT4, TOS1, WAL1*), transcription factors (*CPH2, GAT2,* IPF16124, *MOT1, MSN4, ZCF1*) were down-regulated after 60 min of incubation.

The expression of 29 genes was affected by a factor of ≥ 1.5 after 180 min, with 10 and 19 genes being up- and down-regulated respectively. Of the ten ORFs, two genes

involved in cell wall, *RBT5, YWP1,* two genes encoding for putative acetyl-CoA synthetase *ACH1, ACS1* and the rest were *AOX2* (Alternative oxidase), *FAS1* (beta subunit of fatty-acid synthase), IPF8464, *RPL20B* (predicted ribosomal protein), *RPN12* (26S proteasome regulatory subunit) and *TUB2* (Beta-tubulin). The down-regulated genes were two genes encoding for cell wall and cell surface proteins (*AAF1* and *GSL1*), three genes involved in transport (IPF15119, IPF9079 and *SHA3*) and the rest involved in other functions (Supplementary table 2.8).

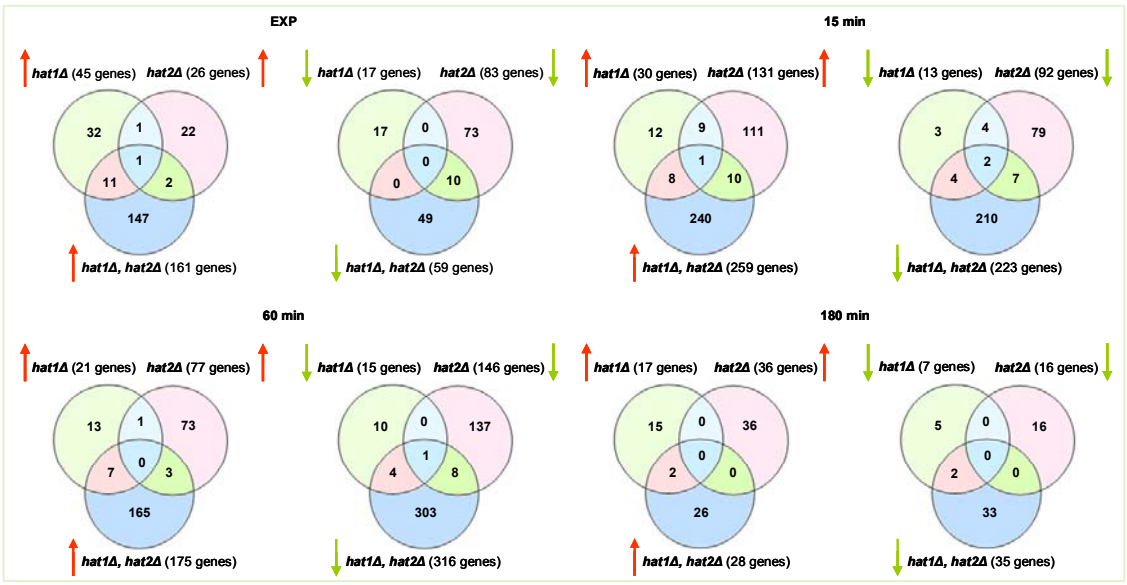

Figure 2.18 Venn diagrams of genes regulated by Hat1p and Hat2p in the simple and double mutants. The CAI4 strain was used as reference for the *hat2Δ* simple and *hat1Δ, hat2Δ* double mutant (MAH4), which were all grown in identical conditions: YNB/28ºC (EXP) and Lee/37ºC (Inducing).

Discussion

In *S. cerevisiae* HAT-B complex is composed of three proteins, Hat1, Hat2 and Hif1 (Ai & Parthun, 2004; Poveda *et al.*, 2004). Hat2p acts as a bridge between Hat1 and Hif1 proteins (Poveda *et al.*, 2004) and is required for high affinity binding of Hat1p to histone H4 (Parthun *et al.*, 1996). It seems to be essential for all the functions carried out by Hat1p because deletion of *HAT2* always produces similar defects to those of *HAT1* (Kelly *et al.*, 2000b; Parthun *et al.*, 1996; Ruiz-Garcia *et al.*, 1998).

In our laboratory the *HAT1* and *HAT2* were deleted in a CAI4 background strain. Morphologically *hat1Δ* mutant is very different to the CAI4 strain (Degano, Ph.D thesis in process) and the culture of the *hat1Δ* mutant in YEPD liquid medium is composed of a mixture of large cells and large chains of elongated, aberrantly shaped cells attached to one another. In contrast, the *hat2Δ* mutant doesn't show any significant difference in growth rate, cell size and morphology.

The focus of our work understood the possible role of *HAT1* and *HAT2* on the expression of genes that are associated with morphogenesis in the *C. albicans*. If Hat2p has additional roles to those described for Hat1p, it seems likely that this fact would be reflected in the transcriptional profile. With the aim of give some light on this, we analysed the transcriptional profile of the *hat2Δ* and *hat1Δ, hat2Δ* mutants. The transcriptional profile of the *hat1Δ* mutant during the yeast mode of growth and during the yeast-hypha transition has been carried out in our laboratory. We have deleted the *HAT1* gene in a *hat2*Δ mutant background strain. As a result, we were able to perform a comprehensive phenotypic and transcriptional analysis of *hat1*Δ and *hat2*Δ simples and double mutants, and in particular we were able to assess the relative contributions of both proteins to growth and morphogenesis in *C. albicans*. Recently, it has been reported that three tagged protein components of the HAT-B enzyme are mainly located in the nucleus (Poveda *et al.,* 2004). However, in several species Hat1p has also been detected in the cytoplasm (Lusser *et al.*, 1999; Ruiz-Garcia *et al.*, 1998; Verreault *et al.*, 1998). Our results are different to those described for *S. cerevisiae*. In this organism deletion of any of *HAT1* or *HAT2* gene produce a similar phenotype (Kelly *et al.*, 2000b; Parthun *et al.*, 1996; Ruiz-Garcia *et al.*, 1998) while we obtained different phenotypes and transcriptomic profiles.

For obtaining the location of Hat1p and Hat2p in *C. albicans*, we carried out a fusion construct to the green fluorescent protein (GFP). This has allowed us to compare our data with the Hat-B proteins of *S. cerevisiae*, which represent the only known complete set of Hat proteins in a fungal species. Our results clearly show that, in *C. albicans* a dominant portion of the putative HAT-B complex is also located in the nucleus.

Next, to further characterize the sub-cellular localization of the two HAT-B components, we asked whether their nuclear residence is interdependent. To address this question and the sub-cellular localization of each fused component, Hat1-GFP and Hat2-GFP, were visualized in yeast cells defective in any of the other opposite proteins. The nuclear residence of Hat1-GFP exhibited no difference between cells with a wild-type. But the nuclear localization of the Hat2-GFP depends on *HAT1*. In the *hat1*Δ strain, the Hat2-GFP signal was visualized throughout the cell. Therefore, Hat1p may participate in the nuclear localization of the catalytic subunit Hat2p. Opposite to our results, it has been reported that in *S. cerevisiae* the nuclear localization of the Hat1p depends on *HAT2* and Hat2p participate in the nuclear localization of the catalytic subunit Hat1p (Poveda *et al.*, 2004).

Our result describes a difference in the interdependency of Hat1p and Hat2p between *S. cerevisiae* and *C. albicans*. In the *C. albicans* like *S. cerevisiae* (Kleff *et al.*, 1995;

Parthun *et al.*, 1996; Poveda *et al.*, 2004) single *hat2*Δ mutant lack of obvious phenotype or growth defects. Contrary to *S. cerevisiae*, the *C. albicans hat1*Δ mutant presents a phenotype with growth defects. Morphologically, the double mutant like the *hat1Δ* simple mutant is very different to CAI4 as is shown in Fig. 2.5. The *hat1Δ, hat2Δ* double mutant like *hat1Δ* simple mutant failed to form true hypha in all the liquid media assayed (Fig. 2.7). We found that the *hat1Δ* and double mutant were more sensitive than the CAI4 and *hat2Δ* to 1 M of NaCl, 1 mg/ml of CFW, 0.5 mg/ml of Congo red and 15 mM of Caffeine (Fig. 2.9). These results suggest that Hat1p play an important role in *C. albicans*. We have identified by two-hybrid experiment that Hat1p and Hat2p doesn't interact directly. We haven't detected any homology of *ScHlf1* in the *C. albicans* Genome Data Base or in any other organism. The results of sub-cellular location and of two-hybrid analysis suggest that there are other protein o proteins that mediate one interaction between Hat1p and Hat2p in *C. albicans*. The determination of the putative bridge protein is under investigation. We analysed the transcriptional profile of *hat2*Δ and *hat1*Δ*, hat2*Δ double mutant during the yeast mode of growth and the yeast-hypha transition at 15, 60 and 180 min. We used CAI4 (Fonzi & Irwin, 1993) as wild-type strain, because all of mutant strains were derived from this strain. Morphologically, deletion of *HAT1* has been known to be exerting relatively strong phenotypic effects upon *C. albicans* (Degano, Ph.D thesis in process). Then, it is surprising that deletion of *HAT1* led to few changes in the transcriptional profile relative to that of the *hat2Δ* mutant. However, this was in contrast with the minimal phenotypic effects of the *hat2Δ* deletion.

109 genes were found to be regulated in the *hat2Δ* mutant during the yeast mode of growth. The major portion of genes up-regulated during the yeast mode of growth in the *hat2Δ* mutant included genes involved in respiration and histone assembly. Seven genes involved in respiration were found to be up-regulated in the *hat2Δ* mutant. We observed eleven down-regulated genes belonging to the glycolysis and TCA cycle functional category. It has been suggested that genes involved in central metabolism and energy generation are stimulated in cells with defects in the cell wall (Lagorce *et al.*, 2003). In the *hat2Δ* strain it is noteworthy that five genes encoding for cell wall proteins show a significant alteration in their expression level. Although the number of down-regulated genes is greater than the number of up-regulated ones, the up-regulated genes show changes of 1.50- to 3.30-fold. In the case of the down-regulated genes, only, *RPS8A* (Niewerth *et al.*, 2003), *RPP0* (Lorenz *et al.*, 2004), *PGA62* (De Groot *et al.*, 2003), IPF6342 (Copping *et al.*, 2005) and *PGA59* show a higher change than 3.70-fold. *YWP1* (Sohn *et al.*, 2003) being the most affected with a 5.88-fold

transcriptional reduction, while the remaining genes only just surpass the threshold of a factor of >1.5. These groups of genes are not reported to be regulated in the *hat1Δ* mutant. It is difficult to predict the overall effect of the deletion of *hat2Δ* on the cell respiration and energy generation, but the 16% of the altered genes belong to this functional group suggesting quite a close relationship between the expression of *HAT2* and some of the central metabolic pathways.

In the *hat1Δ, hat2Δ* double mutant, ten of the genes encoding for cell wall proteins that show an altered expression level were up-regulated including four genes that encode putative GPI-anchored protein (*PGA10, PGA14, RBT5, RHD3*) (de Groot *et al.*, 2004; Kadosh & Johnson, 2001). Since only three genes (*ECM17, IFF5, PGA49*), of this functional group are down-regulated, the data suggest that deletion of the *HAT1* and *HAT2* genes regulate possible cell wall genes involved in the normal cell wall formation.

Also the fact that the *TLO1, TLO3, TLO11, TLO12* and *TLO16* genes, which are members of a family of telomere-proximal genes showed a significantly induced expression level, and that eight genes belonging to this family (*TLO2, TLO3, TLO4, TLO9, TLO10, TLO11, TLO12* and *TLO34*) were up-regulated in *hat1Δ* mutant (Degano, Ph.D thesis in process), could be related to a putative role of *HAT1* in telomeric silencing in *C. albicans*. In *S. cerevisiae* deletion of *HAT1* alone doesn't directly affect on telomeric silencing but in combination with specific histone H3 amino terminal tail mutations, results in a significant defect in telomeric silencing (Kelly *et al.*, 2000b). This result suggested that the function of *C. albicans* Hat1p to regulation of telomeric silencing is distinct of those reported for *S. cerevisiae* Hat-B complex (Poveda *et al.*, 2004).

The transcriptional profile of the *hat2*Δ mutant was very different from the *hat1Δ* mutant during the yeast-hypha transition. Expression of 429 genes (196 up- and 233 down-regulated) was affected after 15, 60 and 180 min of incubation. By contrast a lower number of genes (54 up- and 34 down-) have been reported to be regulated by the deletion of *HAT1* during the same times of incubation and only eighteen genes were common. These results suggest that Hat2p has different roles to those of Hat1p, and this fact is reflected in the transcriptional profile during the yeast-hypha transition present in this work. Deletion of *HAT2* results in only two common genes up-regulated at the three times of incubation. This result indicates a strong sequential alteration of the transcriptional profile in this mutant, a fact that differs from the behavior of other mutants analyzed by our research group. We have detected alteration of nineteen genes encoding for transcription factors, seven up- and twelve down-regulated at any

of the times assayed. Among the up-regulated genes three -*CTA2*, *CTA24, CTA29* (Doedt *et al.*, 2004; Kaiser *et al.*, 1999; van het Hoog *et al.*, 2007; Zakikhany *et al.*, 2007)- encoding for putative transcriptional activators were up-regulated after 180 min and have been reported to be repressed by Efg1p. One gene, IPF19804 (Karababa *et al.*, 2004) is an ortholog of *S. cerevisiae TFC3,* a putative DNA-binding transcription factor; with a decreased transcription upon fluphenazine treatment (Nobile & Mitchell, 2005) were up-regulated at 15 min. The other two genes were *MRR1*, an ortholog of *S. cerevisiae HAP1*, which is a Zinc finger transcription factor (Morschhauser *et al.*, 2007) (at 60 min), *GAL4* (Keng, 1992) which is a putative transcription factor with zinc cluster DNA-binding motif; ortholog of the *S. cerevisiae GAL4* gene (Martchenko *et al.*, 2007) (at 180 min). *GAL4* appeared as up-regulated during the three times of incubation in the *gcn5Δ, hda1Δ* double mutant (Rashki, Ph.D thesis 2009). Of the twelve down-regulated transcription factors, only *HAC1* has been reported to be down-regulated in the *hat1*Δ mutant at 15 min of incubation (Degano, Ph.D thesis in process). We have found this gene also down-regulated in the *hat1Δ, hat2Δ* double mutant confirming both results. However the *HAC1* gene was up-regulated after 15 and 60 min in the transcriptional response of CAI4 to temperature (Rashki, Ph.D thesis 2009). As those are opposite results, the *HAC1* gene is a strong candidate to be used as master gene for analyzing the role of the acetylation process in the yeast-hypha transition.

We have found changes in the transcript levels of eighteen genes encoding for cell wall proteins including nine genes *BMT5* (at 15 min), *IRS4, FGR41* (at 60 min), *HWP1, PGA54* (at 180 min), *BMT9, CHS2, GSL1* (at 15 and 60 min), *PGA45* (at 15 and 180 min) up-regulated; and nine genes *PHR1, TOS1* (at 15 min), *ALS4, ECM31, HYR3, PGA6, PGA63* (at 60 min), *HYR1* (at 180 min), *GSC1* (at 15 and 60 min) down-regulated in the *hat2*Δ mutant. However eight genes, *ALS2, ALS4, FRE10* (at 60 min), *PIR1* (at 180 min), *PGA13* (at 15 and 60 min), *PGA62, YWP1* (at three times), *SIM1* (at 60 and 180 min), and seven genes, *PMT1, SUN41, TOS1* (at 15 min), *ECM33, HWP1, IDH1* (at 60 min), *ALS3* (at 15 and 60 min) were found to be regulated in the same conditions in the *hat1Δ* mutant. As we have not found coincident genes, our results prove again that the *hat2*Δ mutant has a distinct transcriptome of the *hat1Δ* mutant.

The regulation of seventeen genes (13 up-regulated *ADH1, ADH5, FBA1, GPH1, GPM1, GLG1, HXK2, PCK1, PDC11, PFK2, PGK1, TPI1* and *ENO1* after 15 min) involved in the glycolytic pathway was detected. Expression ten of them (*ADH1, ADH5, FBA1, GPH1, GPM1, PDC11, PFK2, PGK1, TPI1* and *ENO1*) have been down-regulated after 15 min in *C. albicans* transcription response to temperature. The genes involved in glycolytic pathway (*FBA1, GPM1, PFK2, PGI1, TPI1* and *HXK2*) have been

reported to be down-regulated in the APSES mutants, *gcn5Δ, hda1Δ* and *odcΔ* mutants during the hypha transition (Doedt *et al.*, 2004; Rashki, Ph.D thesis 2009) suggesting a specific regulation that deserves further investigation.

Fifteen genes encode for ribosomal proteins were found to be regulated in the *hat2Δ* mutant during the yeast-hypha transition. No genes of the ribosomal category have been found to be up- or down-regulated in the *hat1Δ* mutant (Degano, Ph.D thesis in process).

The transcriptional profile of *hat1*Δ, *hat2*Δ double mutant was very different from those found on both the *hat1Δ* and *hat2Δ* simple mutants during the yeast-hypha transition.

Expression of 793 genes was affected after 15, 60 and 180 min of incubation. Forty seven genes involved in ribosomal proteins: cytoplasmic (31 genes) and mitochondrial (7 genes) or putative translation initiation factor (9 genes) were found to be up-regulated in the *hat1Δ, hat2Δ* double mutant (31 at 15 min, 4 at 60 min, 9 at 15 and 60 min, 1 at 60 and 180 min and 2 at three times of incubation) and the expression of almost all of them decreased at 180 min.

None of those genes have been reported to be regulated in the *hat1Δ* mutant, while we have found five genes (*RPL11, RPL20B, RPS4A, RPL10* and *RPL14B*) were down-regulated in the *hat2Δ* mutant.

It is difficult to predict the overall effect of the deletion of both genes, *HAT1* and *HAT2*, on the regulation of genes encoding for ribosomal proteins, but the fact that almost one third of the genes (47 out of 143) encoding for ribosomal proteins and that a 5.9% (47 genes out of the total regulated genes, 793) suggests that Hat1p and Hat2p, jointly regulate expression this set of genes and this point also deserves further investigation.

Regarding the functions of other genes, we observed that expression of 131 common down-regulated genes after 15 and 60 min of incubation. Of those the main representative categories were genes encoding for cell wall proteins (12) -*ALS1* (Hoyer *et al.*, 1995), *ALS3* (Hoyer *et al.*, 1998a), *ALS4* (De Groot *et al.*, 2003), *ECM21* (Karababa *et al.*, 2004; Nobile *et al.*, 2003), *FGR51* (Uhl *et al.*, 2003), *HYR1* (Bailey *et al.*, 1996), *PGA4* (Eisenhaber *et al.*, 2004), *PGA63* (De Groot *et al.*, 2003), *PMT1, PMT2, PMT4* (Cantero *et al.*, 2007; Timpel *et al.*, 1998) and *RBT1* (Sohn *et al.*, 2003)- , seven genes encoding for enzymes glycolytic pathway (*ADH2, PFK1, PFK2, GPM1, GSY1, PGK1*and *TPS2*), three genes involved in glucose transport (*HGT6, HGT7* and *HGT8*), ten genes involved in the cell polarity and signal transduction (*ARP3, ARP8, BEM1, BEM2, BUD2, CLC1, MYO2, RAS1, RGS2* and *TPM2*), six genes involved in the secretory pathway (*ERV46, SEC11, SEC16, SEC24, SEC61, SEC9*) and five transcription factors including *ECM22, HAC1, SSN6, TEC1* and *UME6*.

Down-regulation of nine genes (*ARP3, BUD2, CLC1, MYO2, RAS1, TPM2, ERV46, SEC16* and *SEC24*) involved in the cell polarity and signal transduction and secretory pathway have also been reported in the *odcΔ* mutant in *C. albicans* (Rashki, Ph.D thesis 2009). Those results suggest a coordinate regulation of this set of genes by acetyltranferases and polyamines.

Up-regulation the *HAC1, TEC1* and *UME6* genes have been reported for *C. albicans* as transcriptional response of to temperature (Rashki, Ph.D thesis 2009).

Regulated genes with known functions in the *hat1Δ, hat2Δ* double mutant do not share significant common functional categories (see tables 2.4 and 2.7) with those of *hat1Δ* and *hat2Δ* simple mutants suggesting that Hat1p and Hat2p jointly regulates expression a large number of genes in *C. albicans*.

In summary, despite the fact that both are putative subunits of the HAT-B complex, *hat1*Δ and *hat2*Δ mutants displayed different transcriptional profile in the both the yeast mode of growth and during the yeast-hypha transition suggesting that Hat1p and Hat2p regulate separately different set of genes in *C. albicans*. The study of transcriptional profile of the *hat1*Δ*, hat2*Δ double mutant revealed that a few genes were overlapped with both *hat1*Δ (27 genes) and *hat2Δ* (28 genes) simple mutants. Our results suggest that, in addition of regulation different set of genes, Hat1p and Hat2p function jointly in *C. albicans* regulating another specific set of genes. As in our case, in *S. cerevisiae* it has been reported that *hat1Δ* and *hat2Δ* mutants display different transcriptional profiles during exponential growth in a glucose-rich medium (Rosaleny *et al.*, 2005) and the analysis of the double mutant showed as in our case a higher number of regulated genes than those of the single mutants. In *S. cerevisiae*, the study of the GO categories in the double mutant revealed that their transcriptome is more similar to that observed in the *hat1Δ* mutant however the number of genes and the fold changes observed are higher in the double mutant (Rosaleny *et al.*, 2005). In our case we can not support such a hypothesis because although regarding the phenotypic analysis the double mutant is more similar to the *hat1Δ* mutant this is not the case for the transcriptional profile.

To summarize, in *S. cerevisiae* the proteins Hat1p and Hat2p undoubtedly participate in common processes, such as Lys12 acetylation of free H4 histone (Poveda *et al.*, 2004; Rosaleny *et al.*, 2005). The role of *C. albicans* Hat1p and Hat2p in acetylation of histones is under investigation. However, our data suggested that both proteins may also have functions regulating genes involves in cell physiology as could be deduced by the phenotypes. Up to now it is not clear what those roles could be. Nevertheless, this is not the first case that HAT complexes composed of subunits participate in

different functions. In fact, this appeared to be a general feature of the HATs proteins forming HAT complexes like NuA3, INO80, etc. Our results for the *C. albicans*, HAT-B putative complex suggest, as have been described for other HATs complexes composed by subunits, that it is implicated in more than one function.

Phenotypic, virulence and transcriptome analysis of the *Candida albicans gcn5Δ, sin3Δ* double mutant

Abstract

C. albicans Gcn5p and Sin3p proteins regulate morphogenetic process, including filamentation and chlamidospore formation. The *SIN3* gene of *Candida albicans* was disrupted in a *gcn5Δ* background to investigate the role of a *C. albicans* double mutant *gcn5Δ, sin3Δ* in growth, morphogenesis and pathogenicity. The behavior of the double *gcn5Δ, sin3Δ* mutant resembled that of both simple mutants with respect to hyphal outgrowth and chlamydospore formation. For morphogenesis on solid media and sensitivity to Caffeine, Hygromycin, Amphotrecin B, Itraconazole and Fluconazole, the *gcn5Δ* phenotype appeared as the dominant phenotype in the double mutant. Whole-genome microarray analysis of the *gcn5Δ, sin3Δ* double mutant revealed an altered expression of 558 and 1271 genes during the yeast mode of growth and during the yeast-hypha transition respectively. The main categories of up-regulated genes growing as yeast were oxidative stress response, DNA synthesis and replication, chitin anabolism. The main down-regulated genes correspond to those coding for cell wall proteins, electron transport, ribosomal proteins and transcriptional factors.

Forty-three genes were common to the three times of the yeast-hypha transition assayed (15, 60 and 180 min); 156 genes were common between 15 and 60 min; 47 at 60 and 180 min. Some of genes involved in cell wall (8 genes), fatty acid metabolism (5 genes), oxidase/peroxidase (4 genes) and transcription factors (2 genes) were down-regulated at three times of incubation. The main common genes at 15 and 60 min encode for cell wall proteins (*ALS4, GCS1, HYR1, MNT1, MNT2, PGA54, PMT1, PMT4, RBT4* and *SUN41*), transcriptional factors (*ARG83* and *ECM22*) were down-regulated and genes in the category of mitochondrial structure (*AMI3, MCR1, NAD1, PST2, SOD2* and *YOR100*) and transcriptional factors (*GCN4, PDC2* and *RPN4*) were up-regulated. Finally at 60 and 180 min of incubation, we have found down-regulation of genes involved in electron transport and respiration (*ATP1, ATP14, COX5A, NDE1* and *STF2*), transporters (*CDR1,* IPF12884*, MTR* and *ZRT2*) and a transcription factor (*TYE7*). At one time of incubation, differentially expressed genes represented functions as diverse as mitochondrial, vitamin biosynthesis, ergoestrol biosynthesis, glucose transporters, histone, cell wall, cell polarity, signal transduction and ribosomal proteins. The results presented here include a global transcriptome analysis of the cellular response to the double deletion of the *GCN5* and *SIN3* genes and afford a basis for a

better understanding of the putative interaction between histone acetyltransferases and co-repressors.

Introduction

In eukaryotes, the transition between alternative developmental pathways is mainly attributed to a switch in transcriptional programs. It has been postulated different modes of regulation at a transcriptional level mediated by processes of histone deacetylation and acetylation (Pnueli *et al.*, 2004). There are numerous histone acetyltransferases (HATs) and histone deacetylases (HDACs), even in a simple organism such as yeast. At present, the functions of most of these enzymes and their putative role as transcriptional regulators are not clearly understood. Even less is known about their functions in processes other than transcription. The challenge now is to elucidate, in molecular detail the mechanisms by which and the extent to which, the different HATs and HDACs participate in all the biological processes. In the case of yeast, its small size and the availability of its genome sequence and cDNA microarray have facilitated the recent genome-wide approaches for the comprehensive determination of the functions of HATs and HDACs.

Eukaryotic *GCN5* acetyltransferases influence diverse biological processes by acetylating histones and non-histone proteins and regulating chromatin and gene-specific transcription as part of multi-protein complexes. In lower eukaryotes and invertebrates these complexes include the ADA complex that is still incompletely understood (Wang *et al.*, 2008). In yeast, Gcn5p exists as part of complexes of two basic types: the small ADA and the larger SAGA (Spt-Ada-Gcn5 acetyltransferase) complexes (Lee & Workman, 2007). While in the ADA complex data are scarce, it is known that the yeast SAGA complexes function mostly as coactivators that acetylate nucleosomal histones H3 and H2B and facilitate chromatin remodelling, transcription, nuclear export of mRNAs, and nucleotide excision repair (Baker & Grant, 2007). *C. albicans GCN5* encodes a protein with 449 amino acid and little is known about the regulation of its relative expression in biological process including its role in development. In our laboratory the *GCN5* gene was deleted in a CAI4 background strain. Deletion of *GCN5* appeared to block hyphal formation in a variety of standard inducing conditions, for example in the presence of serum or GlcNAc or during growth on Spider medium (Degano, Ph.D thesis in process). The function of *GCN5* on the alternative pathway of filamentation in *C. albicans* is yet unknown.

SIN3 is a component of histone deacetylase complex known to be important for transcriptional repression and that is conserved from yeast to human (Silverstein &

Ekwall, 2005). While multiple isoforms of *SIN3* have been reported, little is known about their relative expression. *SIN3* is believed to serve as a scaffold protein for assembly of the complex and has been shown to be the major subunit that targets the complex to specific promoters (Silverstein & Ekwall, 2005). Null mutation in *C. albicans sin3Δ* results in a slight growth defect and also fails to undergo hypha formation (switching-defective) under all assayed conditions (Monterola, Ph.D thesis 2002).

In the present work we show that deletion of the *GCN5* and *SIN3* as double mutant like both simple mutants is associated with a difference between the growth rate of the double mutant and CAI4 in liquid medium at 28°C. 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 37°C and in all the solid media assayed. We will try to correlate the molecular basis of those phenotypes with its transcriptome.

Materials and methods

Strains and growth conditions

C. albicans strains, plasmids and bacterial strain used in this study are listed in Table 3.1. Strains were routinely cultured on YEPD or YNB medium at 28°C. Yeast growth was obtained in YNB medium at 28°C supplemented with 0.2 mM of uridine for growth of *URA3*¯ strains. Solid medium was obtained by adding agar (2%).

Solid media for inducing the yeast-hypha transition in *C. albicans* were Lee medium in which glucose was replaced by N-acetylglucosamine (1.25%), Spider medium (1% nutrient broth, 1% mannitol, 0.2% K_2HPO_4), Lee medium plus 4% bovine calf serum (GIBCO), SLAHD and Embedded medium.

Cells were grown at 28°C in YEPD medium, and approximately 50 cells were spread on different agar medium plates. The dimorphic transition in liquid media was induced by growing cells in Lee at 28°C and changing them at a density of 10^7 cells/ml to 37°C in Lee plus 4% bovine calf serum or Lee medium with 1.25% N-acetylglucosamine (GlcNAc)(Sigma-Aldrich) instead of glucose and incubated at 37°C. *Escherichia coli* strain was grown at 37°C in Luria-Bertani medium (LB) supplemented with 100 μg of ampicillin per ml for plasmid selection (Sambrook, 2001).

Table 3.1 *Candida albicans* strains, plasmids and bacterial strain used in this study

	Relevant genotype or characteristics	Parent	Reference
***C. albicans* strains**			
SC5314	Clinical isolated (Wild type)	-	(Gillum, 1984)
CAI4	*ura3Δ::imm434/ura3Δ::imm434*	CAF2-1	(Fonzi and Irwin, 1993)
CAMR	*ura3Δ::imm434/URA3*	CAI4	(Rodrigues and Dominguez, 2005)
RDG4	*gcn5Δ::hisG /gcn5Δ::hisG ura3Δ::imm434/ura3Δ::imm434*	RDG3	(Degano and Dominguez, 2005)
SFC4	*sin3Δ::hisG /sin3Δ::hisG ura3Δ::imm434/ura3Δ::imm434*	SFC3	(Monterola and Dominguez, 2002)
ZAH1	*sin3Δ::hisGURA3hisG/SIN3 ura3Δ::imm434/ura3Δ::imm434 gcn5Δ::hisG/gcn5Δ::hisG*	RDG4	present work
ZAH2	*sin3Δ::hisG/SIN3 ura3Δ::imm434/ura3Δ::imm434 gcn5Δ::hisG/gcn5Δ::hisG*	ZAH1	present work
ZAH3	*sin3Δ::hisG/sin3Δ::hisGURA3hisG ura3Δ::imm434/ura3Δ::imm434 gcn5Δ::hisG/gcn5Δ::hisG*	ZAH2	present work
ZAH4	*sin3Δ::hisG /sin3Δ::hisG ura3Δ::imm434/ura3Δ::imm434 gcn5Δ::hisG/gcn5Δ::hisG*	ZAH3	present work
ZAHR4	*sin3Δ::hisG /sin3Δ::hisG ura3Δ::imm434/URA3 gcn5Δ::hisG/gcn5Δ::hisG*	ZAH4	present work
EVA1	*ura3Δ::imm434/URA3 sin3Δ::hisG/sin3Δ::hisG*	SFC4	present Work
Plasmids			
pSNC3	Plasmid pBluescript KS^+ containing the disruption cassette of the *SIN3* gene	pSNC1	(Monterola, 2002)
pGEM-T	Plasmid containing ampicillin resistance gene, T7 and SP6 RNA Polymerase transcription initiation site, T7 and SP6 RNA Polymerase promoter, multiple cloning site, *lacZ* start codon, lac operon sequences, lac operator, β-lactamase coding region, phage f1 region, binding site of pUC/M13 Forward Sequencing Primer, binding site of pUC/M13 Reverse Sequencing Primer		Promega (Robles, 1994)
pMR1	Plasmid containing the reinsertion cassette of the *URA3* gene		(Rodriguez and Dominguez)
Bacterial strain			
DH5α	*FΦ 80lac ZΔM15, Δ (lacZYA-argF), U169, deoR, recA1, endA1, hsdR17, phoA, supE44, (rk^-, mk^+), gyrA96, Δthi-1, gyrA96relA*		(Hanahan and Meselson, 1983)

Reagents

Agar, yeast extract, bactopeptone, yeast nitrogen base, cornmeal agar and glucose were purchased from Difco Laboratories (Detroit, MI. USA); DNA restriction and modification enzymes were purchased from Roche, Invitrogen; Fermentas and Promega, unless otherwise stated. 10× buffers for restriction enzymes were those supplied by manufacturers. For the double digestions, Carlos Buffer (CB 10×), DTT 10× (DTT 10 mM) and spermidin 10× were used. The usual chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Merck (Merck and Co. Inc).

Strain construction

To investigate the function of *GCN5* and *SIN3* in *C. albicans*, the gene *SIN3* was deleted in the *gcn5*Δ background strain (Degano, Ph.D thesis in process). The plasmid pSNC3 was constructed in our laboratory by ligating the PCR products generated from the up-stream (600 bp) and down-stream (588 bp) flanking regions of *CaSIN3* to a *hisG-URA3-hisG* cassette in pSNC1. The pSNC3 was linearized by *Sac*I and *Kpn*I and from the resulting plasmid; a 5.1-kb *Sac*I-*Kpn*I fragment was isolated and used to transform strain RDG4 (*Δgcn5*). The *C. albicans* strain, heterozygous for the first *CaSIN3* disrupted allele, were further selected on the medium containing 5-fluoroorotic acid to get Ura^- cells (Fonzi & Irwin, 1993). The positive clone was utilized for the deletion of the second remaining wild-type allele. The verification of *CaSIN3* deletion in the heterozygous and homozygous strains was monitored by PCR and Southern blotting using a Rediprime^{Tm} II Random Prime labelling system (Amersham-Biosciences). The 588 bp hybridization probe corresponding to down-stream flanking regions of *CaSIN3* was used. Chromosomal DNA isolated from all strains was digested with *Nde*I for Southern blotting.

DNA manipulation

Restriction analysis, DNA cloning, agarose gel electrophoresis were performed using standard procedures (Sambrook, 2001). Polymerase chain reactions were carried out in an Eppendorf Master Cycler gradient and PERKIN ELMER Gene Amp PCR System 2400.

Spot assay for analyzing sensitivity to different substances on plates

Methods for testing the *C. albicans* strains were similar for all effectors. Cultures were grown in 50 ml of YEPD medium until the exponential phase and then diluted to an optical density at 600_{nm} of 0.4. Five microliters of undiluted cell culture and 1/10 serial dilutions of each cell culture were spotted onto YEPD plates containing the following: NaCl (0.5 to 1 M), Calcofluor white (0.1 to 0.5 mg/ml), Caffeine (8 to 15 mM), Sodium dodecyl sulfate (SDS; 0.005 to 0.01%), Fluconazole (2 to 4 μg/ml), Hygromycin (100 to 300 μg/ml), Amphotericin B (50 to 100 μg/ml), Itraconazole (4 μg/ml), $CaCl_2$ (0.5 to 1.5 M), KCl (1.5 M), and Congo red (0.1 to 0.5 mg/ml). Differences in growth were recorded after incubation of the plates at 28°C for 96 h. Zymolyase, Alcian blue binding assay and RNA isolation were performed as described in Chapter II.

cDNA preparation and microarray hybridization also were carried out as described in previous chapter.

Virulence studies

The mouse strain employed in the infection model was male CD1. Mice were infected through the intra-peritoneal mode with a reconstructed *URA3 sin3Δ* and *sin3Δ, gcn5Δ* double mutant. The *C. albicans* cells were injected with 10^8 (data not shown) and 5×10^8 cells. The mortality rate was monitored for 30 days.

Results

Construction of *C. albicans gcn5Δ, sin3Δ* double mutant by gene disruption

The "Ura blaster" technique was used to sequentially disrupt both copies of the *C. albicans SIN3* gene in the *gcn5Δ* mutant background strain. Chromosomal DNA isolated from wild-type and transformant strains were digested with *Nde*I and analyzed by Southern hybridization (Fig. 3.1B). A 588-bp *Sal*I-*Kpn*I DNA fragment derived from plasmid pSNC3-3′ was used as a specific probe. The wild-type *SIN3* allele showed a 3.6-kb band (Fig. 3.1B, Lane 1 and 3). A new band which represented the "Ura blaster" integrated into one *SIN3* allele appeared in the transformants (Fig. 3.1B, Lane 4). After selection on 5-fluoroorotic acid-containing medium, the loss of the *URA3* gene and one copy of the *hisG* element resulted in a 9.8-kb band (Fig. 3.1B, Lane 5). The remaining intact *SIN3* allele was disrupted similarly, leading to homozygous *sin3*::*hisG/*Δ*sin3*::*hisG-URA3-hisG* strains (Fig. 3.1B, Lane 6) and corresponding Ura$^-$ derivatives (Fig. 3.1B, Lane 7). Phenotypes reported in this chapter were observed in at least two independently isolated disrupted. Our ability to generate viable *gcn5*Δ*, sin3*Δ double mutant indicates that both genes are not essential in *C. albicans*.

Table 3.2 Oligonucleotides used in this study

Primer	Sequence 5′-3′	Primer	Sequence 5′-3′
RD17	GGTGACAAACTAGTTCAATGC	**RD57**	ACTGAAGTAGACTTCCCACT
RD18	GCTTATCAAGCTTTGGAAG	**URA1**	GGATACTATCAAACAAGAGG
RD19	CCACTCGAGACGTAATGG	**URA2**	AATGCTGGTTGGAATGCTTA
RD24	GCGGCCGCCACAAGAAT	**AMH1**	CCGAACATCAACCAATCGT
RD32	GGCAATCACATCAAGACT	**IP2**	TTACAATCAAAGGTGGTCC
RD33	TGTTCAACTTCAAGTCTC	**IP3**	GGTACAGTTGTTCCTCACA
RD50	GGTCAAGGTACCAACAGATT	**SINCA10**	GGTTCAACATCCCATCGATCA
RD51	CATCGCTAGTAGTTCTGACT	**SINCA15ter**	CATGGCAATCTGTTTCATCAA
RD54	ATTGAAAGACTGCAGTTTTGT	**SINCA15orf**	AATGGAACACAGTTGCCG
RD55	CTTATTCTCGAGCAATGCTT	**SINCA16**	TCTCTGTCATGTACTGAG
RD56	TATCCATTGTCCAGGTGGTT		

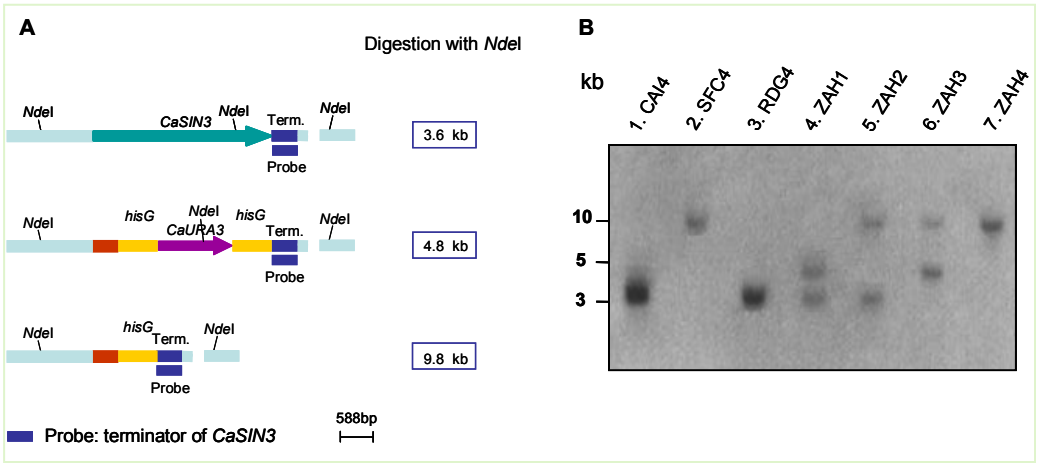

Figure 3.1 Deletion of *CaSIN3* alleles. (**A**) Structure of different alleles.The wild-type *SIN3* gene and the alleles disrupted by the *hisG-URA3-hisG* cassette or by *hisG* alone are shown. (**B**) Southern blot analysis of genomic DNA was performed with the following strains digested with *Nde*I.
Lane 1, CAI4 (*GCN5/GCN5; SIN3/SIN3*)
Lane 2, SFC4 (*GCN5/GCN5; sin3Δ::hisG/sin3Δ::hisG*)
Lane 3, RDG4 (*gcn5Δ::hisG/gcn5Δ::hisG; SIN3/SIN3)*
Lane 4, ZAH1 (*gcn5Δ::hisG/gcn5Δ::hisG; SIN3/sin3Δ:hisGURA3hisG*)
Lane 5, ZAH2 (*gcn5Δ::hisG/gcn5Δ::hisG; SIN3/sin3Δ::hisG*)
Lane 6, ZAH3 *(gcn5Δ::hisG/gcn5Δ::hisG; sin3Δ::hisGURA3hisG/sin3Δ::hisG)*
Lane 7, ZAH4 (*gcn5Δ::hisG/gcn5Δ::hisG; sin3Δ::hisG/sin3Δ::hisG*)
The 588 bp fragment was used as a probe.

Growth curves of the CAI4, both single and double mutants showed difference in beginning of the exponential growth phase in either YEPD (Fig. 3.2) or YNB medium. The beginning of the exponential growth phase in the double mutant was delayed by up to 18 h. The growth phenotype observed in the *C. albicans gcn5*Δ*, sin3*Δ mutant is not as severe as the defect displayed by the *gcn5Δ* mutant that grows at a rate about one-half that of the CAI4 (Degano, Ph.D thesis in process). Since the growth defect of *gcn5Δ* mutant was slightly suppressed by deletion of the *SIN3* gene. Our results suggest that the deletion of *SIN3* in a *gcn5Δ* background mutant slightly compensate the growth defect of the *gcn5Δ* mutant.

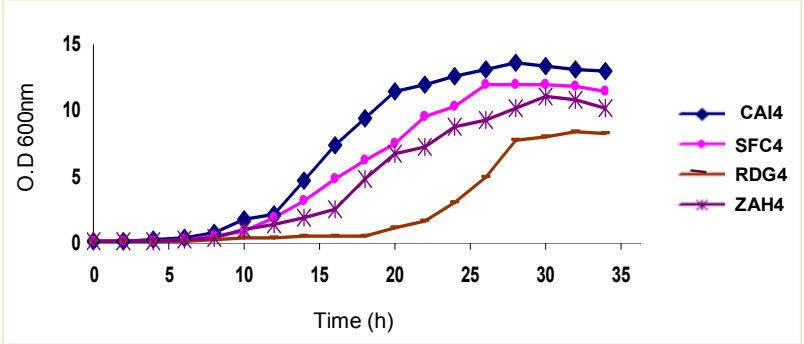

Figure 3.2 Growth curves of CAI4, RDG4 (*gcn5Δ*), SFC4 (*sin3Δ*) and ZAH4 (*gcn5Δ, sin3Δ*). Strains were pre-grown on rich medium containing 1% glucose and inoculated at an O.D at 600nm of 0.1 YEPD. Growth was monitored over a period of 34 h by measuring the optical density of the cultures.

Morphologically, the double mutant likes both simple mutants are very different to CAI4 strain as show in Fig. 3.3.

The culture of the *gcn5Δ, sin3Δ* double mutant in YEPD liquid medium was composed of a mixture of rode or cigarette shape cells and short chains of elongated, aberrantly shaped cells attached to one another, suggesting an intermediate phenotype between the one displayed by the *gcn5Δ* and *sin3*Δ mutants.

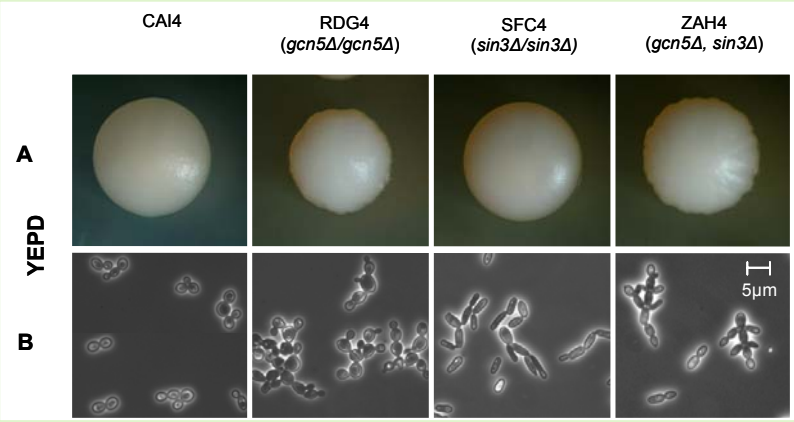

Figure 3.3 (A) Colony growth and (B) morphological characteristics of *C. albicans* homozygous strains CAI4, RDG4, SFC4 and ZAH4 in YEPD medium.

Behavior of the *gcn5Δ, sin3Δ* double mutant in solid media

In order to determine whether the absence of *C. albicans* Gcn5p and Sin3p 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. Cells were grown at 28°C in YEPD medium and approximately 50 cells were spread on different agar media plates containing Spider, Lee plus GlcNAc, and Lee plus 4% bovine calf serum (GIBCO), SLAHD and Embedded medium. We found that the homozygous mutant lacked the ability to form lateral hypha at 37°C on all solid media, even in the presence of serum, a strong inducer of the dimorphic transition. The appearance of colonies after 4 days of growth at 37°C in all solid media can be seen in Fig. 3.4.

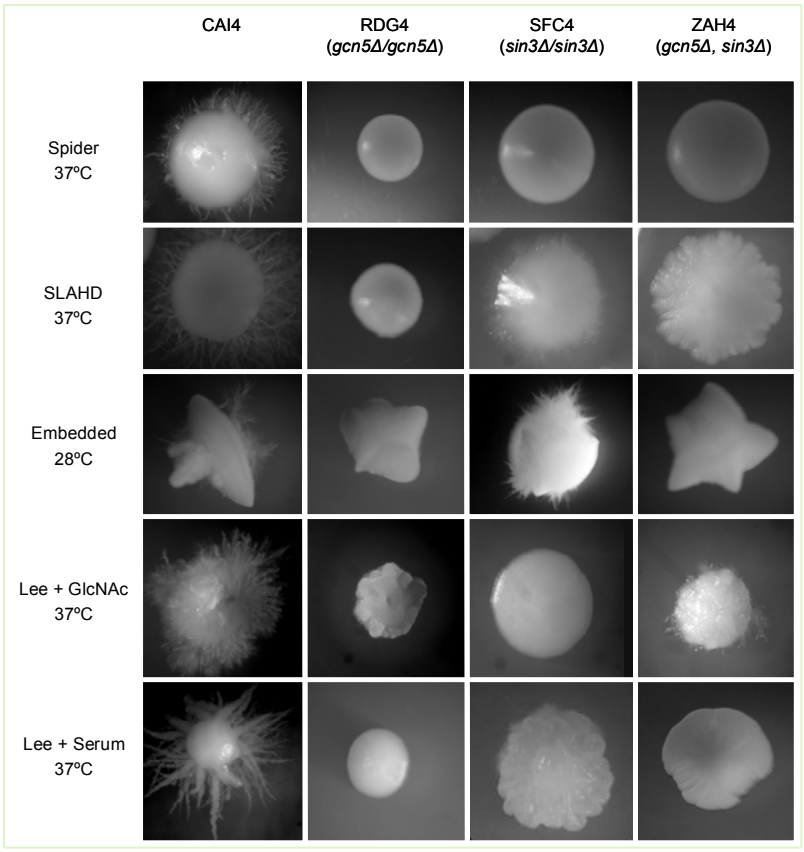

Figure 3.4 Colony growth of the *gcn5Δ, sin3Δ* double mutant in solid media. All mutants and CAI4 were grown on solid media which induce hyphal development. The plates were incubated for 4 days at 37°C.

Behavior of the *gcn5Δ, sin3Δ* double mutant in liquid media

The behavior of the double mutant strain was next investigated by assessing hyphal formation in Lee (pH 6.8, 37°C) (data not shown) or in serum inducing media.

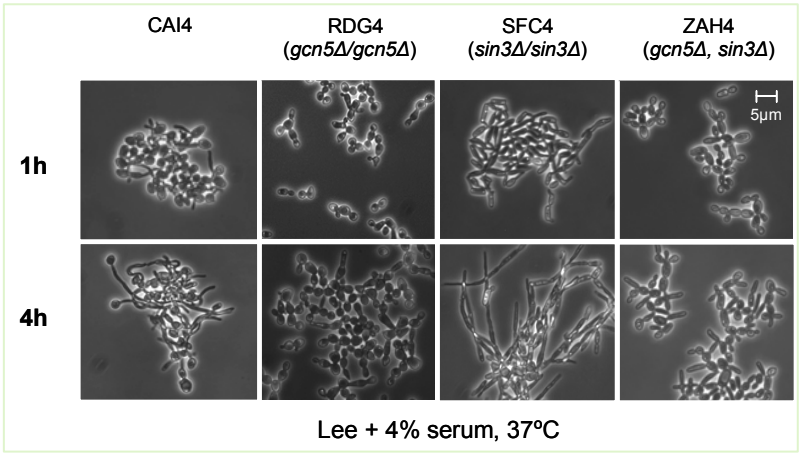

Figure 3.5 Cellular morphology of CAI4, RDG4, SFC4 and ZAH4 strains during the yeast-hypha transition in Lee medium supplemented with 4% serum at 37°C after 1 and 4 hours.

Fig. 3.5 shows that the double *gcn5Δ, sin3Δ* mutant like both simple mutants failed to form germ tubes in the liquid media assayed, indicating that one or more of the pathways involved in the yeast-to-hypha transition didn't function in the double and both single mutants.

Chlamydospore formation

We attempted to determine whether the deletion of both genes might be involved in the formation of chlamydospores. Therefore, a CAI4 strain, the double and both simple mutants was streaked out lightly on cornmeal agar (Difco)–0.33% Tween 80, covered by cover slips, and incubated at 25°C for 7 to 14 days.

The results are shown in Fig. 3.6. The CAI4 strain was able to form chlamydospores. Chlamydospores formed as terminal thick-walled cells on short side branches (suspensor cells) or as terminal cells on filaments. But the double mutant like both simple mutants was completely deficient in chlamydospore formation. These results demonstrate that both proteins are needed in chlamydospore formation.

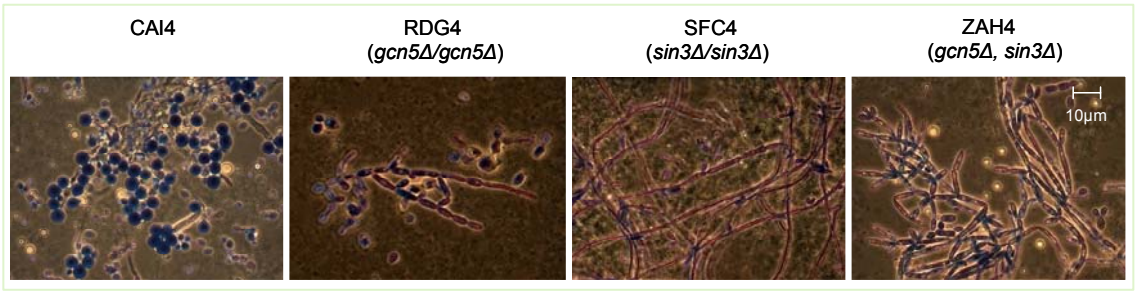

Figure 3.6 Chlamydospore formation of *C. albicans* in the *gcn5Δ, sin3Δ* double mutant

Phenotype analysis of the *gcn5Δ, sin3Δ* double mutant

Possible defects in the cell wall of double mutant were first investigated by experiments including sensitivity to different effectors, using a screening method designed to identify genes involved in cell surface assembly (Lussier, 1997).

We found that the double mutant showed sensitive to Caffeine, Congo red, NaCl, KCl, Fluconazole, Itraconazole and Hygromycin. No difference in sensitivity was found towards Calcoflour white and SDS when compared to CAI4 strain (Fig. 3.7).

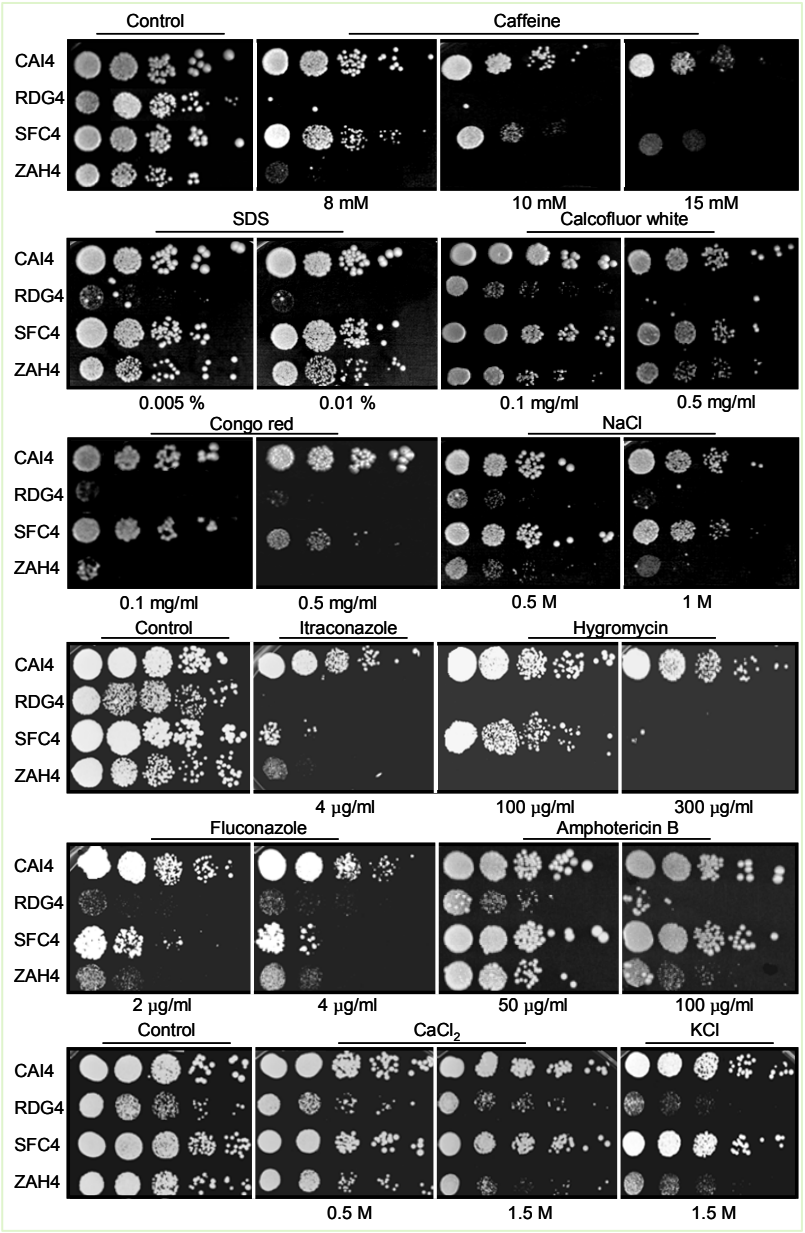

Figure 3.7 Sensibility assays of CAI4, RDG4 (*Δgcn5*), SFC4 (*Δsin3*) and ZAH4 (*gcn5Δ, sin3Δ*) to different effectors. All strains were grown in liquid YEPD. The optical density at 600nm of the cultures were adjusted to 0.4 with the same medium and 5µl aliquots from the cultures and from 10-fold serial dilutions were spotted onto YEPD plates supplemented with different agents. Plates were incubated at 28°C for 4 days.

The phenotype observed in the double and *sin3*Δ single mutant is not as severe as the defect displayed by C. *albicans gcn5Δ* mutant, suggesting that Gcn5p is required to maintain normal cell physiology and deletion of *SIN3* compensate sensitivity of *gcn5Δ* mutant in Calcoflour white and SDS. The sensitivity of yeast cells to Zymolyase has been carried out to uncover changes in cell wall composition and arrangement (Ram *et al.*, 1994). Our results indicated that there were no significant differences between the double, *sin3Δ* simple mutants and the CAI4 strain as regards sensitivity to Zymolyase (Fig. 3.8).

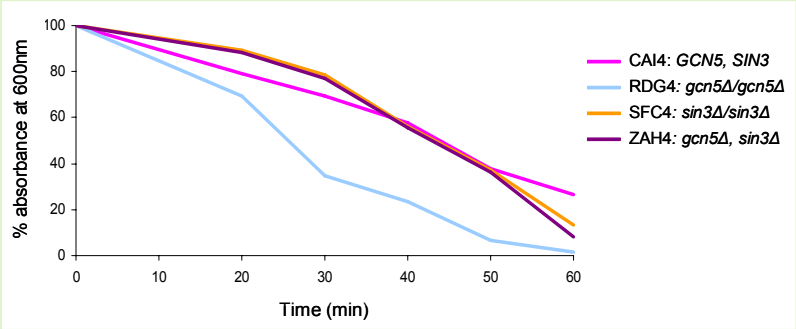

Figure 3.8 Resistance to a cell wall-degrading enzymatic complex of *C. albicans*. All strains were grown until the exponential phase in YEPD at 28°C. Aliquots (2×10^7 cells) were resuspended in 10 mM Tris-Hcl pH 7.5 and treated with 10 μg of Zymolyase per ml. The decrease in optical density (O.D) (percentage of resistant cells) is monitored for 60 min.

Alcian blue binding assay

We then determined the level of Alcian blue binding assay of CAI4 and three mutant strains of *C. albicans,* using the method previously described by Herrero et al (Herrero *et al.*, 2002). Both double and *sin3Δ* simple mutants showed a significant decrease in Alcian blue binding suggesting a modification of the cell wall composition (Fig. 3.9).

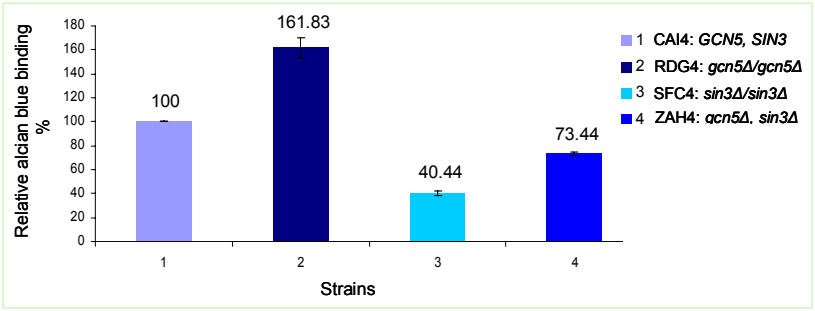

Figure 3.9 Cell surface charges in *C. albicans* strains. Alcian blue binding assays. Relative dye binding was calculated as the percentage of dye bound compared with results for the parental strains (CAI4, RDG4 and SFC4). Results are average of two independent determinations; bars indicate standard deviations.

Virulence assay

To test whether *C. albicans* Gcn5p and Sin3p influence virulence in systemic animal models, 10^8 (Data not show) and 5×10^8 cells of a reconstructed *URA3* in the *sin3Δ* and *gcn5Δ, sin3Δ* double mutants and CAI4 strain were injected intra-peritoneally of CD1 mice and monitored mouse survival for 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 shown) and rapid mortality. In contrast, 80% of mice injected with the double, *sin3Δ* and *gcn5*Δ (Rashki, Ph.D thesis 2009) simple mutants survived for this observed period (Fig. 3.10).

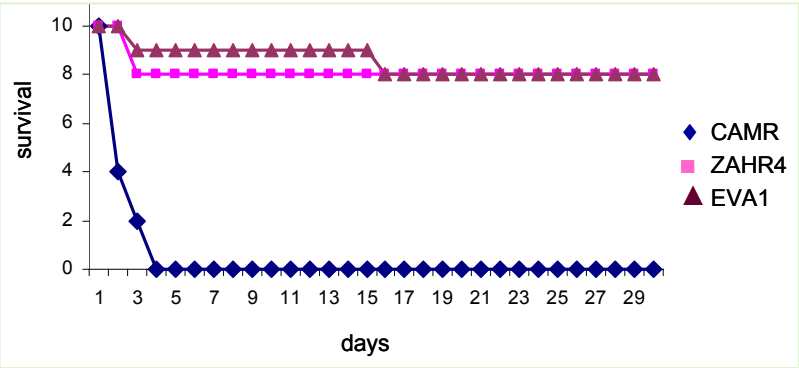

Figure 3.10 Virulence assays. Survival curves for mice (n = 10 for each *C. albicans* strain at each inoculation dose) infected with 5×10^8 cells of *C. albicans*. ♦CAMR (CAI4-*URA*$^+$), ■ ZAHR4 (*gcn5Δ, sin3Δ-URA*$^+$) and ▲EVA1 (*sin3Δ-URA*$^+$).

Global transcriptional responses of the *C. albicans gcn5Δ, sin3Δ* double mutant

A transcriptional profiling analysis was performed to further examine the roles of Gcn5p and Sin3p proteins in *C. albicans*. The transcriptome of the *gcn5Δ, sin3Δ* double mutant was compared with the CAI4 strain during the yeast mode of growth and during the yeast-hypha transition. Previously, in our laboratory we had performed experiments in which the transcriptomes of the *gcn5Δ* and *sin3Δ* 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 3.1-3.4). To explore the functions of the *gcn5Δ, sin3Δ* double mutant under condition that promoted growth in the yeast form, we compared the transcriptomes of CAI4 and the double mutant strain in YNB medium at 28°C. In the *gcn5Δ, sin3Δ* double mutant we detected an altered expression of 558 genes by ≥ 1.5-fold: 294 genes being up-regulated and 264 down-regulated. When transcriptome of cells growing in Lee medium at 37°C during the hypha transition were compared, 1271 genes modified their expression level, including 690 up- and 581 down-regulated genes. Approximately 30% of the *C. albicans* genes showed significant changes in their expression levels (ρ -value ≤ 0.02), indicating a global transcriptional response.

Differential gene expression in exponential growth phase

The analysis of the microarray data of cells in the exponential growth phase showed 558 affected genes (Supplementary tables 3.1 and 3.2). Among the total number of altered genes, 131 (23.47%) were genes with unknown functions. Amongst the rest of the genes, *HSP12*, encode a heat shock protein with homology to the *S. cerevisiae*

HSP12, showed the highest fold change (6.40-fold), whereas the lowest expression level found in this experiment was observed for IPF191 (-5.88 -fold), which encodes a putative transporter. Due to the large number of genes found to be up- or down-regulated, this analysis was focussed on a few of the representated functional categories.

Genes involved in DNA synthesis and replication

Our microarray analysis indicated that thirteen genes involved in DNA synthesis and replication e.g. *CDC21, DPB2*, *HYS2, POL2, POL3, RAD6* and *TOP1* were up-regulated (Table 3.3). In addition, two genes -*RFA1* and *RFA2*- that function as part of DNA replication factor also were up-regulated. It has been described that over-expression of *RFA1* may functions in the cell cycle delay at G2/M phases by disrupting spindle attachment to chromosomes and activating the DNA damage checkpoint (Niu *et al.*, 2008), which may be responsible for the elongated cell in the double mutant. Up-regulation of *RFA1* in the *sin3Δ* simple mutant has been reported (Martin, Ph.D thesis 2006) suggesting that Sin3p may play a role in the regulation of cell cycle progression. In agreement with our data, expression of genes playing a role in the G2 cell cycle progression in *SIN3*-deficient cells has also been reported in *Drosophila* (Pile *et al.*, 2003). Twelve genes encoding for oxidative stress including *AHP2* (Fernandez-Arenas *et al.*, 2007), *GAD1* (Bensen *et al.*, 2004), *GLO1* (Wang *et al.*, 2006), *GLR1* (Alarco & Raymond, 1999; Fradin *et al.*, 2005; Wang *et al.*, 2006), *GRE3* (Ramsdale *et al.*, 2008), *GRP2* (Enjalbert *et al.*, 2006), *GRP6, GRX3* (Yin *et al.*, 2004), *MCR1* (Bensen *et al.*, 2004) *SOD2, TRX1* and *TSA1* (Seneviratne *et al.*, 2008) were found to be up-regulated and three of them, *AHP2, MCR1* and *TSA1* overlapped with the *sin3Δ* simple mutant.

Table 3.3 Differentially expressed genes in *gcn5Δ, sin3Δ* mutant during the yeast growth phase

Gene name	ORF	Description	Fold regulation
DNA synthesis and replication			
CDC21	orf19.3549	DNA polyamerase III	1.59
DBP3	orf19.12334	DNA -directed DNA polymerase epsilon subunit B	1.76
HYS2	orf19.3960	DNA-directed DNA polymerase delta	2.01
POL2	orf19.2365	DNA-directed DNA polymerase epsilon,	1.74
POL3	orf6.3494	DNA polymerase III	1.88
RAD51	orf19.3752	DNA repair protein	2.16
RAD6	orf19.7195	Ubiquitin protein ligase	1.71
RFA1	orf19.2093	DNA replication factor	1.62
RFA2	orf19.2267	DNA replication factor A	2.36
RNR1	orf19.5779	Ribonucleoside-diphosphate reductase	2.94
RNR21	orf19.5801	Ribonucleoside-diphosphate reductase	1.77
RNR22	orf19.1868	Ribonucleoside-diphosphate reductase	3.03
TOP1	orf19.7742	DNA topoisomerase I	2.58
Oxidative stress response			
AHP2	orf19.6470	Macrophage-downregulated gene	2.37
GAD1	orf19.1153	Glutamate decarboxylase	2.02
GLO1	orf19.6058	Oxidative stress-induced via Cap1p	1.69

GLR1	orf19.11623	Glutathione reductase; oxidative stress-induced via Cap1p	1.73
GRE3	orf19.4317	D-xylose reductases	1.78
GRP2	orf19.4309	Similar to *S. cerevisiae* Gre2p (methylglyoxal reductase); induced in core stress response or by oxidative stress	5.54
GRP6	orf19.3151	Putative reductase	1.84
GRX3	orf19.2727	Glutaredoxin-like protein	2.25
MCR1	orf19.3507	Protein described as NADH-cytochrome-b5 reductase	2.33
SOD2	orf19.3340	Mitochondrial manganese-containing superoxide dismutase	1.81
TRX1	orf19.7611	Thioredoxin	1.79
TSA1	orf19.7417	Protein of TSA/alkyl hydroperoxide peroxidase C (AhPC) family	1.92
Glycolisis			
ALD5	orf19.13228	Protein described as an aldehyde dehydrogenase	3.10
FBA1	orf19.4618	Putative fructose-bisphosphate aldolase; enzyme of glycolysis	2.02
FBP1	orf19.6178	Fructose-1,6-bisphosphatase	1.72
PCK1	orf19.7514	Phosphoenolpyruvate carboxykinase	4.15
Oxidation of fatty acids			
ECI1	orf19.6445	Protein similar to *S. cerevisiae* Eci1p, which is involved in fatty acid oxidation	1.99
POT1	orf19.7520	Putative peroxisomal 3-oxoacyl CoA thiolase	1.94
POX1-3	orf19.9221	Predicted acyl-CoA oxidase; farnesol regulated	1.83
SPS20	orf19.4157	Peroxisomal 2,4-dienoyl-CoA reductase	1.96
Siderophore-iron transport			
FET33	orf19.943	Putative multicopper ferro-O_2-oxidoreductase	1.70
FRE10	orf19.1415	Major cell-surface ferric reductase under low-iron conditions	1.97
FTR1	orf19.7219	High-affinity iron permease	3.56
FTR2	orf19.7231	High-affinity iron permease;	2.22
Pentose-phosphate pathway			
PRS1	orf19.969	Ribose-phosphate pyrophosphokinase	2.24
TKL1	orf19.5112	Putative transketolase	1.87
ZWF1	orf19.12218	Putative glucose-6-phosphate dehydrogenase	1.79
Chitin anabolism			
CHS3	orf19.4937	Major chitin synthase of yeast-form and hyphal cells	1.91
CHT2	orf19.3895	Chitinase; putative N-terminal catalytic domain	2.17
CHT3	orf19.7586	Chitinase	2.34
Nuclear migration			
NUM11	orf19.4715	Nuclear migration protein	1.92
NUM12	orf19.2924	Nuclear migration protein	2.21
TUB1	orf19.7308	Alpha-tubulin	1.73
TUB2	orf19.6034	Beta-tubulin	1.58
Tricarboxylic-acid pathway			
FUM12	orf19.6725	Protein described as similar to fumarate hydratase, enzyme of citric acid cycle	1.76
IDP2	orf19.3733	Putative isocitrate dehydrogenase	2.19
LSC2	orf19.710	Protein described as beta subunit of succinate-CoA ligase	2.03
MDH1	orf19.7481	Protein described as malate dehydrogenase	1.77
Glucose transporter			
HGT6	orf19.2020	Putative glucose transporter of the major facilitator superfamily	2.43
HGT8	orf19.2021	Putative glucose transporter of the major facilitator superfamily	2.11
SHA3	orf19.3669	Protein similar to *S. cerevisiae* Sha3p, which is a serine/threonine kinase involved in glucose transport	1.69
Cell wall			
PGA14	orf19.968	Putative GPI-anchored protein of unknown function	2.69
PGA23	orf19.11225	Putative GPI-anchored protein of unknown function	1.76
PGA4	orf19.4035	Cell-surface protein; predicted glycosylphosphatidylinositol (GPI) lipid anchor	2.75
PGA54	orf19.2685	Putative GPI-anchored protein; hyphal induced	2.10
PGA56	orf19.1105.2	putative GPI-anchor	
PMI1	orf19.8968	Phosphomannose isomerase; cell wall biosynthesis enzyme	1.81
PMT2	orf19.6812	Essential protein mannosyltransferase with roles in hyphal growth	1.77
Transcription factor			
BDF1	orf19.978	Putative transcription factor	2.04
TYE7	orf19.4941	Putative bHLH (basic region, helix-loop-helix) transcription factor	1.84
ZCF1	orf19.255	Predicted zinc-cluster protein of unknown function	1.95
ZPR1	orf19.3300	Protein with putative zinc finger; regulated by Gcn4p	1.58

Down-regulated genes			
Cell wall			
ALS1	orf19.5741	Adhesion; ALS family of cell-surface glycoproteins	0.54
ALS2	orf19.2122	ALS family protein which includes cell-surface glycoproteins	0.61
ALS3	orf19.2355	Agglutinin like protein	0.42
ALS4	orf19.4556	ALS family protein; role in adhesion and wild-type germ tube induction	0.19
ALS9	orf19.5742	ALS family protein; expressed during infection of human epithelial cells	0.47
BGL22	orf19.7339	Putative glucanase; induced during cell wall regeneration	0.54
CHS7	orf19.2444	Protein required for wild-type chitin synthase III activity	0.51
CWH41	orf19.4421	Processing alpha glucosidase I	0.42
EAP1	orf19.1401	Cell wall adhesin required for cell-cell adhesion and biofilm formation	0.29
ECE1	orf19.3374	hyphal-specific expression increases with extent of elongation of the cell	0.24
PGA16	orf19.8468	Putative GPI-anchored protein of unknown function	0.36
SCW1	orf19.9345	Cell surface manno-protein	0.60
Electron transport			
ATP1	orf19.6854	Protein similar to alpha subunit of ATP synthase	0.43
ATP2	orf19.5653	Protein described as a similar to F1 beta subunit of F1F0 ATPase complex	0.54
ATP6		Subunit 6 of the F0 sector of mitochondrial F1F0 ATP synthase	0.21
COB		Cytochrome b	0.29
COX3A		Subunit III of cytochrome c oxidase	0.37
COX3B		Subunit III of cytochrome c oxidase	0.46
CYC1	orf19.1770	Cytochrome c	0.49
PET117	orf19.6225.1	Cytochrome c oxidase assembly factor	0.60
PMA1	orf19.5383	Plasma membrane H(+)-ATPase	0.35
Transport			
BET5	orf19.302	Targeting and fusion of ER to Golgi transport vesicles	0.56
FCY24	orf19.7331	Putative transporter; more similar to *S. cerevisiae* Tpn1p	0.54
HAK1	orf19.6249	Putative potassium transporter	0.31
HOL2	orf19.4889	Predicted membrane transporter	0.22
HOL4	orf19.12021	Protein described as an ion transporter	0.37
IFC1	orf19.11233	Oligopeptide transporter	0.41
IFC3	orf19.3749	Oligopeptide transporter	0.48
IPF1471	orf19.304	Putative transporter similar to MDR proteins	0.43
IPF1524	orf19.341	Putative spermidine export pump	0.62
IPF191	orf19.3232	Putative transporter	0.17
IPF9079	orf19.4550	Predicted membrane transporter	0.59
SSU1	orf19.7313	Protein similar to *S. cerevisiae* Ssu1p sulfite transport	0.56
Ribosomal proteins			
MRPL27	orf19.3064	Putative ribosomal protein	0.50
MRPL6	orf19.7486	Putative mitochondrial ribosomal protein	0.56
MRS4	orf19.2178	RNA splicing protein and member of the mitochondrial carrier family (MCF)	0.52
PRP18	orf19.2112	U5 snRNA-associated protein	0.20
PRP31	orf19.1296	Pre-mRNA splicing protein	0.37
PRP5	orf19.6831	Protein described as a pre-mRNA processing RNA-helicase	0.60
RPL14B	orf19.4931.1	Ribosomal protein L14B (by homology)	0.52
RPL18	orf19.5982	Predicted ribosomal protein;	0.56
RPS620A	orf19.6300	Downregulation correlates with clinical development of fluconazole resistance	0.52
RPS620B	orf19.6301	Clade-associated gene expression	0.54
Transcription factors			
GLN3	orf19.11393	GATA transcription factor involved in regulation of filamentous growth	0.51
HAC1	orf19.2432	Putative transcription factor involved in unfolded protein response	0.64
HAP2	orf19.8814	CCAAT-binding factor regulates low-iron (chelation) induction of *FRP1*	0.61
IPF1674	orf19.6559	Putative transcription initiation factor	0.54
IPF1731	orf19.3088	Putative transcription factor with bZIP DNA-binding motif	0.49
IPF29	orf19.5975	Putative transcription factor with zinc finger DNA-binding motif	0.60
LEU3	orf19.11700	Predicted zinc-finger protein	0.38
PPR1	orf19.3986	Putative transcription factor with zinc cluster DNA-binding motif	0.43
RIM101	orf19.7247	Transcription factor involved in alkaline pH response	0.36

ZCF2	orf19.431	Putative transcription factor with zinc cluster DNA-binding motif	0.55
ZCF20	orf19.11621	Predicted zinc-finger protein of unknown function	0.40
ZCF32	orf19.5940	Predicted zinc-finger protein of unknown function	0.58

We determined whether up-regulation of these genes may affect the growth of double and *sin3Δ* simple mutants on hydrogen peroxide. In the exponential phase of growth, 10^7 cells/ml of each strain were spotted on YEPD medium supplemented with different concentration of H_2O_2. We observed that the double mutant like *sin3Δ* simple mutant showed more resistance to hydrogen peroxide (H_2O_2) (Fig. 3.11) suggesting that *SIN3* plays a role in regulation this set of genes.

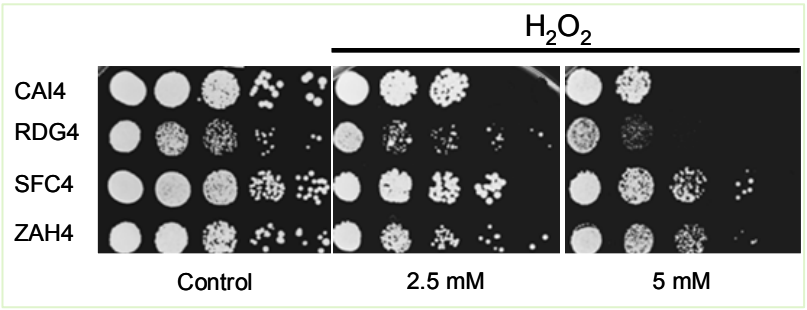

Figure 3.11 Sensitivity of the ZAH4 (*gcn5Δ, sin3Δ*) to the hydrogen peroxide (H_2O_2)

The transcription level four genes involved in the oxidation of fatty acids and peroxisomal activity including *ECI1* (Lan *et al.*, 2002; Uhl *et al.*, 2003), *POT1* (Enjalbert & Whiteway, 2005), *POX1-3* and *SPS20* were increased. It has been observed that the β-oxidation of fatty acids is associated with the activity of peroxisomes (e.g. with the onset of the response to oxidative stress) (Koerkamp *et al.*, 2002; Lan *et al.*, 2002) and up-regulation of numerous genes involved in fatty acid oxidation (Pile *et al.*, 2003) has been reported in *Drosophila* in agreement with our results.

Genes coding for transcription factors

Sixteen genes encode transcriptional regulators were found to be regulated by double mutant (Table 3.3). Four of them were up-regulated and twelve were down-regulated. Two of the four up-regulated, *ZCF1* (Park *et al.*, 2003) and *ZPR1* (Rogers & Barker, 2003), and six of twelve down-regulated, IPF1731, IPF29, *PPR1, ZCF2, ZCF20, ZCF32* transcriptional regulators are encoded by genes that previously characterized as transcription factors with a zinc cluster DNA-binding motif. This family of transcriptional regulators may be a major target for both proteins in *C. albicans* and their role deserves further analysis. Other transcriptional factors (Table 3.3) belong to disperse families of regulators that respond to different factors and regulate a variety of

genes with different functions. None of them are regulated in the *gcn5Δ* and *sin3Δ* simple mutants except of *RIM101, PPR1* and *LEU3* which have been reported to be up-regulated in the *gcn5Δ* simple mutant.

Other transcripts

We have detected 4 genes, including *NUM11, NUM12, TUB1* and *TUB2* up-regulated in the double mutant, two of them -*NUM12* and *TUB1*- have been reported to be up-regulated in the *sin3Δ* simple mutant. *S. cerevisiae num1* and *tub1* mutants showed a defect in correct nuclear migration (Heil-Chapdelaine *et al.*, 2000; Schatz *et al.*, 1988). Over-expression of alpha- and beta-tubulin genes in *S. cerevisiae,* separately or together, leads to accumulation of large excesses of each of the polypeptides, arrest cell division and forms abnormal structures. Excess beta-tubulin also might prevent microtubule elongation and over-expression of *ScTUB1* temporally blocked normal cell cycle during the logarithmic growth phase (Weinstein & Solomon, 1990). This result also could be explaining large cell morphology of the double and *sin3Δ* simple mutant.

Transcript levels of seven genes coding for cell wall proteins including *PGA4, PGA14, PGA23, PGA54, PGA56* (Castillo *et al.*, 2008)*, PMI1* (Yin *et al.*, 2004), *PMT2* (Prill *et al.*, 2005) and three genes involved in the chitin anabolism: *CHS3* (Mio *et al.*, 1996)*, CHT2* (McCreath *et al.*, 1995) and *CHT3* (Dunkler *et al.*, 2005; McCreath *et al.*, 1995; McCreath *et al.*, 1996) were up-regulated. None of them has been reported to be regulated in the both simple mutants, suggested that both genes regulate in a join way, an important set of cell wall genes.

Four of the seven genes coding for regulatory enzymes of the citric acid cycle, including succinate-CoA ligase, *LSC2*, (Kadosh & Johnson, 2005; Lan *et al.*, 2004; Murad *et al.*, 2001a), malate dehydrogenase, *MDH1*, (Maglott *et al.*, 2007), isocitrate dehydrogenase, *IDP2*, (Lan *et al.*, 2002) and fumarate hydratase, *FUM12* and four genes involved in glycolysis including *ALD5* (Brand *et al.*, 2004)*, FBA1* (Doedt *et al.*, 2004; Garcia-Sanchez *et al.*, 2004; Nantel *et al.*, 2002)*, FBP1* (Doedt *et al.*, 2004) and *PCK1* (Leuker *et al.*, 1997) were up-regulated in the double mutant. Some of genes involved in glycolysis pathway including *ALD5, GPH5, GLK4, PDC11* and *PYC2* has been reported to be up-regulated in the *gcn5Δ* simple mutant (Degano, Ph.D thesis in process). None of them except *IPD2* has been regulated in the *sin3Δ* mutant under yeast mode of growth. Our results remarkably are in agreement with those reported in the *Drosophila sin3Δ* mutant (Pile *et al.*, 2003).

Overview of changes in global gene expression during the yeast-hypha transition

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 *gcn5Δ, sin3Δ* double mutant in Lee medium for 15, 60 and 180 min at 37°C using the CAI4 strain as reference. A total of 1271 genes were found to change at the consensus level of ≥ 1.5 fold, including 690 up- and 581 down-regulated genes. The distribution of genes altered at the three points is represented in the Venn diagram, and the clustering of genes regulated by deletion of *GCN5* and *SIN3* is represented in Fig. 3.12 (A and B). We found that 43 genes were common to the three times assayed (10 and 33 up- and down- respectively), 156 genes at 15 and 60 min (102 up- and 54 down-) and 47 genes at 60 and 180 min (9 up- and 38 down-). 324 genes (227 up- and 97 down-), 645 genes (315 up- and 330 down-) and 51 genes (24 up- and 27 down-) varied only at one time of induction (at 15, 60 and 180 min respectively). However, only 5 genes were common between 15 and 180 min validating our experimental approach. The elevated number of regulated genes detected only at one time suggests that transitory changes occur sequentially in response to both mutations during the morphogenetic switch (Fig. 3.12).

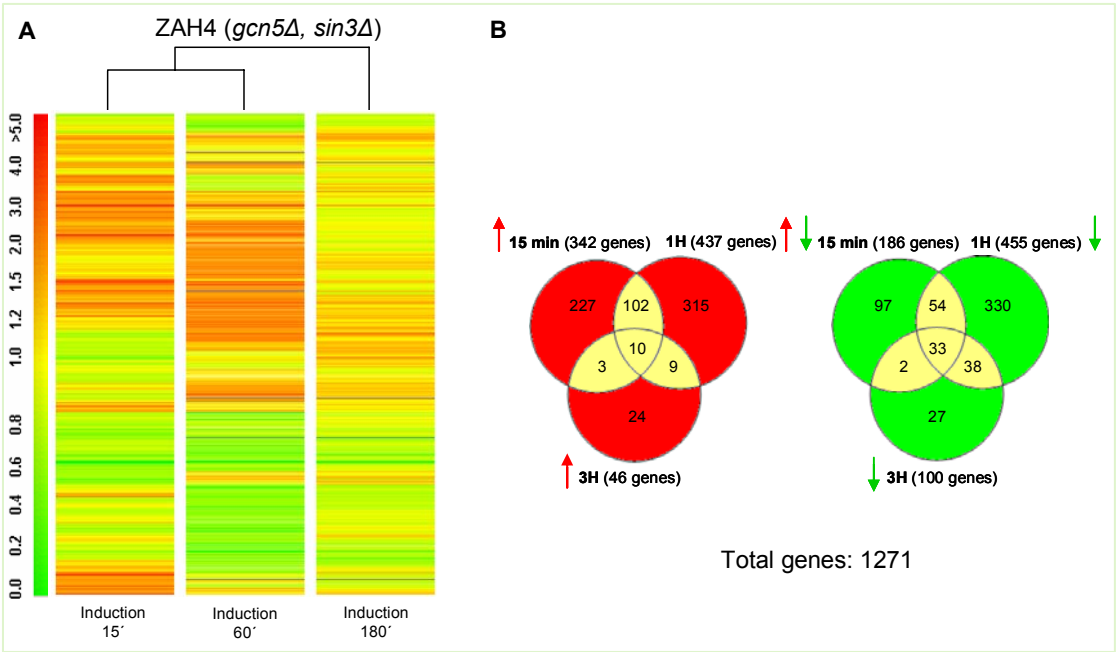

Figure 3.12 Transcript profiles of *C. albicans gcn5Δ, sin3Δ* double mutant during the yeast-hypha transition (A). Venn diagrams of genes regulated by Gcn5p and Sin3p in the double mutant (B). The CAI4 strain was used as reference, which all were grown in identical conditions: Lee/37°C (inducing).

Forty three genes common at the three times are listed in Table 3.4. Only 2 of the common genes correspond to genes of unknown function. Of the rest of the genes, 16 belong to disperse functional categories i.e., *HSP12* heat-shock protein, *ACC1* protein with similarity to acetyl-coenzyme-A carboxylases, etc., and 25 genes can be grouped in seven functional categories. The main category corresponds to cell wall proteins and includes the down-regulation of *HWP1* a well known cell wall protein (Staab *et al.*, 1999), *ECE1* a protein involved in cell elongation (Bennett *et al.*, 2003; Birse *et al.*, 1993) and *RBT1* (Bennett *et al.*, 2003; Sohn *et al.*, 2003) a putative cell wall protein with similarity to Hwp1p, two genes belonging to the *ALS* family, including *ALS1* and *ALS3*. All these down-regulated genes have been described previously as being up-regulated during the hypha transition (Braun *et al.*, 2005; Nantel *et al.*, 2002) and some are regulated by Efg1p and Tup1p (Doedt *et al.*, 2004; Kadosh & Johnson, 2005; Sohn *et al.*, 2003). Another class of well-known genes regulated at three times consists of genes belonging to the following categories: fatty acid metabolism (5 genes), electron transporters (3 genes), oxidases/peroxidses (4 genes), vitamin biosynthesis (2 genes) and carbohydrate metabolism (3 genes).

Table 3.4 Commom differentially expressed genes in *gcn5Δ, sin3Δ* mutant at the three times of incubation (at 15, 60 and 180 min)

Gene name	ORF	Description	15 min	60 min	180 min
Down-regulated genes					
Cell wall					
ALS1	orf19.5741	Adhesin; ALS family of cell-surface glycoproteins	0.10	0.11	0.20
ALS3	orf19.2355	Agglutinin like protein	0.21	0.33	0.09
ECE1	orf19.3374	Protein comprising eight 34-residue repeats	0.03	0.06	0.05
HWP1	orf19.1321	Hyphal cell wall protein	0.08	0.23	0.29
MP65	orf19.9345	Cell surface mannoprotein	0.33	0.29	0.50
PGA58	orf19.4334	Putative GPI-anchored protein of unknown function	0.59	0.50	0.53
RBT1	orf19.1327	Putative cell wall protein with similarity to Hwp1p	0.40	0.29	0.52
RHR2	orf19.5437	Putative glycerol 3-phosphatase	0.25	0.24	0.48
Fatty acid metabolism					
ERG251	orf19.12101	Ketoconazole-induced	0.44	0.11	0.48
FAD2	orf19.7765	Delta-12 fatty acid desaturase	0.53	0.46	0.60
FAS2	orf19.5948.1	Fatty-acyl-CoA synthase, alpha chain, internal fragment	0.22	0.43	0.51
HET3	orf19.6327	Putative sphingomyelin transfer protein	0.41	0.39	0.57
OLE1	orf19.5117	Fatty acid desaturase (stearoyl-CoA desaturase)	0.31	0.39	0.57
Oxidase/peroxidase					
FET3	orf19.11687	Multicopper oxidase	0.62	0.24	0.52
FET34	orf19.4215	Protein similar to multicopper ferroxidase	0.48	0.49	0.52
GPX2	orf19.85	Similar to glutathione peroxidase	0.15	0.25	0.51
SOD5	orf19.2060	Copper- and zinc-containing superoxide dismutase	0.52	0.14	0.32
Transcription factors					
GAT2	orf19.4056	Putative DNA-binding transcription factor	0.54	0.37	0.46
UME6	orf19.9381	Transcription factor; required for wild-type hyphal extension	0.37	0.25	0.62
Charbohydrate metabolism					
ADH1	orf19.3997	Alcohol dehydrogenase; at surface of yeast-form cells but not hyphae	0.32	0.08	0.24
HGT7	orf19.2023	Putative glucose transporter, major facilitator superfamily	0.40	0.07	0.23

PFK2	orf19.6540	Beta subunit of phosphofructokinase (PFK)	0.35	0.12	0.49
Vitamin biosynthesis					
FCY24	orf19.7331	Putative transporter	0.45	0.28	0.54
THI13	orf19.7324	Protein not essential for viability; similar to *S. cerevisiae* Thi13p	0.50	0.16	0.50
Other function					
ACC1	orf19.7466	Protein with similarity to acetyl-coenzyme-A carboxylases	0.30	0.40	0.47
ARP3	orf19.2289	Shows Myo5p-dependent localization to cortical actin patches at hyphal tip	0.18	0.16	0.41
CAN5	orf19.3641	Expression is regulated upon white-opaque switching	0.57	0.26	0.50
DEF1	orf19.7561	Protein required for filamentous growth and for escape from epithelial cells	0.38	0.24	0.48
DUR3	orf19.6656	Putative protein of unknown function	0.38	0.27	0.54
IPF1548	orf19.951	Transcription downregulated upon yeast-hyphal switch	0.44	0.43	0.49
IPF8762	orf19.822	Protein detected in some, not all, biofilm extracts	0.49	0.16	0.44
SAM4	orf19.386	Alkaline upregulated	0.54	0.39	0.43
YHB1	orf19.3707	Nitric oxide dioxygenase	0.40	0.27	0.26
Up-regulated genes					
Electron transport					
ATP6		Subunit 6 of the F0 sector of mitochondrial F1F0 ATP synthase	3.73	2.53	1.76
COX1		Subunit I of cytochrome c oxidase	2.93	1.78	1.86
COX3A		Subunit III of cytochrome c oxidase	2.51	2.07	1.87
Other function					
ALD5	orf19.13228	Protein described as an aldehyde dehydrogenase	3.12	1.77	2.12
HSP12	orf19.3160	Heat-shock protein; induced upon osmotic/oxidative/cadmium stress	24.53	7.23	2.23
IPF15217	orf19.3779	Unknown function	2.94	7.83	1.85
IPF4065	orf19.1862	Possible stress protein	10.87	4.16	2.46
IPF4510	orf19.6999	Unknown function	1.53	2.30	1.83
IPF6881	orf19.4246	Putative phosphatidyl synthase	7.32	3.01	2.14
PUT2	orf19.3974	Alkaline upregulated	4.47	4.32	2.24

Expression of transcription factors

Only two transcription factor was down-regulated at the three times of incubation; namely, *GAT2*, which has been described as a putative DNA-binding transcription factor with unspecified role in morphogenesis, that await further characterization and *UME6* is a transcription factor; required for wild-type hyphal extension (Banerjee *et al.*, 2008). Deletion of *UME6* gene produces clear defects in hyphal formation and extension, and hence our results are in agreement with phenotype of double mutant during the hypha transition. Two transcriptional factors -*ARG83* and *ECM22*- were down-regulated at two times: 15 and 60 min. Six genes including *BDF1* (Bensen *et al.*, 2002; Marchais *et al.*, 2005), *HIR2* (DeSilva *et al.*, 1998; Spector *et al.*, 1997), IPF1731 a putative transcription factor transcriptionally regulated upon hyphal formation (Murillo *et al.*, 2005; Nobile & Mitchell, 2005; Park *et al.*, 2003), *NRG1* (Murad *et al.*, 2001b), *NUT2* (Gustafsson *et al.*, 1998) and *SWI4* (Castillo *et al.*, 2006) were up- and two genes including *LEU3* (Bensen *et al.*, 2004; Maicas *et al.*, 2005; Ramsdale *et al.*, 2008), *ZCF32* (Maicas *et al.*, 2005; Znaidi *et al.*, 2007) and *ZFU2* a putative transcription factor with zinc finger DNA-binding motif (Mitrovich *et al.*, 2007; Nobile &

Mitchell, 2005) were down-regulated at 15 min. Some of transcription factors are regulated in the yeast-hypha transition, as described from previous studies (Banerjee *et al.*, 2008; Murad *et al.*, 2001a; Murillo *et al.*, 2005; Nobile & Mitchell, 2005; Park *et al.*, 2003; Rashki, 2009) and differentially expression of these genes in the both simple mutants and CAI4 strain are shown in Fig 3.13. Together these results, which fit nicely with previous data regarding hypha-transition, strongly support the validity of our microarray analysis.

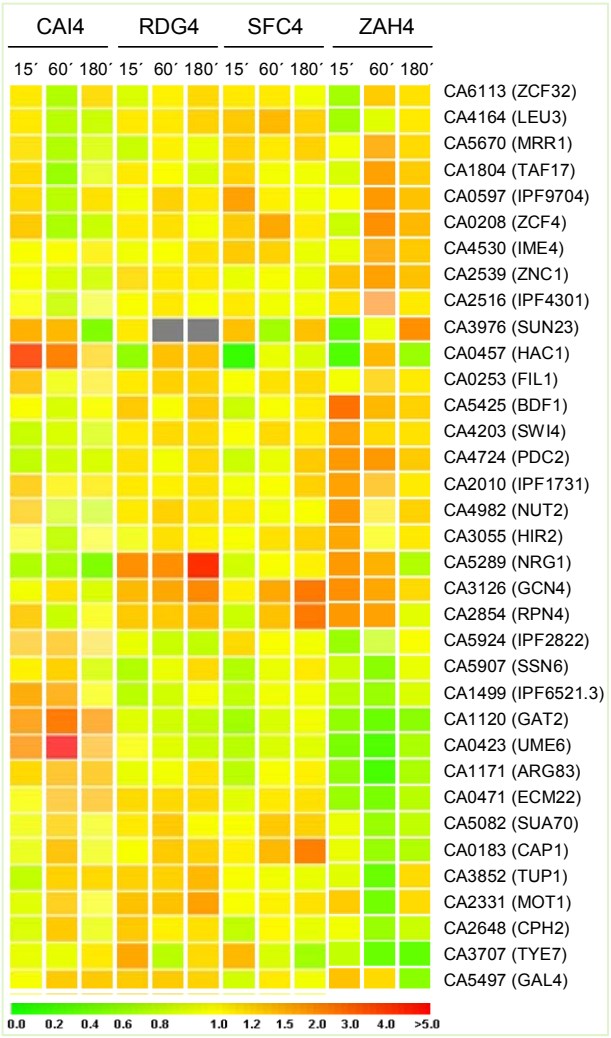

Figure 3.13 Cluster analysis of genes encoding for transcriptional factors in the CAI4, RDG4 (*gcn5Δ*), SFC4 (*sin3Δ*) and ZAH4 (*gcn5Δ, sin3Δ*) strains during the yeast-hypha transition.

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 by the double mutant. Many genes were could not be

readily categorized into functional groups 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 Sin3p affect a large number of cell functions and most likely serve to globally reprogram of *C. albicans* physiology that allow the double mutant to adapt to this environment.

Cell wall genes: Genes encode cell wall proteins were also altered by loss of Gcn5p and Sin3p during hypha transition. For example, the ALS family includes 8 genes that five of them were down-regulated included *ALS1* and *ALS3* at the tree times, *ALS2* (Hoyer *et al.*, 1998b; Zhao *et al.*, 2005b), *ALS9* (Zhao *et al.*, 2003) at 15 min and *ALS4* (De Groot *et al.*, 2003) at 15 and 60 min (Fig. 3.14A). *ALS1* and *ALS3* has been reported to be up-regulated during the hypha transition in the CAI4 strain at 28-37°C temperature shifts in the Lee medium (Rashki, Ph.D thesis 2009).

HYR1 a predicted cell wall protein which described as glutathione peroxidase and regulated by Rfg1p, Efg1p, Nrg1p, Tup1p, Cyr1p (Fernandez-Arenas *et al.*, 2007; Kadosh & Johnson, 2001; Murad *et al.*, 2001b) is down-regulated at 15 and 60 min. Two genes, *CHS3* (Mio *et al.*, 1996; Munro *et al.*, 1998), at 60 min, and *YWP1*, at 180 min, were up-regulated. Down-regulation of *YWP1* has been reported in the CAI4 strain during hypha transition (Rashki, Ph.D thesis 2009). This finding is consistent with the phenotypic results, which indicated that the double mutant failed to form true hypha in either liquid or solid media.

GPI anchored proteins: Our results indicate that the transcript levels of seventeen 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, *PGA1* (Castillo *et al.*, 2006; De Groot *et al.*, 2003), *PGA49* (De Groot *et al.*, 2003), *CWH41* (Mora-Montes *et al.*, 2007), *ECM1* (Harcus *et al.*, 2004), *FGR12* (Uhl *et al.*, 2003), *FGR41* (De Groot *et al.*, 2003), *IFF5* (De Groot *et al.*, 2003) were up-regulated and *KRE1* (found highly regulated during *C. albicans* protoplast regeneration) (Castillo *et al.*, 2006), *PGA6, PGA45, PGA53, PGA62* and *PGA63, ECM21, ECM33, FGR6*, *PHR1* were down-regulated at 60 min (Fig. 3.14B). The transcript levels of *PGA37* (De Groot *et al.*, 2003) at 15 min and *PGA54* a putative GPI-anchored protein (De Groot *et al.*, 2003), *RBT4* which required for virulence in mouse systemic (Garcia-Sanchez *et al.*, 2005; Sohn *et al.*, 2003) at 15 and 60 min were down-regulated (Fig. 3.14B).

Regulation a large number of genes coding for putative GPI-anchored proteins in the *C. albicans* CAI4 (22 genes), *gcn5Δ* (10 genes), *hda1Δ* (5 genes) and *gcn5Δ, hda1Δ*

double mutant (16 genes) has been reported in our laboratory. From our results and those described by other, it may be concluded that the morphogenetic switch involves a strong remodelling of the yeast cell wall and membrane.

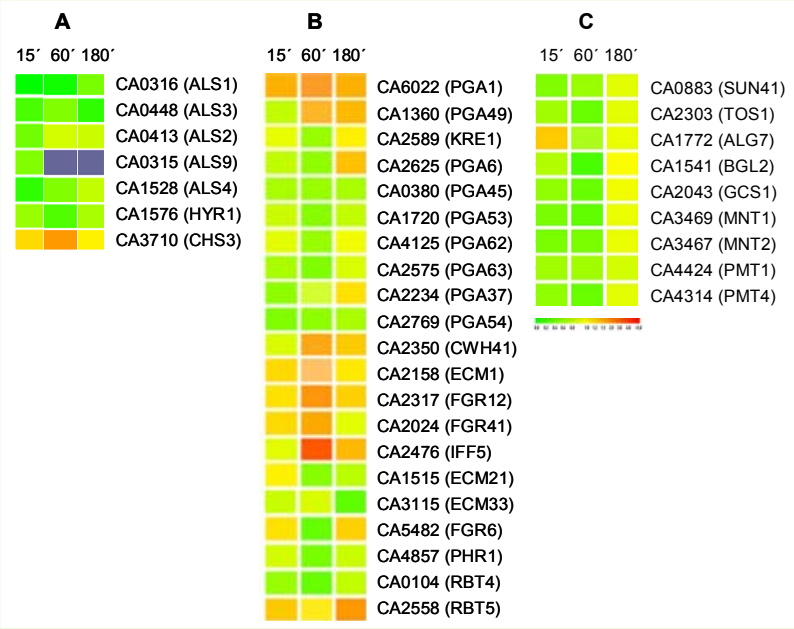

Figure 3.14 Transcriptional **response of genes involved in the cell wall in the *Δgcn5, Δsin3* double mutant.** Representative genes responsible for specific cellular functions are grouped and their regulation during 15, 60 and 180 min of yeast-hypha transition in the *Δgcn5, Δsin3* double mutant is shown in the color bar diagrams.

Glucanases, glucosidases and mannosyltransferases: Our microarray analysis indicated that seven genes encoding glucanases, glucosidases and mannosyltransferases including *SUN41* a putative cell wall glycosidase which is involved in biofilm formation and required for cell separation (Firon *et al.*, 2007) and *TOS1* a protein described as similar to alpha agglutinin anchor subunit (Bader *et al.*, 2008), *GCS1* subunit of beta-1,3-glucan synthase (Braun *et al.*, 2005; Mio *et al.*, 1997), *MNT1* a Alpha-1,2-mannosyl transferase (Braun *et al.*, 2005; Buurman *et al.*, 1998), *MNT2* a Alpha-1,2-mannosyl transferase (Braun *et al.*, 2005; Munro *et al.*, 2005), *PMT1* a mannosyltransferase (Prill *et al.*, 2005; Timpel *et al.*, 1998) and *PMT4* a mannosyltransferase required for normal cell wall composition and full virulence in mouse systemic infection (Prill *et al.*, 2005) were down-regulated at 15 and 60 min. Deletion of both alleles of the *SUN41* resulted in defects of hyphal formation (Firon *et al.*, 2007) that is in agreement with phenotype of double mutant during the hypha transition (Fig. 3.14C).

Genes involved in protein synthesis and ribosomal proteins: A second important category includes up-regulation of *NOC4, NOP2, NOP10, RPB8, RPC10, RPC31,*

RPC82, RPL25, RPL26A, RPL27A, RPL35, RRP1, RRP3, RRP9, RRS1, RSA2, SIK1 and down-regulation of *RPL2, RPL7A, RPL13, RPL14, RPL15A, RPL18, RPL30, RPN2, RPP2A, RPP2B, RPS4A, RPS16A, RPS17B, RPS20* genes at 60 min of hypha transition. No genes of this category were found up- and down-regulated at other time point. Differentially expressions of some of them are reported in the *gcn5Δ* and *sin3Δ* simple mutants during hypha transition. 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. Three of them (*RPL27A, RPS4A* and *RPS20*) have been described as up-regulated under standard conditions of the yeast-hypha transition (temperature) in CAI4 strain (Rashki, Ph.D thesis 2009). Five of them (*RPL13, RPL25, RPL26A, RPL30* and *RPS4A*) have been reported up-regulated by comparison of *Cacwt1* mutant with a wild type strain (Reuss & Morschhauser, 2006) and finally ten of them were down-regulated in the *C. albicans gcn5Δ* mutant during yeast form growth (Degano, Ph.D thesis in process). Differencially expression of this set of genes in the CAI4 and both simple mutants is shown in Fig. 3.15.

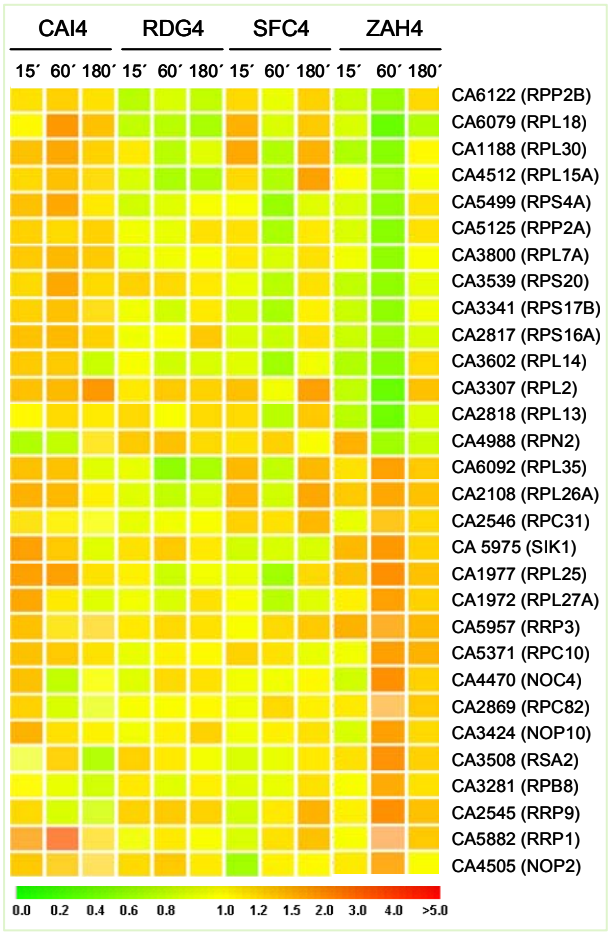

Figure 3.15 Cluster analysis of genes encoding for ribosomal proteins in the CAI4, RDG4 (*gcn5Δ*), SFC4 (*sin3Δ*), ZAH4 (*gcn5Δ, sin3Δ*) strains during the yeast-hypha transition.

Central carbon metabolism: The regulation of 34 genes (27 down-regulated after 60 min of incubation) involved in the glycolytic pathway, the TCA and glyoxylate cycles and gluconeogenesis (see Fig. 3.16A and B; Fig. 3.17) were detected. Expression some of them has been reported to be decreased in the *gcn5Δ* (180 min) and *sin3Δ* (60 and 180 min) (Fig. 3.17). In general our results are in agreement with those described in the *gcn5Δ, hda1Δ* double mutant during the yeast-hypha transition (Rashki, Ph.D thesis 2009). These changes reflect differences in the modulation of metabolic genes during the hypha transition.

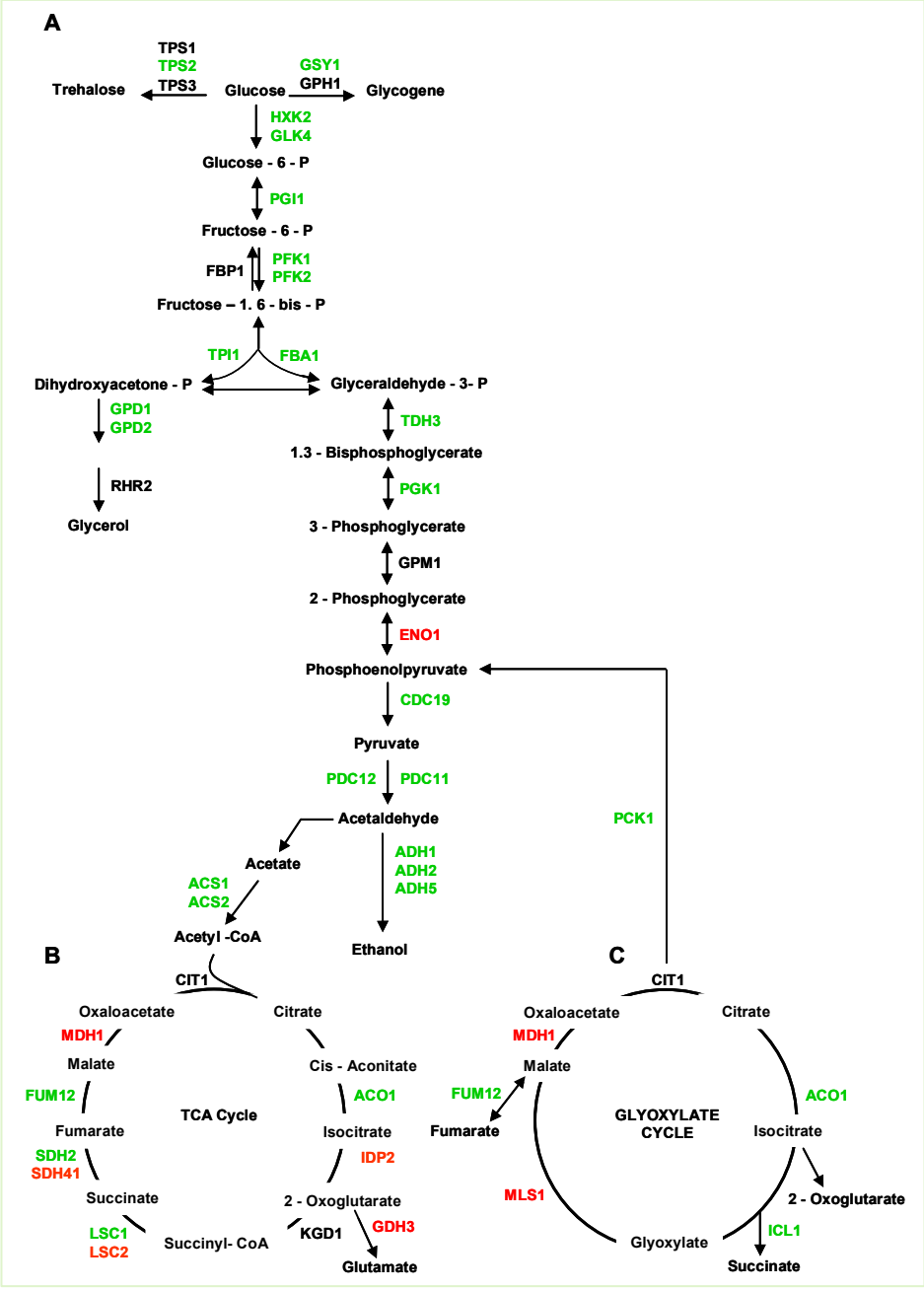

Figure 3.16 Transcriptional regulation of genes involved in the glycolitic pathway (A), TCA cycle (B) and glyoxylate cycle (C) in the *gcn5Δ, sin3Δ* double mutant.

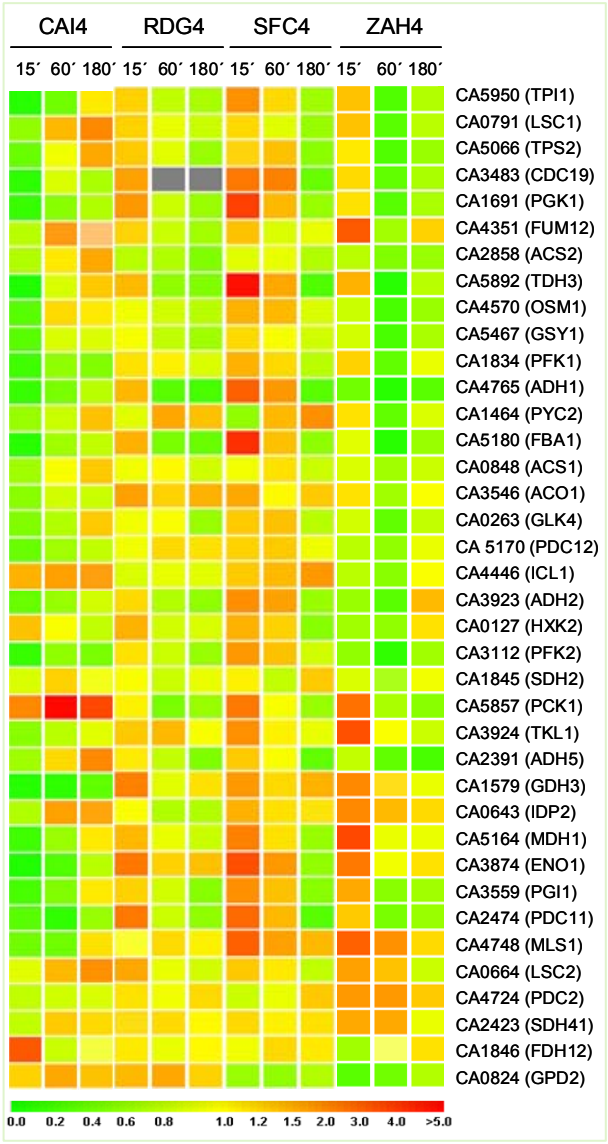

Figure 3.17 Cluster analysis of genes involved in carbohydrate metsabolism in the CAI4, RDG4 (*gcn5Δ*), SFC4 (*sin3Δ*) and ZAH4 (*gcn5Δ, sin3Δ*) mutants during the yeast-hypha transition.

However, their relationship to morphogenesis has not been clarified; even it has been suggested to be due to physiological changes underlying the shift in growth conditions and not to the morphogenetic process itself (Swoboda *et al.*, 1994). We have found down-regulation of five genes belonging to the glucose transporter *HGT1* (Walker *et al.*, 2009), *HGT2* (Fan *et al.*, 2002), *HGT6* (Fan *et al.*, 2002), *HGT7* and *HGT8* (Fan *et al.*, 2002) at 60 min (Fig. 3.18).

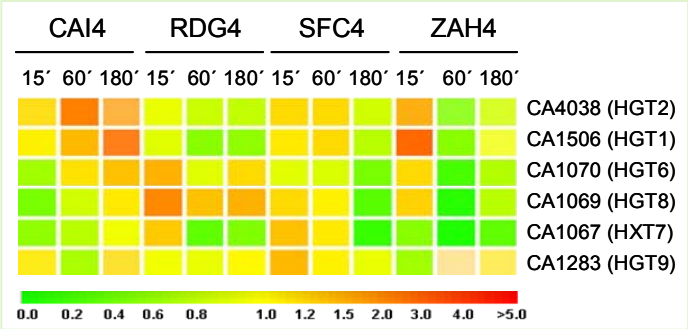

Figure 3.18 Cluster analysis of genes inolved in glucose transporter in the CAI4, RDG4 (*gcn5Δ*), SFC4 (*sin3Δ*), ZAH4 (*gcn5Δ, sin3Δ*) strains during the yeast-hypha transition.

Cell polarity and signal transduction: A number of genes involved in cell polarity and signal transduction e.g. *AIP2, RAC1, RAS1, RGS2, RHO3* and *RSR1* were down-regulated at 60 min (Supplementary table 3.4). Among these, *CDC42* (Bassilana *et al.*, 2005; Mirbod *et al.*, 1997; Ushinsky *et al.*, 2002), *GPR1* (Maidan *et al.*, 2005; Miwa *et al.*, 2004; Prigneau *et al.*, 2003), *RAC1* (Bassilana & Arkowitz, 2006) and *RSR1* (Bassilana *et al.*, 2003; Yaar *et al.*, 1997) has been described to be required for wild-type hyphal growth.

The down-regulation of three components of the secratory pathway IPF1022, *SEC24* possible role in ER to Golgi transport and induced upon yeast-hyphal switch (Nantel *et al.*, 2002) and *SEC61,* similar to *S. cerevisiae* Sec61p act in protein translocation from the endoplasmic reticulum (de la Rosa *et al.*, 2004; Ruiz *et al.*, 2001) were detected at 15 and 60 min of incubation. Down-regulation this set of genes has been reported in the *gcn5Δ, hda1Δ* double mutant during one time of hypha transition. None of them were regulated in the *sin3Δ* and *gcn5Δ* simple mutants. Transcription level genes including *GPA2, GPR1, CA2348, RAC1, RGS2, AIP2, RHO3, RAS1* and *RSR1* have been reported that increased in the CAI4 strain and decrease in the *gcn5Δ* and *sin3Δ* simple mutants during hypha transition (Fig. 3.19).This result suggesting that Gcn5p and Sin3p have a role in the regulation of these genes. It appears that the physiological reprogramming by loss of Gcn5p and Sin3p during hypha transition involves in pathways that decreases transcription of cell polarity and signal transduction and secretory pathway genes, although we do not presently know the functional reasons for this phenomenon.

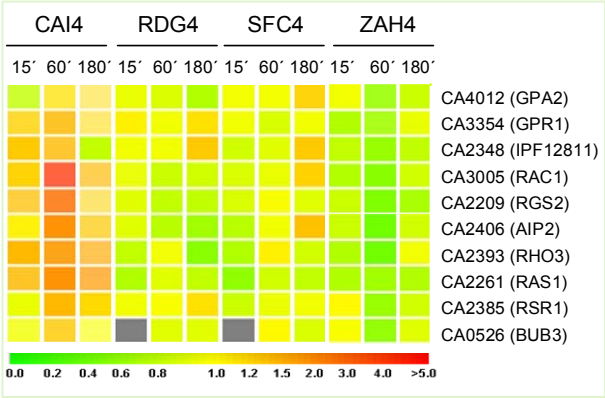

Figure 3.19 Cluster analysis of genes involved in signal transduction in the CAI4, RDG4 (*gcn5Δ*), SFC4 (*sin3Δ*) and ZAH4 (*gcn5Δ, sin3Δ*) strains during the yeast-hypha transition

Vitamin biosynthesis: We have detected the down-regulation five genes involved in vitamin biosynthesis -*BIO2* (Bruno & Mitchell, 2005), *BIO3* (Maglott *et al.*, 2007), *BIO4* (Maglott *et al.*, 2007), *THI4* (Braun *et al.*, 2005; Zhao *et al.*, 2005a) and *THI20* (Braun *et al.*, 2005)- at 60 min, one of them that is fungal specific,*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 (Muller *et al.*, 1999). The down-regulation of *BIO2* at 180 min and two genes involved in thiamine biosynthesis (*THI4* and *THI13*) at one and two times of incubation in the *gcn5Δ* and *sin3*Δ simple mutants has been described suggested role of Gcn5p and Sin3p in regulation of genes involved in vitamin biosynthesis. Expression of three genes involved in thiamine biosynthesis -*THI4*, *THI6*, and *THI13*- has been reported to be up-regulated in the CAI4 hyphal growth (Rashki, Ph.D thesis 2009).

Set of genes regulated only at one incubation time: After 15 and 60 minutes of incubation at 37°C, several sets of genes were either up- or down-regulated. The main groups of up-regulated genes (at 15 min) included four genes contributing to oxidative stress response (*GAD1, GRX3*, *SOD1* and *TSA1*) and *CTA1* (Lan *et al.*, 2004; Niewerth *et al.*, 2003), *MCR1* (Bensen *et al.*, 2004), *NAD1* (Karababa *et al.*, 2004; Wang *et al.*, 2006), *SOD2* (Bensen *et al.*, 2004; Enjalbert *et al.*, 2006) and *TRX1* at 15 and 60 min. Other up-regulated genes at 15 min were four genes involved in biosynthesis of glutamate, aminoadipic acid pathway (*LYS2, LYS9, LYS21* and *LYS22*) (Fig. 3.20), four genes of the F_1F_0 ATPase complex (*ATP1, ATP5*, *ENA2* and *VMA2*) and five genes of proteasome subunits (*PR26, PRE1, PRE4, PRE5* and *PRE6*). The genes up-regulated at 60 min were five genes involved in peroxisomal assembly (*PER3, PEX3, PEX10, PEX17* and *SPS20*), eight genes of mitochondrial (*ALD4, ISA2, MDM12, MTF1, NAM2, NUC1, TIM9* and *TIM10*) (Fig. 3.20).

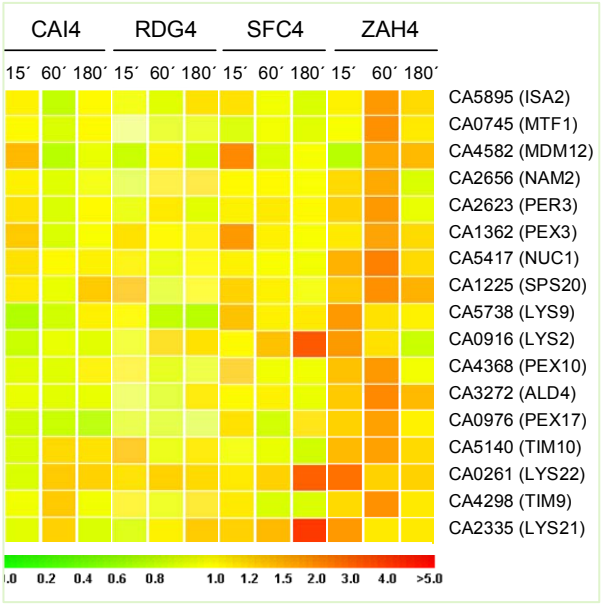

Figure 3.20 Cluster analysis of gene expression in the CAI4, RDG4 (*gcn5Δ*), SFC4 (*sin3Δ*) and ZAH4 (*gcn5Δ, sin3Δ*) strains during the yeast-hypha transition.

Among the down-regulated genes after 60 min, we noted six genes corresponding to ergosterol biosynthesis (*ERG1, ERG3, ERG4, ERG10, ERG11* and *ERG13*) (Fig. 3.21). The down-regulation most of them have been reported in the *gcn5Δ, hda1Δ* double mutant and CAI4 strain during the yeast-hypha transition (Rashki, Ph.D thesis 2009) together with this result suggested a change in membrane composition during hypha formation.

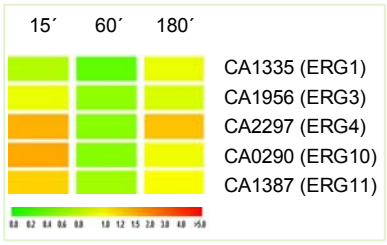

Figure 3.21 Differential expression of the genes involved in ergosterol biosynthesis

Clustering analysis these sets of genes in the double, both simple mutants and CAI4 strain during hypha transition (Fig. 3.22) was showed that transcription level genes including *SOD1, SOD2, TRX1, PRE4, GAD1, CTA1* and *MCR1* are decreased in the CAI4 (at 15 and 60 min) and increased in both simple and double mutants indicate role of *GCN5* and *SIN3* in regulation genes involved in oxidative stress. As sowed in Fig. 3.22 transcription two genes involved in sodium transport, *ENA21* and *ENA22* are increased in the *gcn5Δ* simple, *gcn5Δ*, *sin3Δ* double mutant and CAI4 strain and decreased in the *sin3Δ* mutant during the hypha transition suggested role of Sin3p in the regulation of sodium transporter.

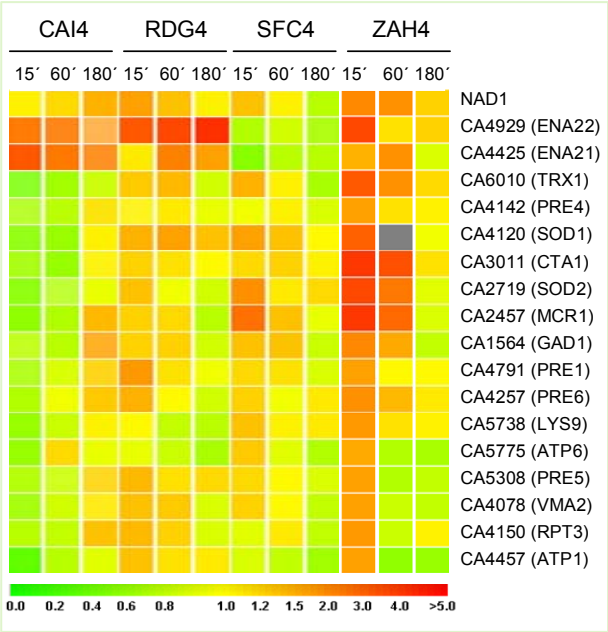

Figure 3.22 Cluster analysis of genes involved in oxidative stress and intracelular transporter in the CAI4, RDG4 (*gcn5Δ*), SFC4 (*sin3Δ*) and ZAH4 (*gcn5Δ, sin3Δ*) strains during the yeast-hypha transition.

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 a component of histone deacetylase (*SIN3*) by analyzing the expression of genes associated with virulence and pathogenicity in *C. albicans*, such as those involved in the yeast-hypha transition. In our laboratory the *GCN5* and *SIN3*, were deleted in a CAI4 background strain. In this work, we deleted the *SIN3* gene in a *gcn5Δ* mutant background strain. Deletions of *GCN5* and *SIN3* 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 (Degano, Ph.D thesis in process; Monterola, Ph.D thesis 2002). During the yeast-hypha transition, both proteins seem to suppress transcription of genes that directly or indirectly control hypha switch, which seems to be operative in both liquid and solid media assayed. On the other hand Gcn5p and Sin3p are positively required for maintenance of the yeast growth form. In the absence of Gcn5p, cells change to a pseudohypha form (Degano, Ph.D thesis in process) and loss of Sin3p caused cells change to a cigarette-like form (Monterola, Ph.D thesis 2002). Furthermore, Gcn5p and Sin3p are required for the competence of yeast cells to undergo hyphal morphogenesis in standard (aerobic) conditions. The functions of *GCN5* and *SIN3* on the known filamentation pathways in *C. albicans* are as yet unknown. In present work, we show

that deletion of *C. albicans SIN3* in a *gcn5Δ* background strain compensate growth defect of the *gcn5Δ* during exponential growth in rich liquid medium at 28°C. Morphologically, like both simple mutants, double mutant is very different to CAI4 strain and was showed an intermediate phenotype of the *gcn5Δ* and *sin3Δ*. This result suggested that in the standard condition, both proteins control independently expression of different set of genes involved in morphogenesis. The main phenotype of the double mutant is the inability to switch from the yeast to the filamentous form in liquid medium containing serum or at 37°C and in all the solid media assayed. The results also demonstrating that the *gcn5Δ, sin3Δ* double mutant as well as both simple mutants completely abolished chlamydospore formation. Recent studies have been shown that in *C. albicans*, genes like *CYP56, ISW2, MDS3, RIM101, RIM13, SCH9* and *SUV3* are required for efficient chlamydospore formation. Our results indicate that either deletion any of both genes mimic the effect described previously (Melo *et al.*, 2008; Nobile *et al.*, 2003). In the double mutant a total of 558 genes, out of 6039 present in the microarray, changed in a minimal medium (YNB) at 28°C during the yeast mode of growth. During the yeast growth form a number of genes involved in DNA synthesis and replication was up-regulated. One of them -*RFA1*- overlapped between the double and *sin3Δ* simple mutant and recently has been implicated in cell cycle progression (Niu *et al.*, 2008). It has been described that over-expression of *RFA1* delayed the cell cycle at G2/M phases by disrupting spindle attachment to chromosomes and activating the DNA damage checkpoint (Niu *et al.*, 2008). Morphologically the double and *sin3Δ* simple mutants were showed a cigarette shape phenotype suggested Sin3p control cell shape through regulation of *RFA1* in *C. albicans*. None of genes involved in the DNA synthesis and replication category (except of *RFA1* in *sin3Δ*) appeared regulated in any *gcn5Δ* or *sin3Δ* simple mutants. This result suggested that this group of genes is regulated jointly by Gcn5p and Sin3p under standard condition. Upon comparing our results concerning oxidative stress genes with those described in the *sin3Δ* simple mutant during the yeast growth form, we observed up-regulation genes involved in the oxidative stress response (Table 3.3) and some of them have been regulated in the *sin3Δ* simple mutant consistent with the phenotype of both strains (*sin3Δ* and double mutant) in a medium containing hydrogen peroxide (H_2O_2). Furthermore, the up-regulation of genes involved in DNA polymerase and DNA repair including *CDC21* (Liu *et al.*, 2005), *DPB2* (Backen *et al.*, 2000), *RAD51* (Liu *et al.*, 2005) and *RAD6* (Leng *et al.*, 2000) maybe play a role in DNA repair, by bypassing DNA damage caused by hydroxyl radicals (OH), are also in agreement with resistance phenotype of the double and *sin3Δ* simple mutants to hydrogen peroxide

(H_2O_2). Loss of *GCN5* and *SIN3* results in the up-regulation of numerous genes involved in glycolysis and TCA cycle. The metabolism of glucose to pyruvate during glycolysis produces energy in the form of ATP (Pile *et al.*, 2002). Of the ten enzymes in the glycolytic pathway, four are transcriptionally up-regulated in the double mutant. In addition, the expression of genes involved in tricarboxylic-acid cycle also was up-regulated (Table 3.3) indicate a metabolic change in the double mutant. Several authors have been described the up-regulation genes involved in citric acid and glyoxylate cycles under many different conditions, for example in the APSES mutants and *Drosophila sin3Δ* mutant cells (Doedt *et al.*, 2004; Pile *et al.*, 2003). Deletion of *GCN5* and *SIN3* also results in the up-regulation a number gene involved in fatty acid oxidation (*ECI1* and *POX1-3*). Thus, under standard conditions *GCN5* and *SIN3* jointly could act as transcriptional regulators of genes playing roles in glycolysis, fatty acid oxidation and the citric acid cycle. We found at least, that nineteen genes corresponding to well known cell wall proteins were regulated during the yeast mode of growth in the double mutant (Table 3.3) suggesting a cell wall remodelling. Phenotype analysis of the double mutant in medium containing Caffeine, Congo red, NaCl, SDS and Zymolyase confirms that the double mutant cells undergo a change in cell wall composition (Fig. 3.7 and 3.8). Another group of regulated genes included sixteen genes coding for transcription factors (Table 3.3). None of them are regulated in the *gcn5Δ* and *sin3Δ* simple mutants except *RIM101, PPR1* and *LEU3* that have been reported to be up-regulated in the *gcn5Δ* simple mutant (Degano, Ph.D thesis in process). Six of them have been characterized as transcription factors with a zinc cluster DNA-binding motif (Park *et al.*, 2003) and the rest belong to disperse family of regulators that respond to different factors and regulate a variety of genes with different functions. Our results suggest that under standard condition Gcn5p and Sin3p jointly regulate expression a number of transcriptional regulators. During the hypha transition, a total of 1271 genes, out of 6039 present in the microarray, changed due to deletion *GCN5* and *SIN3*, in a minimal medium (Lee) at one of the three incubation times assayed (15, 60 and 180 min). These represent a percentage of 21% of *C. albicans* genome indicating an important role for both genes. 35 genes encoding for transcription factors were regulated during at least one time of the yeast-hypha transition. Of those two genes including *GAT2* and *UME6* were down-regulated at the three times (Table 3.4) and others were regulated at one or two times of incubation (Supplementary table 3.4). *GAT2* similar to *S. cerevisiae* Gat2p, transposon mutation affects filamentous growth (Uhl *et al.*, 2003) and *UME6* is a transcription factor; required for wild-type hyphal extension (Banerjee *et al.*, 2008; Uhl *et al.*, 2003).

Deletion of two genes produces clear defects in hyphal formation and extension. Regulation of *GAT2* and *UME6* has been reported to be decreased in the *gcn5Δ, sin3Δ* simple and in the *gcn5Δ, hda1Δ* double mutant and increased in the CAI4 strain during the hypha transition. Thus, *SIN3* and *GCN5* regulate specifically, in up to now an unknown way, both transcription factors. We have found regulated at least 37 genes corresponding to well characterize cell wall proteins and many genes coding for glucanases, glucosidases, mannosyltransferases and GPI anchored proteins. Some of those genes overlap with both *gcn5Δ* and *sin3Δ* simple mutants at different time points. Different regulation of cell wall genes (e.g. *ALS1, ALS3, ECM33* and *SUN41*) have been described by our group during induction of the morphogenetic switch by temperature in CAI4 strain and some of them were also observed in other double mutant (e.g. *gcn5Δ, hda1Δ*) suggested that the physiological reprogramming by loss of Gcn5p and Sin3p involves in remodelling of cell wall composition. We found *PHR1* and *PGA62* genes to be down-regulated in the *gcn5Δ* simple and double mutants at the three times of induction, while an up-regulation was described in the *sin3Δ* and CAI4 strain in the same conditions. We have detected up- and down-regulation of genes related to the translation apparatus at 60 min. Regulation of this group of genes have not been reported in previous works describing the yeast-hypha transition (Kadosh & Johnson, 2005; Nantel *et al.*, 2002; Singh *et al.*, 2005) although it have been found in both simple mutants. We have detected a decrease in the transcript of genes involved in ergoestreol biosynthesis at 60 min. Ergosterol genes were mainly up-regulated after exposition to antifungal agents (Liu *et al.*, 2005; Moreno *et al.*, 2007) and some of them has been reported to be down-regulated at 15 min of incubation in the *gcn5Δ, hda1Δ* double mutant and CAI4 strain suggesting that both mutations do not affect directly the expression of those genes. We have detected down-regulation genes involved in cell polarity (e.g. *CDC42, GPA2* and *GPR1*) and signal transduction at 60 min of incubation. Several authors have been described changes of genes involved in cell polarity and signal transduction under hypha development and described that expression some of genes like *CDC42* is required for budding and maintenance of hyphal growth (Bassilana *et al.*, 2005; Mirbod *et al.*, 1997; Ushinsky *et al.*, 2002), *GPA2* that regulates filamentous growth (Miwa *et al.*, 2004; Sanchez-Martinez & Perez-Martin, 2002), *GPR1* required for wild-type hyphal growth (Maidan *et al.*, 2005; Miwa *et al.*, 2004; Prigneau *et al.*, 2003). It has been reported that expression most of them to be increased in CAI4 strain and decreased in both simple mutants. Thus our result is in agreement with phenotype of double mutant under the morphogenetic switch. No previous data have been reported for the other sets of genes described in this Chapter;

namely, those coding for vitamin biosynthesis, genes of the proteasome subunits, genes involved in peroxisomal and for the F_1F_0 ATPase complex.

Introducción

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 específica ante un cambio de las condiciones del medio (Berman, 2004). El hecho de que los hongos sean organismos eucariotas con un desarrollo rápido y que resulten más fáciles de manipular que los organismos pluricelulares, ha estimulado la utilización de algunas especies de levaduras como organismos modelo en estudios genéticos y de biología molecular. Especialmente este es el caso de *Saccharomyces cerevisiae*, sin embargo, esta especie no es patógena, por lo que no resulta útil a la hora de analizar mecanismos de virulencia, ni presenta la plasticidad morfológica característica de otras especies fúngicas. Un hongo que resulta de gran utilidad como modelo para analizar ambos tipos de procesos es *Candida albicans.*

Candida albicans

Se trata de un patógeno fúngico oportunista de alta incidencia en humanos, sobre el que es necesario realizar nuevos trabajos de investigación, con el fin de desarrollar tratamientos terapéuticos eficaces. Estos conocimientos, además pueden ayudar a comprender la biología molecular de otros patógenos fúngicos más virulentos cuyo manejo en laboratorio resulta complicado. *C. albicans* es capaz de crecer adoptando diferentes morfologías, según las condiciones del medio, por lo que resulta útil como modelo en estudios de diferenciación celular. Tradicionalmente se ha clasificado a *C. albicans* como un hongo dimórfico por su capacidad de intercambiar la morfología de hifa y la de levadura. Al igual que otras especies englobadas dentro de este grupo es capaz de adoptar más de dos morfologías, por lo que debería considerarse en realidad como un hongo polimórfico. En España los datos obtenidos entre 1990 y 1999 indican que los agentes fúngicos provocan entre el 4.5% y el 7% de las infecciones totales, siendo *C. albicans* el principal patógeno fúngico. Esta especie se encuentra entre los 10 microorganismos más frecuentes del total de aislamientos tanto de infecciones comunitarias como nosocomiales (estudio EPINE**,** proyecto sobre la “Evolución de la Prevalecía de las infecciones nosocomiales en los hospitales españoles”, 1990-1999). Según un estudio publicado recientemente, la tasa de mortalidad asociada a candidemias sistémicas en España se encuentra en 40.6%, valor similar al estimado en otros países (Peman *et al.*, 2005; Peman, 2005). En todos los estudios realizados se ha puesto de manifiesto que aunque la mayor parte de las candidiasis se deben a *C.*

albicans, en los últimos años se ha detectado un aumento en la incidencia de otras especies del género, especialmente *C. parasiliensis* y *C. krusei* (Ruiz, 2004). Estos datos son importantes dado que la especie que se ha asociado con mayor mortalidad y que presenta mayor resistencia a los antifúngicos de elección, los azoles, es *C. krusei* (60% en seguimientos realizados en España) (Peman *et al.*, 2005).

Características generales y 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 se 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. Siguiendo recientes clasificaciones (Calderone, 2002): el género *Candida* se encuadra dentro del Reino Fungi, Phylum *Ascomycota*, Clase *Ascomycetes*, Orden *Saccharomycetales*, Familia *Candidaceae***.** La comparación de secuencias de diferentes genes indican que filogenéticamente el grupo más próximo evolutivamente es el de los ascomicetos. Respecto a *C. albicans*, en los últimos años se han publicado diferentes trabajos sobre la posible existencia de un ciclo sexual en esta levadura (Hull & Johnson, 1999; Hull *et al.*, 2000; Magee & Magee, 2000; Magee & Magee, 2004; Soll *et al.*, 2003). Las características más destacadas 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 ADN (Odds, 1988).

Características de las diferentes morfologías

C. albicans es un organismo polimórfico, ya que es capaz de crecer en forma de hifa, levadura, pseudohifa o dar lugar a la formación de clamidosporas (Fig. 1.2). Las levaduras o blastosporas tienen forma elipsoidal con un diámetro aproximado de 5 μm, y se dividen por gemación. 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 (Odds, 1988; Sudbery *et al.*, 2004). Las clamidosporas son esporas asexuales con una pared gruesa que se forman mediante

el recubrimiento de las células preexistentes, tienen forma redondeada 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 (Fig. 1.2). *In vivo* rara vez se detectan estas estructuras (Cole *et al.*, 1991). En el laboratorio su formación se induce en medios sólidos pobres en nutrientes, suplementados con detergentes, en condiciones microaerófilas y a temperaturas entre 20°C y 30°C, mientras que su formación se reprime en presencia de glucosa (Calderone, 2002; Nobile *et al.*, 2003).

El genoma de *Candida 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 ADN 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* tiene una longitud de aproximadamente 16 Mb, siendo por tanto un 33% mayor que el de *S. cerevisiae*. 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*. El ADN mitocondrial de esta especie ha sido también secuenciado, estimándose un tamaño total de 40420 pares de bases (Número de accesión GeneBank: AF285261). A partir del ensamblaje 19 varias organizaciones llevaron a cabo una anotación de las posibles fases de lectura abierta (ORFs) presentes en su genoma. Se identificaron 6244 ORFs. Se ha propuesto la función de algunos de esos genes basándose en un elevado porcentaje de identidad con genes previamente caracterizados de *S. cerevisiae*, que corresponderían a sus homólogos funcionales.

Relación entre morfología y ciclo celular

El estudio detallado de las características de las diferentes morfologías de *C. albicans* ha permitido determinar que la organización del ciclo celular de las hifas difiere sustancialmente del de pseudohifas y levaduras. Tanto en levaduras como en pseudohifas, antes de que aparezca la yema se forma un anillo de septinas en el sitio de gemación, marcando la posición del cuello entre la célula madre y la célula hija. Durante la mitosis, la división nuclear ocurre en el plano delimitado por este anillo, y una vez completada el anillo se divide en dos, formándose entre ambos el septo primario. En ambas morfologías la división celular supone la aparición y constricción de

un anillo de actomiosina. El ciclo celular de pseudohifas difiere del de levaduras en la mayor duración de la fase G2, lo que permite una mayor elongación de la yema, y en el mantenimiento de la unión celular tras la formación del septo (Sudbery, 2001). En el desarrollo inicial de las hifas se forma una banda basal de septinas que se dispone de forma longitudinal al tubo germinativo (Sudbery *et al.*, 2004; Sudbery, 2001). A diferencia de los anillos de septinas de los septos, la banda basal no contiene la septina CaCdc3p y en su formación no interviene CaGin4p (Wightman *et al.*, 2004). El núcleo de la célula madre migra hacia el interior del tubo y es allí donde se divide (Gow *et al.*, 1986; Warenda & Konopka, 2002). A lo largo de la hifa aparecen sucesivos anillos de septinas, organizando los septos de separación celular. A diferencia de lo que ocurre en levaduras, en las hifas los anillos de septinas no se desensamblan en cada ciclo (Sudbery *et al.*, 2004)(Fig. 1.3). Se ha observado que las levaduras de *C. albicans* pueden seguir un patrón de gemación bipolar, con la yema surgiendo en el polo opuesto a la anterior célula hija, o axial, con la yema formándose junto a la cicatriz anterior. Estos patrones están influenciados por la temperatura, por debajo de 25°C hay mayor proporción de células con patrón axial y a altas temperaturas hay mayor proporción de células con gemación bipolar (Berman & Gow, 2004; Chaffin, 1984; Gale *et al.*, 2001; Herrero *et al.*, 1999a). Durante el crecimiento como pseudohifas, cada célula del filamento continúa su ciclo y desarrolla un patrón de gemación unipolar/axial. La célula hija gema en el polo opuesto al de su punto de nacimiento por lo que se forma una estructura ramificada. Durante los primeros ciclos se mantiene la división sincrónica entre las células del filamento (Gimeno *et al.*, 1992; Sudbery *et al.*, 2004; Yokoyama & Takeo, 1983). El crecimiento en forma de hifa tiende a ser por tanto más linear y los patrones de ramificación tienden a ser menos regulares que los de pseudohifas (Barelle *et al.*, 2003; Gow, 1997; Sudbery *et al.*, 2004; Whiteway & Oberholzer, 2004).

Tipos de patologías causadas por *C. albicans*

C. albicans se encuentra como comensal inocuo en la cavidad oral y los tractos digestivo y genital de humanos y otros mamíferos. Habitualmente se encuentra en equilibrio con la microbiota normal y con el sistema inmune del hospedador. Aunque altamente adaptada como organismo comensal, esta especie presenta un gran número de elementos virulentos que ante determinados estímulos le permiten colonizar y producir daños directos en el hospedador (Hube & Naglik, 2001). Las patologías que *C. albicans* puede causar se engloban dentro de tres tipos: cutáneas, mucosas y sistémicas. La candidiasis cutánea se desarrolla generalmente a partir de una

alteración de la barrera de la piel. Existen varios tipos, el intertrigo, la onicomicosis (infecciones de las uñas y el tejido circundante) y el granuloma candidiásico (Martinez Roig, 2003). Las candidiasis mucosas pueden afectar a la mucosa oral (muguetis bucal), a la esofágica (esofagitis candidiásica), a la intestinal o a la genital. Las infecciones sistémicas son las más peligrosas, se producen por invasión de uno o varios órganos internos, afectando fundamentalmente a individuos inmunocomprometidos.

Factores de virulencia de *Candida albicans*

El espectro de afecciones causadas por *C. albicans* es más amplio que el de la mayoría de patógenos oportunistas. Esto es atribuible a la enorme plasticidad de su fenotipo que le permite sobrevivir en diferentes tejidos del hospedador (Calderone & Fonzi, 2001). Nuestros conocimientos sobre la biología de *C. albicans* como comensal son muy reducidos, tan sólo se ha descrito que pequeñas variaciones en el sistema inmune del hospedador son capaces de alterar 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 (Naglik *et al.*, 2003).

Rutas de señalización celular

La supervivencia de *C. albicans* en el cuerpo humano, se comporte como comensal o como patógeno, depende no sólo de su capacidad de respuesta, sino también de su capacidad para detectar y transmitir los estímulos del ambiente exterior. Las rutas de señalización celular son uno de los mecanismos celulares más conservados en todos los organismos (Lengeler K.B., 2000; Lengeler *et al.*, 2000). Están formadas por una sucesión de proteínas que se van activando unas a otras en cascada. Una característica importante es la regulación transversal entre ellas para asegurar que no son activadas por la señal errónea, ya que algunas de las proteínas. El cambio entre la forma de crecimiento de levadura y la de hifa puede considerarse una respuesta adaptativa por la que determinados hongos ajuntan su morfología a una nueva situación ambiental. Se desarrolla por la sucesión de una serie de procesos

encadenados: percepción de un condicionante externo, traducción de la señal en un mensaje bioquímico, expresión génica diferencial y desarrollo de un patrón de crecimiento polarizado (Chio, 2004)(Fig. 1.5).

Rutas de transducción de señales de la transición dimórfica

Existen múltiples rutas de señalización en *C. albicans* que regulan la transición levadura-hifa. Entre las que destacan 4 vías que inducen la formación de hifas: la ruta de proteínas quinasas activada por mitógeno vía CaCph1p (ruta MAPK), la ruta de la proteína quinasa A dependiente de cAMP mediada por CaEfg1p (ruta PKA-cAMP), la ruta de respuesta a pH a través de CaRim101p y la ruta de respuesta a una matriz extracelular mediada por CaCzf1p. Existen además varias vías represoras de la filamentación que actúan a través de CaTup1p o bien a través de CaRbf1p (Fig. 1.6).

- Ruta de proteínas quinasas activada por mitógeno (ruta MAPK)

En *S. cerevisiae* la ruta MAPK (mitogen activated protein kinases) activada en respuesta a feromonas, esta implicada en el proceso de conjugación, en el desarrollo de pseudohifas y en el crecimiento invasivo. En *C. albicans* se han identificado proteínas homólogas a las que conforman esta ruta en *S. cerevisiae* y se ha comprobado que están relacionadas con filamentación bajo condiciones de limitación de nutrientes (Ernst, 2000b).

- Ruta de la proteína quinasa A dependiente de cAMP (ruta PKA-cAMP)

La ruta PKA-cAMP juega un papel crucial en el control de la filamentación en *C. albicans, S. cerevisiae* y otros hongos. En *C. albicans* la actividad de esta ruta es necesaria para la filamentación bajo múltiples estímulos, como la carencia de nutrientes o la presencia de inductores químicos como la NAcGlc o el suero (Chio, 2004 ; Ernst, 2000b).

- Ruta de inducción por pH

En *C. albicans* el cambio de pH ácido a neutro induce la formación de hifas a 37°C. Este organismo posee una ruta de respuesta a pH conservada con respecto a otros hongos. Al igual que en *S. cerevisiae* y *Aspergillus nidulans,* el factor de transcripción CaRim101p (CaPrr2p) regula la expresión de genes de respuesta a pH, al ser activado por proteolisis dependiente de la proteínas CaRim8p (CaPrr1p) y CaRim20p (CaEnx3p) (Calderone, 2002; Davis, 2003; Ernst, 2000b; Liu, 2001).

- Otras rutas inductoras de la filamentación

La capacidad de *C. albicans* de filamentar cuando crece dentro de una matriz sólida se considera una respuesta que facilita la penetración del hongo en los tejidos del hospedador. Se ha identificado una ruta que induce la formación de hifas en estas condiciones, en la que participa la proteína CaCzf1p. Esta proteína contiene un dominio "Zinc finger", generalmente implicado en la interacción con secuencias de ADN. El gen *CaCZF1* no es necesario para la respuesta a suero, pH, NAcGlc, prolina o medio Spider pero si es necesario para la correcta filamentación en medio embedded, por lo que se ha relacionado con la respuesta al estrés mecánico provocado por el crecimiento dentro del agar (Brown *et al.*, 1999) y con la respuesta a una baja concentración de oxígeno que viene provocada por esta forma de crecimiento (Ernst, 2000a).

- Regulación negativa de la filamentación

CaTUP1 codifica un factor transcripcional determinante en el mantenimiento del crecimiento en forma de levadura. La deleción de *CaTUP1* provoca crecimiento en forma de pseudohifas en todos los medios ensayados, tanto en condiciones inductoras como no inductoras de la miceliación (Braun & Johnson, 1997). En *S. cerevisiae*, la proteína ScTup1p interacciona de forma indirecta con los promotores de determinados genes, a través de su unión a diferentes proteínas de unión a ADN (Smith & Johnson, 2000). Una de las proteínas de *S. cerevisiae* capaces de interaccionar con ScTup1p es ScNrg1p, que es un factor transcripcional con un dominio de "Zinc finger" de unión a ADN, que dirige el complejo ScSsn6p-ScTup1p a determinados genes diana. En *C. albicans* se ha demostrado que la transcripción de *CaNRG1* es menor durante la formación de las hifas en suero a 37°C (Braun *et al.*, 2001; Liu, 2001) y que la inactivación de esta proteína causa crecimiento filamentoso e invasivo en condiciones no inductoras. A diferencia del mutante *Catup1*, el mutante *Canrg1* es capaz de formar hifas auténticas en condiciones de inducción (Murad *et al.*, 2001b). La asociación con CaTup1p parece clara, ya que se ha demostrado que CaNrg1p reprime la expresión de genes relacionados con virulencia y morfogénesis que son también reprimidos por CaTup1p y que la mayoría de ellos contienen en su promotor un elemento NRE (Nrg1 response element) (Murad *et al.*, 2001b).

Papel de los procesos de acetilación-desacetilación de histonas en la regulación transcripcional

Las cuatro histonas (H2A, H2B, H3 y H4) que constituyen los nucleosomas poseen un dominio globular y uno o dos dominios amino-terminales flexibles, que junto con la región carboxilo terminal de la histona H2A, median las interacciones internucleosomales, favoreciendo la compactación del ADN (Wolffe & Guschin, 2000). En los dominios terminales de las histonas se encuentran residuos de lisinas susceptibles de ser acetilados de forma reversible. Estos aminoácidos se encuentran en posiciones muy conservadas en todos los organismos eucariotas, especialmente en las histonas H3 y H4. El estado de acetilación es regulado por la acción opuesta de dos tipos de enzimas, las histonas acetiltransferasas (HATs) y las histonas desacetilasas (HDACs). Actualmente se sabe que algunas de estas enzimas son capaces de actuar sobre otras proteínas diferentes de las histonas (Yang & Gregoire, 2005). Se ha sugerido que los grupos acetilo (cargados negativamente) contrarrestan la carga positiva de los residuos de lisina y por tanto disminuyen la interacción de las histonas con el ADN (cargado negativamente) del propio nucleosoma y de nucleosomas próximos. De esta forma se facilita el acceso de la maquinaria de transcripción general y de proteínas reguladoras. Siguiendo este modelo, la desacetilación tendría el efecto contrario y ya que al aumentar el grado de empaquetamiento se dificultaría la transcripción (Fig. 1.8) (Kurdistani & Grunstein, 2003). De forma alternativa se ha sugerido que el efecto de la acetilación sobre la transcripción podría deberse, no a modificaciones de la estructura de la cromatina, sino a que los residuos acetilados proporcionan superficies de anclaje apropiadas para determinados componentes de la maquinaria de transcripción u otros reguladores de la expresión génica (Kurdistani & Grunstein, 2003). Las histonas acetiltransferasas (HATs) y las histonas desacetilasas (HDACs) actúan junto a otras proteínas formando complejos multiproteicos. Se han detectado interacciones de algunos de estos complejos con proteínas de unión a ADN que dirigen las HDACs y las HATs a determinadas secuencias de los promotores, actuando sobre los nucleosomas adyacentes al sitio de unión. Este modo de acción se ha denominado acetilación-desacetilación específica (Kurdistani & Grunstein, 2003; Vogelauer *et al.*, 2000). Los enzimas histonas acetiltransferasas y también las histonas desacetilasas, *in vivo* forman parte de complejos multiproteicos de elevada masa molecular. Los complejos HAT se han clasificado en dos tipos A y B en función de su localización subcelular y de su habilidad para modificar o no las histonas. Las HATs tipo A son enzimas nucleares capaces de acetilar histonas ensambladas en cromatina.

Los complejos HAT tipo B se han descrito como complejos de localización preferente en el citoplasma que únicamente acetilan histonas libres, probablemente para su posterior deposición en cromatina.

Características del gen *GCN5*

El co-activador transcripcional *GCN5* (general control nonrepressed) se ha descrito como HAT de tipo B, una histona acetiltransferasa por su similitud con la proteína p55, un activador HAT nuclear aislado de *Tetrahymena* (Georgakopoulos, 1992). Estudios genéticos sobre *GCN5* habían puesto de manifiesto su función en la regulación transcripcional de algunos genes, por tanto, el descubrimiento de la capacidad de acetilar histonas de esta proteína supuso un nexo importante entre la acetilación de histonas y la regulación transcripcional. La lisina 14 de H3 es una de las principales dianas de acetilación de Gcn5p (Kuo *et al.*, 1996; Kuo & S. Y. y Allis, 1996), aunque su especificidad varía dependiendo de las proteínas que le acompañen. Hasta el momento se ha identificado que Gcn5p es la subunidad catalítica de al menos tres complejos tipo A diferentes de levadura: ADA (0.8 MDa), SAGA (1.8 MDa) y HAT-A2 (180 kDa) (Carrozza, 2003). Gcn5 forma la subunidad catalitica junto a Ada2 y Ada3. Gcn5 libre es capaz de acetilar histonas libres preferemente en la lisina 14 de H3, mientras que en el complejo Ada, acetila las lisinas 9, 14 y 18 de esa misma histona (Grant *et al.*, 1997).

Características de los genes *HAT1* y *HAT2*

La proteína Hat1p de levadura fue la primera proteína de tipo HAT identificada y fue descrita como HAT de tipo B. Las proteínas HATs de tipo B usan histonas recién sintetizadas como sustratos; esas histonas son acetiladas antes de ser transportadas al núcleo. Hat1p se purifica por tanto en las fracciones citoplasmáticas junto con Hat2p, un homólogo de RbAp48 (una proteína asociada a Rb) que actúa como una posible chaperona de la histona H4 y es también una subunidad del factor 1 de ensamblaje de la cromatina (CAF1), lo que refuerza la idea de la relación entre la actividad acetiltransferasa de Hat1p y el ensamblaje de la cromatina. RbAp48 también interactúa con subunidades catalíticas de enzimas desacetilasas. Esto sugiere que RbAp48 dirige el tetrámero acetilado de histonas H3/H4 hacia las horquillas de replicación del ADN durante el proceso de replicación. Además, los complejos de desacetilación también son dirigidos, probablemente vía interacción con homólogos de RbAp48, hacia los nucleosomas recién ensamblados eliminando grupos acetato (Kuo & Allis, 1998). Así, Hat1p y Hat2p forman un complejo de acetiltransferasas de histonas de tipo B, donde Hat1p es la subunidad catalítica de la enzima que acetila específicamente los residuos

de lisina de las posiciones 5 y 12 de las histona H4 libre y Hat2p es la subunidad regulatoria que aumenta la actividad de Hat1p promoviendo la interacción entre Hat1p y el extremo amino terminal de la histona H4 (Qin & Parthun, 2002). Se ha demostrado que la acetiltransferasa Hat1p participa en el silenciamiento de los telómeros. El silenciamiento de los telómeros implica una represión transcripcional de los genes próximos a estas regiones (los genes subtelomérico) y está mediado por la formación de heterocromatina. Cuando se delecionan los genes *HAT1* o *HAT2* no se observa ningún cambio en el nivel de silenciamiento de los genes subtelomérico pero sí se observa un defecto significativo en el silenciamiento cuando las mutaciones en los genes *HAT1* y *HAT2* se combinan con mutaciones en el extremo amino terminal de la histona H3. En la histona H4, el papel de Hat1p en el silenciamiento subtelomérico está mediado exclusivamente por la lisina 12. Además, en contraste con otras acetiltransferasas la actividad Hat1p es necesaria para la represión transcripcional más que para la activación génica (Kelly *et al*., 2000a). El papel de la proteína Hat1p se ha relacionado con la reparación del ADN debido principalmente a dos observaciones: en primer lugar, algunos mecanismos de reparación de daño en el ADN requieren la síntesis de nuevo ADN, que necesita ser ensamblado en la cromatina y, en segundo lugar, se ha descubierto que algunos factores reguladores de levaduras afectan tanto al silenciamiento de los telómeros como a la reparación del daño en el ADN. Así, se ha demostrado que la proteína Hat1p y residuos específicos de lisina en el extremo amino terminal de la histona H3 están implicados en la reparación del daño en el ADN. Concretamente, se ha visto que la histona H3 y la proteína Hat1p contribuyen a la reparación por recombinación de las roturas en el ADN de doble cadena (Qin & Parthun, 2002).

Características del gen *SIN3*

La proteína Sin3p de diferentes especies constituye la plataforma sobre la cual se ensambla un complejo con actividad histona desacetilasa denominado complejo SIN3/HDAC. En mamíferos, el núcleo del complejo está formado por 8 proteínas mSin3, RbAp46, RbAp48, Sap30, Sap18, Sds3 y las histona desacetilasas HDAC1 y HDAC2. En *S. cerevisiae*, hasta el momento, sólo se ha detectado interacción de ScSin3p con una histona desacetilasa, ScRpd3p, por lo que el complejo se conoce como Sin3/Rpd3. Sin embargo, no se descarta que ScSin3p pueda interaccionar con otras histona desacetilasas (Kadosh & Struhl, 1997; Rundlett *et al*., 1998; Silverstein & Ekwall, 2005). Dentro del complejo de *S. cerevisiae* se aislaron otras proteínas como ScSap30p, ScSds3p, ScPho23p y ScUme6p (Kurdistani & Grunstein, 2003) (Fig. 4.1).

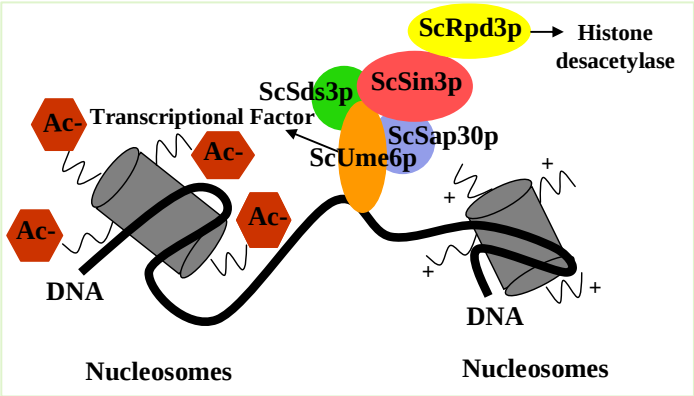

Figura 4.1 Esquema del complejo de Sin3/Rpd3 en *S. cerevisiae*

Se ha observado que la proteína ScSin3p no es necesaria para la actividad histona desacetilasa *in vitro* pero si *in vivo*, probablemente debido al papel de ScSin3p en dirigir a ScRpd3p a determinados promotores (Wu *et al.*, 2001). En nuestro laboratorio por la Dra. Monterola se ha caracterizado el gen de *C. albicans* homólogo al gen *SIN3* de *S. cerevisiae* y se ha analizado su papel en morfogénesis. A 28°C, las células carentes de *sin3*Δ son más alargadas que las de la cepa CAI4 y en todos los medios de inducción de la filamentación ensayados, la cepa delecionada no es capaz de formar auténticas hifas, sólo pseudohifas. En el mismo trabajo fue aislado el gen *SIN3* de otra levadura dimórfica, *Yarrowia lipolytica*, observándose que, al igual que en *C. albicans*, la cepa delecionada no era capaz de formar hifas (Monterola, 2002; Tebarth *et al.*, 2003). La ausencia del gen *SIN3* de *C. albicans* altera la expresión de un factor clave en las rutas de señalización de la transición morfológica, CaEfg1p. En las cepas silvestres de *C. albicans*, el nivel de expresión de *EFG1* disminuye rápidamente en los primeros minutos tras la inducción de la filamentación y a continuación va aumentando progresivamente. En el mutante nulo *sin3Δ* la expresión se mantiene constante, de forma que durante las dos primeras horas de la transición dimórfica el nivel de expresión de *EFG1* es mayor en la cepa delecionada que en la cepa silvestre. Se ha demostrado que CaSin3p es capaz de interaccionar con el promotor del gen *EFG1* y que en la represión de *EFG1* interviene la propia proteína CaEfg1p. Considerando estos datos, junto con la observación de que el fenotipo mostrado por la deleción de *SIN3* es similar al de la sobreexpresión de *EFG1*, se ha propuesto que CaSin3p interviene en la autorregulación negativa de *EFG1* (Monterola, 2002; Tebarth *et al.*, 2003).

Objetivos

La capacidad de *C. albicans* de llevar a cabo la transición levadura-hifa es una de sus características más estudiadas, ya que se ha visto que las hifas están implicadas en procesos de virulencia y que cepas incapaces de llevar a cabo la formación de hifas son avirulentas (Lo *et al.,* 1997; Calderone, 2002). Nos planteamos los siguientes objetivos:

1. Determinar, bajo diferentes condiciones de inducción, si las histonas acetiltransferasas CaHat1p y CaHat2p, en el doble mutante de *C. albicans,* son necesarias para el desarrollo de procesos morfogenéticos, tales como la formación de hifas, pseudohifas y clamidosporas.

2. Determinar el sitio de comienzo del transcrito en los genes *HAT1* y *HAT2*.

3. Determinar la localización subcelular de las proteínas Hat1p y Hat2p.

4. Comprobar si la deleción de los genes *hat1Δ y hat2Δ* en los mutantes simples y en un doble mutante afectan a la virulencia de *C. albicans* en un modelo de infección sistémica de ratón.

5. Analizar el transcriptoma de los mutantes *hat2Δ* y del doble mutante *hat1Δ, hat2Δ* durante el crecimiento levaduriforme y durante en el proceso de transición levadura-hifa.

6. Determinar la participación de CaGcn5p y CaSin3p en el doble mutante ZAH4, en la regulación de la transición levadura-hifa en diferentes condiciones de inducción, así como en la formación de clamidosporas.

7. Analizar el transcriptoma del doble mutante *gcn5Δ, sin3Δ* durante el crecimiento en fase exponencial a 28°C y durante la transición levadura-hifa a 37°C.

8. Comprobar si la deleción de los genes *sin3Δ* como mutante simple y *gcn5Δ, sin3Δ* doble mutante afectan a la virulencia de *C. albicans* en un modelo de infección sistém

Medios de cultivo y condiciones de crecimiento

Medios de cultivo para *C. albicans*

En la Tablas 2.1 y 3.1 se muestran los genotipos más relevantes de las cepas de *C. albicans* utilizadas y obtenidas en el presente trabajo. Los medios de cultivo empleados para el crecimiento de levaduras fueron: medio YEPD y YNB. Para los medios de cultivo sólidos añadiendo agar al 2%. 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 28°C. El crecimiento se determinó midiendo la absorbancia en un espectrofotómetro (Hitachi modelo U-2001) 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 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 37°C, excepto en el caso del medio Embedded en el que la suspensión de células se mezcló con este medio previamente mantenido a 50°C. A continuación se sirvió en placas, se dejaron solidificar y se incubaron a 28°C.

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 y sobre ellas se dispusieron cubreobjetos con el fin de crear condiciones de crecimiento microaerófilas. Las placas se incubaron en oscuridad a 25°C, durante 7-14 días y se tomaron muestras de cada una de ellas. 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 28°C durante 48 horas en el caso de *C. albicans* y a 37°C durante 12 horas en el caso de *E. coli*. Posteriormente, se conservaron a 4°C. Con la finalidad de mantener las células durante largos periodos del tiempo, los microorganismos se almacenaron en viales con glicerol al 30% en ultra congeladores a -80°C.

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).

Medio de cultivo para *E. coli*

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

Detección de secuencias de ADN específicas (Southern-blot)

Una vez digerido el ADN genómico de *C. albicans* (10 μg) con las enzimas de restricción adecuadas, los fragmentos de ADN se separaron, junto a un marcador de peso molecular, mediante electroforesis en geles de agarosa al 1%. El ADN 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 (Hybond-N^{TM} de Amersham Pharmacia Biotech) se dejó hibridando durante aproximadamente 16 horas a 65°C. 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 o IP (Image Plate) que esta recubierta por una capa de fósforo foto-estimulable, durante un período de tiempo que osciló entre 1 y 5 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 ADN que habían hibridado específicamente con la sonda.

RLM-RACE (RNA Ligase Mediated Rapad Amplification of cADN Ends)

El ARN total de *C. albicans* fue mapeado usando el kit “FirstChoice RLM-RACE” de Ambion. El ARN se aisló de la cepa CAI4, creciendo en fase exponencial rompiendo las células en un “micro-dismembrator” (Braun, Melsungen). Después empleamos el método de extracción con trizol descrito en “Galar Fungal standared operating procedures”. El proceso consiste en tratar la muestra de ARN total con un fosfato alcalino (CIP) que elimina los fosfatos 5´ libres de ARN degradado o ADN contaminante. A continuación la estructura cap del ARN de longitud completa es eliminada con la enzima pirofosfatasa ácido del Tabaco (TAP), quedando un grupo monofosfato libre en el extremo 5´. En este momento se liga a este grupo un adaptador (5´GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA 3´)

utilizando la ligasa de ARN T4. Seguidamente se procede a una trascripción reversa y el cADN obtenido es amplificado mediante PCR anidada. El producto de la segunda PCR puede ser clonado en pGEM-T y secuenciado.

Ensayo de sensibilidad

Las células se recogieron en fase exponencial, se prepararon suspensiones a una D.O 600_{nm} de 0.4 y a partir de ellas se realizaron diluciones seriadas de 1/10. Se tomaron 5 µl de cada una de las diluciones y se sembraron en placas de medio YEPD conteniendo diferentes concentraciones de los compuestos ensayadas Anfotericina B, Higromicina; Itraconazol, Flocunazol, NaCl, Calcofluor white, Rojo congo, Cafeína, SDS, KCl, $CaCl_2$ y Zimoliasa.

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 (Herrero *et al.*, 2002). Este ensayo permite detectar defectos 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 1 ml a una D.O de 2.5 por duplicado 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

Una vez obtenidas las cepas, 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 hasta fase exponencial de crecimiento a 28°C con el fin de obtener células en forma de levadura. Las células se recogieron, se lavaron dos veces con PBS 1× y se resuspendieron a una concentración de 10^8 y 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 en 10 ratones CD1 con 10^8 y 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.

Microscopía

La observación de los microorganismos se realizó con un microscopio DMRXA (Leica) y un microscopio Leica DFC 350 FX acoplados a una cámara digital. La morfología de las colonias de *C. albicans* se examinó en un estereomicroscopio ZEISS Stemi SV-6.

Aislamiento de ARN, síntesis de cADN e hibridación

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 -80°C 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). 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 retro transcripción en la cual se incorporan los nucleótidos marcados durante la síntesis de las moléculas de cADN. En cada reacción se utilizaron 15 μg de ARN total a los que se añadió 1 μl del inhibidor de ribonucleasas ARNsin (Promega) y agua destilada tratada con DEPC hasta completar un volumen de 21 μl. Las soluciones de ARN se dejaron en un baño a 42°C 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) (1 μl), Oligo dT (12-18) (Sigma) (0.5 μg/μl) (1 μl), Mezcla de dNTPs excepto dCTP (Sigma) (10 mM) (3μl), 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 ARN se añadió a la mezcla de reactivos y seguidamente se incubó 5 minutos a 65°C y 5 minutos a 42°C. Se añadió 1 μl de transcriptasa reversa (Invitrogen) y se incubó otra hora a 42°C, 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 65°C. La purificación de las moléculas de cADN 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 cADN 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 cADN que se obtiene, la absorbancia de la muestras se midió directamente, sin diluciones, utilizando ultramicrocubetas (Hellma). 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 cADN procedentes de cada una de las dos cepas a comparar (marcada una con Cy3 y otra con Cy5) y 5 μl de ADN de esperma de salmón (Sigma-Aldrich) previamente desnaturalizado por calor. El cADN se desnaturalizó manteniendo la muestra 2 minutos a 95°C 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 ADN y una vez que la muestra se extendió por aproximadamente la mitad del cubreobjetos se dió la vuelta al microarray y se colocó en una cámara de hibridación (Corning), que se sumergió en un baño a 42°C. 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 lavó dos veces con 50 ml SSC 2×, en el último de ellos se mantuvo 5 minutos con agitación ocasional. Por último, el microarray se secó mediante centrifugación a 1100 rpm durante 5 minutos a temperatura ambiente.

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 37°C. Para cada comparación mutante *vs.* CAI4 se han realizado cuatro hibridaciones correspondientes a dos réplicas biológicas, en los que el ARN de la cepa mutante procede de cultivos independientes y dos hibridaciones para cada uno de ellos en los que el cADN se marcó de forma inversa (dye swap). El cADN de la cepa parental, CAI4, se sintetizó en cada condición a partir de una mezcla de ARN 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 (Fig. 4.2).

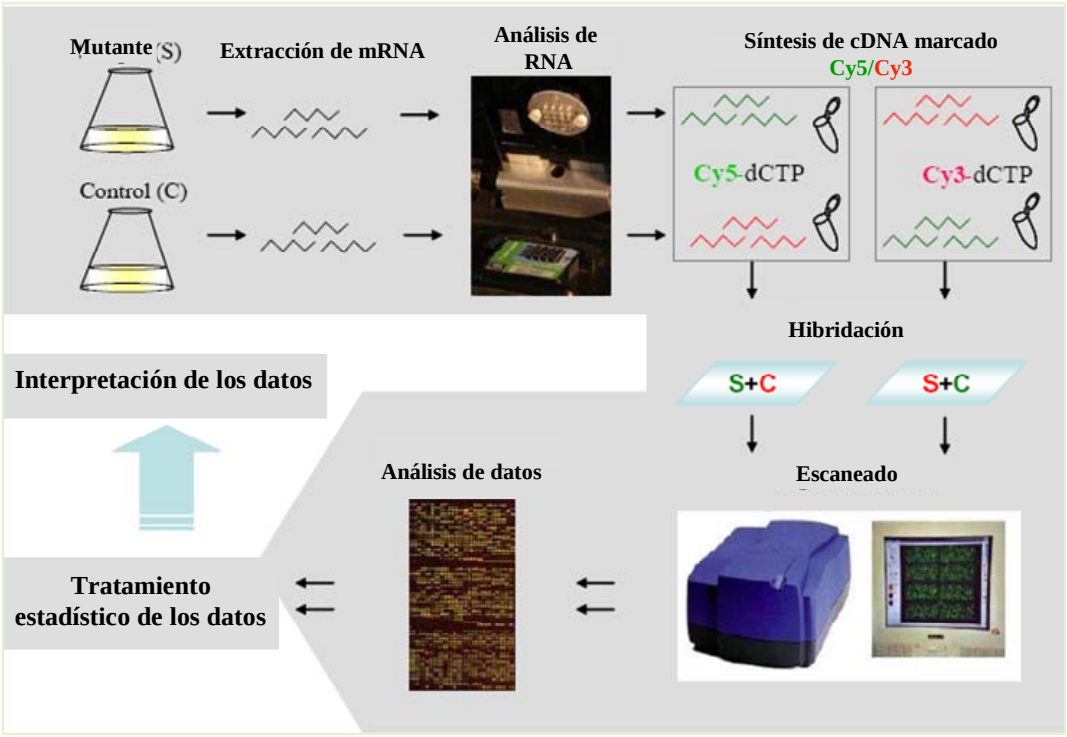

Figura 4.2 Esquema del protocolo seguido para el análisis comparativo del transcriptoma de dos cepas de *C. albicans*, mediante microarray de cADN de vidrio

Análisis de los datos obtenidos

La adquisición de las imágenes correspondientes a cada microarray se realizó en el Servicio de Genómica del Centro de Investigación del Cáncer (USAL-CSIC) con el escáner GenePix4000B y el programa asociado al mismo GenePix Pro 4.0 (Axon Instruments Inc.). Se llevaron a cabo varios escaneados, modificando los valores de fotomultiplicación (PMTs) asociados a cada fluorocromo, hasta obtener una imagen en la que las intensidades medias de ambos canales estuvieran equilibradas (ratio medio

Cy5/Cy3 próximo a 1). El programa GeneSpring 5.0.3 (Agilent technologies, CA) fue utilizado a continuación para normalizar, integrar, realizar análisis de significación estadística y comparar los datos de diferentes microarrays. Los datos se sometieron a una normalización per spot y per chip dependiente de intensidad (Lowess) aplicada a cada uno de los bloques del microarray. Esta normalización permite comparar los datos de los diferentes genes, tanto si proceden del mismo como de diferentes microarrays, y al ser dependiente de intensidad, corrige las variaciones no significativas que dependen del nivel de expresión de cada gen. La detección de genes que se expresan de forma diferencial en el mutante en cada una de las condiciones analizadas se llevo a cabo según el test estadístico de comparación de grupos "Wilcoxon-Mann-Whitney" y la corrección para test múltiples "Benjamini and Hochberg FDR" con un valor de $\rho \leq 0.02$. De esta forma la probabilidad de error es de un 2% del total de los genes que pasan el test. El análisis de significación se realizó sólo sobre los genes que presentaban un factor de variación mínimo de 1.5 en al menos una hibridación, este punto de corte se introdujo para controlar la astringencia de la corrección "Benjamini and Hochberg FDR", que está influenciada directamente por el número de genes (Garcia-Sanchez *et al.*, 2004).

Resultados

Determinación del sitio de inicio de la transcripción de los genes *HTAT1* y *HAT2* por método de RLM-RACE

Para identificar los sitios de inicio de la transcripción del gen *HAT1* realizamos una RLM-RACE. El resultado se muestra en la Fig. 2.1A una banda de ~450 pb, tres sitios putativos de inicio de la transcripción fueron identificadospara. Estos productos pudieron originarse a partir de tres sitios distintos de inicio de la transcripción o quizás las bandas más pequeñas pueden representar la terminación prematura de la reacción de transcripción reversa. Las tres posiciones de 21, 24 y 48 fueron consideradas como el sitio de inicio de la transcripción del gen *HAT1* (Fig. 2.1B). El sitio de inicio de la transcripción del gen *HAT2* aparentemente se encuentra en el interior de la fase codificante descrito por la aproximación “in silico”. Experimentos de secuenciacion de la proteina purificada o análisis de la ORF en otras cepas de *Candida* son necesario para elucidar este punto (Fig. 2.1C).

Interrupción del gen *HAT1* en la cepa RDH4 (*hat2Δ*)

Nuestro grupo, dentro del proyecto de investigación en el que se incluye el presente trabajo, está llevando a cabo el análisis sistemático de la implicación de diferentes histonas acetiltransferasas e histonas desacetilasas en la regulación de la transición levadura-hifa y de otros procesos morfogenéticos. El primer objetivo ha sido la interrupción de los dos alelos del gen *HAT1* en la cepa RDH4 (con el gen *HAT2* delecionada que había sido obtenida previamente en nuestro laboratorio). Ello se llevó a cabo siguiendo la estrategia del “Ura-blaster” (Fonzi & Irwin, 1993). En la Fig. 2.3B se muestra la comprobación del genotipo de las cepas generadas mediante Southern. El ADN genómico fue digerido con *Ssp* I y como sonda se utilizó la secuencia de 459 pb correspondiente a la región 3´ no codificante (promotor) del gen *HAT1.* Tal y como puede apreciarse en la Fig. 2.3B, las cepas CAI4, RDH4 y heterocigotas para la deleción del gen presentan la banda de 0.64 kb correspondiente al alelo silvestre de *HAT1.* Esta banda no aparece en las cepas homocigotas para la deleción de *HAT1* (MAH3 y MAH4) 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 *hat1Δ, hda2Δ* 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 retraso en el crecimiento del doble mutante *hat1Δ,*

hat2Δ en medio YEPD respecto a la cepa parental CAI4. Se observó que en fase exponencial el tiempo de generación de las cepas MAH4 era mayor al de la cepa parental CAI4. Estimamos que en este medio la cepa MAH4 iniciaba la fase exponencial tras aproximadamente 16 horas de crecimiento (Fig. 2.4).

Efecto de la doble deleción *hat1Δ, hat2Δ* en la filamentación en medios sólidos

Hemos analizado el análisis del fenotipo del doble mutante *hat1Δ, hat2Δ* (cepa MAH4), de los dos mutantes simples y de la cepa CAI4 durante crecimiento de forma levadura e hifa. En la Fig. 2.6 se puede apreciar claramente que el doble mutante presenta una morfología hiperfilamentosa como el mutante simple *hat1Δ* en los medios inductores de filamentación. El aspecto de las colonias en el medio sólido YEPD se muestra en la Fig. 2.5A y presenta mayor rugosidad.

Efecto de la doble deleción *hat1Δ, hat2Δ* en la transición levadura-hifa en medio líquido

Después de inducir la transición dimórfica en medio líquido Lee con suero al 4% a 37°C se observó que el mutante doble *hat1Δ, hat2Δ* es incapaz de formar hifas, igual el mutante simple *hat1Δ*. Ambas cepas forman en lugar de hifas, pseudohifas. El doble mutante se comporta así, de diferente forma que el mutante simple *hat2Δ*, ya que en medios líquidos si es incapaz de formar hifas (Fig. 2.7). En la inducción en medio Lee a 37°C observamos el mismo efecto (datos no mostrados).

Efecto de las deleción de los genes *HAT1* y *HAT2* en la formación de clamidosporas

A continuación se analizó el efecto de la deleción de los genes *HAT1* y *HAT2* en la formación de clamidosporas. Las cepas se incubaron en medio cornmeal agar bajo condiciones microaerófilas y a temperatura 25°C, hemos observado que la cepa doble mutante *hat1Δ, hat2Δ* es capaz de formar clamidosporas al igual que ambos mutantes simples (Fig. 2.8).

Ensayo de sensibilidad

Hemos analizado la sensibilidad de estas cepas a los siguientes compuestos: Cafeína, Calcofluor white, NaCl, SDS, Rojo congo, Fluoconazol, Itraconazol, Higromicina y Anfotericina B. Como se puede observar en la Fig. 2.9 se encontraron diferencias de sensibilidad a las concentraciones señaladas de Calcofluor white, NaCl, Cafeína y Rojo congo entre el doble mutante y el mutante *hat1Δ* con respecto a la cepa CAI4. 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.

Para el ensayo de sensibilidad a Zimoliasa, tomamos como referencia estudios previos realizados en nuestro laboratorio (Herrero *et al.*, 1999b). El resultado obtenido se muestra en la Fig. 2.10. Como puede observarse, la caída de la densidad óptica a largo del tiempo es más rápido en el doble mutante y mutante sencillo *hat1Δ*. Este resultado demuestra que el doble mutante *hat1Δ, hat2Δ* y el mutante *hat1Δ* son más sensibles a la Zimoliasa. La mayor sensibilidad del mutante *hat1Δ y hat1Δ*, *hat2Δ*, que la cepa CAI4, frente a la β-1,3 glucanasa (Zimoliasa) sugiere una alteración de la pared celular de estos mutantes.

Ensayo de afinidad a Alcian blue

Uno de los ensayos utilizados para determinar la existencia de posibles alteraciones en pared celular, es el ensayo de "Alcian blue". En la Fig. 2.11 se muestran los valores del colorante fijado por la cepa CAI4 y los mutantes de *C. albicans.* Si tomamos como 100% la cantidad de colorante fijado por las células de la cepa CAI4, las células del doble mutante fijan un 92.36%. El porcentaje de colorante fijado por las cepas RDH4 y RGL4 es de un 118,94% y 48,14% respectivamente. En *C. albicans*, la capacidad de adhesión depende de su pared celular (Sundstrom, 1999). Estudios han revelado que las manoproteínas una vez glicosiladas interaccionan directamente con los células del hospedador es decir, el proceso de glicosilación es un factor importante para la virulencia (Timpel *et al.*, 2000). Durante el proceso de transición levadura-hifa existe un rápida remodelación y expansión de la pared celular; por esta razón, determinadas mutaciones en los genes que intervienen en la formación de la pared celular dan lugar a cepas con defectos en la filamentación (Herrero *et al.*, 2002; Timpel *et al.*, 2000). Además, los genes específicos para la formación 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 levadura-hifa, se vean reflejados en la alteración de algunos componentes de la pared celular.

Virulencia

El ensayo de virulencia se llevó a cabo con las cepas: RGLR6 obtenida tras la reconstrucción de uno de los locus *URA3* de la cepa RGL4 (*hat1Δ*), RDHR9 obtenida de la cepa RDH4 (*hat2Δ*) y MAHR1 obtenida de la cepa MAH4 (*hat1Δ, hat2Δ*). Se inyectó por vía intra-peritoneal a cada uno de los ratones una suspensión de 5×10^8

células en 200 μl de PBS 1×. 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.
La curva de supervivencia se muestra que efectivamente no hay diferencias significativas entre los resultados obtenidos para el mutante simple *hat2Δ* y la cepa CAI4. La deleción del gen *HAT2* no parece causar, por tanto, alteraciones en la virulencia de *C. albicans*, al menos bajo las condiciónes ensayadas.
Tal y como se muestra en la grafica (Fig. 2.12), el resultados obtenidos con las cepas RGLR6 y MAHR1 no es similar al obtenido con la cepa control CAMR. En los mutantes simple *hat1Δ* y doble *hat1Δ, hat2Δ* se muestró una reduccion (40%) y (30%) en la virulencia respectivamente.

Localización subcelular de la proteína CaHat1p y CaHat1p

Para la fusión de ambas proteínas, la ORF de *HAT1* y *HAT2* se amplificaron desde el codón de inicio hasta el codón previo al de terminación mediante PCR usando como molde ADN de la cepa CAI4 y como oligonucleótidos HAT1-GFPF y HAT1-GFPR (la ORF del gen *HAT1*) y HAT2-GFPU y HAT2-GFPR (la ORF del gen *HAT2*). El fragmento de 1260 pb (gen *HAT1*) y 1473 pb (gen *HAT2*) se clonó en el plásmido pAG1 (Gonzalez-Novo *et al.*, 2004). Entre los transformantes obtenidos se seleccionó uno de aquellos en los que el gen *HAT1* y *HAT1* quedó insertado en el mismo sentido que el gen *GFP3*. Se obtuvo así los plásmidos pAHR1 y pZRG1 en el que las secuencias *HAT1-GFP3* y *HAT2-GFP3* constituyen una única fase de lectura abierta. El vector pAHR1 y pZRG1 se utilizó para transformar células de las cepas CAI4, RGL4 (*hat1Δ*), RDH4 (*hat2Δ*) y MAH4 (*hat1Δ, hat2Δ*). Se seleccionaron clones de cada una de las transformaciones y se dejaron crecer en YNB a 28°C. Los cultivos se refrescaron mediante una dilución 1/5 del cultivo en medio YNB y se incubaron durante 5 horas a 28°C. Se tomó una muestra de cada uno de los transformantes y se observó en un microscopio óptico DRMX (Leica) con un fuente de UV Leica ebq 100, utilizando los filtros adecuados para la visualización de la fluorescencia de la GFP y del colorante Hoechst. Se observó que las proteínas Hat1p y Hat2p se localizaban en un punto discreto dentro de cada célula. La tinción con Hoechst, colorante fluorescente que se une al ADN, permitió determinar que se trataba del núcleo (Fig. 2.13 y 2.14). En la segunda parte de este trabajo, hemos transformado el vector pAHR1 para transformar células de la cepa RDH4 (*hat2Δ*). En los clones analizados se observó que la proteína Hat1p se localizaba en el núcleo (Fig. 2.15A). Por ultimo, hemos transformado con el vector pZRG1 la cepa RGL4 (*hat1Δ*). En los clones analizados se observó la proteína Hat2p, no se localizaba en el núcleo (Fig. 2.15B). Ello indica que la localización nuclear de Hat2p depende de Hat1p.

Ensayo de doble híbrido en *Candida albicans*

Hemos realizado un estudio por el sistema de doble híbrido para identificar sí proteína Hat1p tiene interacción directa con Hat2p. Para este rastreo se construyó un plásmido (Tabla 2.1) que contenía la secuencia que codifica Hat1p y Hat2p. Se confirmó que la interacción entre las proteínas Hat1 y Hat2 no existe en el ensayo de doble híbrido. Lo que sugiere que debe existir una proteina que haga de puente similar a la Hif1 de *S. cerevisiae* aun que esto no le ha detectado mediante analisis blott en ninguna otra levadura.

Análisis del transcriptoma de mutante *hat2Δ* durante crecimiento en forma de levadura

A partir de células recogidas en fase de crecimiento exponencial como levaduras, se detectaron 109 genes cuya expresión se alteraba significativamente en el mutante *hat2Δ.* Se detectaron dos genes comunes (*HHF1* y *STF2*) entre *hat1Δ* y *hat2Δ*. Dentro del grupo de genes sobreexpresados se detectaron genes implicando en las siguientes categorías: respiración (ATP6, *COX3A, COX3B, NAD2, NAD5*, *NAD6* y *STF2*) y ensamblaje de histonas (*HHF1, HTA1* y *NHP6A*). No hemos detectado genes comunes relacionados con las mismas funciones en los mutantes *hat1Δ* y doble *hat1Δ, hat2Δ* durante el crecimiento en forma de levadura. Además se detectaron genes subexpresados relacionados con glicólisis (*CDC19, FBA1, PDB1, PFK2* y *PYC2),* ciclo TCA *(ACO1, ACO2, CIT1, GDH3, MDH1-1* y *MIS11*), genes que codifican para proteínas ribosomales (*RPL8B, RPL13, RPL16A, RPL81, RPP0, RPS4A, RPS8A, RPS15, RPS23A* y *YST1*), genes que codifican proteínas de pared celular (*PGA45, PGA59, PGA62, TOS1* y *YWP1*), genes de respuesta a estrés (*SOD1, SOD2, SSB1* y HSP90) y factores de transcripción (*EFG1, NRG1, RGT1* y *SKO1*). *CaSKO1* codifica un factor transcripcional cuyo homólogo en *S. cerevisiae* tiene un efecto represor de la actividad de la ruta PKA-cAMP y que es capaz de unirse al promotor de *ScSOK2* (homólogo de *CaEFG1*) (Nehlin *et al.*, 1992; Proft *et al.*, 2005). En conjunto estos datos demuestran que el perfil transcripcional del mutante *hat2Δ* es diferente del de los mutantes *hat1Δ* y doble *hat1Δ*, *hat2Δ* lo que sugiere que Hat2p tiene funciones celulares especificas.

Análisis del perfil de la transcripción de *hat2Δ* durante la transición levadura-hifa

El análisis del perfil transcripcional de la cepa *hat2Δ* en comparación con el de la cepa parental indicó que durante la transición levadura-hifa, la deleción del *HAT2* altera la

expresión de al menos 429 genes, con un factor de variación mutante *vs* cepa control ≥ 1.5. De éstos, 196 genes son sobreexprsados y 233 genes subexpresados. 2 genes son comunes a los tres tiempos (sobreexpresados), 54 genes entre los 15 y 60 min (35 sobre- 19 sub-expresados) y 11 genes entre los 15 y 180 min (9 sobre- y 2 sub-expresados). 156 genes (85 sobre- y 71 sub-expresados), 167 genes (40 genes sobre- y 127 sub-expresados) y 39 genes (25 sobre- y 14 sub-expresados) solo varian en un tiempo de inducción (15, 60 y 180 min respectivamenete). Considerando datos publicados por diversos autores sobre la modulacion de la expresión genica durante la transición levadura-hifa, se trató de determinar que genes implicados en diversos categoria se encontraban alterados en el mutante *hat2*Δ durante la transición levadura-hifa. Se ha detectado la sobreexpresion de 17 genes relacionados con metabolismo de carbohidratos (principalmente genes relacionados con la ruta de glicolisis) (e.g *ADH1, ADH5, ENO1, FAB1* y *PCK1*) durante 15 min, 9 genes de pared celular (e.g *BMT5, BMT9, CHS2, GSL1, HWP1, PGA45* y *PGA54*), 5 genes de transporte de azúcares (*HGT1, HGT7*, *HGT8, HGT9* y *SHA3*), 3 genes de la familia de la telomerasa (*TLO3, TLO4* y *TLO12)* y 5 genes de proteínas ribosimales (*RPL4B, RPL5, RPL33, RPL42* y *RPS6A*) (Tabla 2.4). La regulación de la expresión de genes de glicolisis 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 glicolisis se reduce drásticamente y a continuación aumenta progresivamente, llegando a ser en algunos genes mayor en hifas que en levaduras. Sin embargo, su relación con 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 (Rashki, Tesis doctoral 2009; Swoboda *et al.*, 1994). Ademas, observándose que en el inicio de la transición levadura-hifa (15 min) la expresión de un número de los genes que codifican enzimas de la ruta de glicolisis se aumente en el mutante *gcn5*Δ (Degano, Tesis doctoral en proceso). Dentro del grupo de los genes subexpresados, se ha observado 7 genes relacionados con respiración (*COX1, COX3A, COX3B, COX6, COX15, NAD2* y *NAD6*), 9 genes de pared celular (*ALS4, ECM31, GCS1, HYR1, HYR3, PHR2*, *PGA6, PGA63* y *TOS1*), 12 genes relacionados con proteínas ribosomales (*MRPL19, MRPL38, RPL8B, RPL10, RPL11*, *RPL14B, RPL16A, RPL20B, RPL82, RPS4A, RPS15* y *RPS16*), 8 genes que codifican para factores de transcripción (*CTA4, CTA8, CPH1, HAC1, SPT20, TBP1, ZCF20* y *ZPR1*) y por último 4 genes relacionados con biosíntesis de ergosterol (*ERG4, ERG7, ERG9* y *ERG26*) (Tabla 2.4). *HAC1* codifica el homólogo en *C. albicans* del activador transcripcional ScHac1p de *S. cerevisiae*. ScHac1p es capaz de interaccionar con el

motivo UPRE, asociado a proteínas de respuesta a choque térmico implicadas en el plegamiento de proteínas, y con el motivo de respuesta a cAMP (secuencia CRE) (Mori *et al.*, 1996; Nojima *et al.*, 1994). El cAMP actúa como segundo mensajero en una de las principales rutas de transducción de señales que controlan la filamentación en *C. albicans*. La expresión de *CaHAC1* se induce durante la miceliación a 37°C en la cepa silvestre de forma coordinada con otros reguladores morfogenéticos, por lo que la activación de este gen en *C. albicans* podría constituir una respuesta al aumento de la temperatura de incubación. Hasta el momento no se ha analizado su posible asociación con la regulación de la transición dimórfica. El estudio de un mutante nulo *Cahac1* podría resultar interesante ya que permitiría determinar si la regulación dependiente de Hac1p juega un papel relevante en la transición levadura-hifa inducida por temperatura.

Análisis del transcriptoma del doble mutante *hat1Δ*, *hat2Δ* durante la fase de levadura

Los perfiles de transcripción se utilizaron para examinar más a fondo el papel de ambas acetiltransferasas en *C. albicans*. El transcriptoma del mutante doble *hat1Δ, hat2Δ* se compararó con la cepa CAI4 durante la crecimiento levadura y durante la transición levadura-hifa. Anteriormente en nuestro laboratorio se han llevado a cabo experimentos, en los que se ha analizado los transcriptomas del mutante simple *hat1Δ*. Tal y como se ha señalado anteriormente, en las 4 condiciones examinadas, crecimiento exponencial como levaduras (en YNB a 28°C) y a tres tiempos (15, 60 y 180 min) durante la transición dimórfica en medio líquido Lee a 37°C, el mutante doble *hat1Δ, hat2Δ* muestra diferencias morfológicas respecto a la cepa parental. A partir de células recogidas en fase exponencial de crecimiento como levaduras, se detectó que la expresión de 219 genes cuya expresión se encontraba alterada por la deleción de *HAT1* y *HAT2* en el crecimiento en forma de levadura. El número de genes que presentan expresión diferencial en el mutante doble *hat1Δ, hat2Δ* es mayor que el detectado en los mutantes simples *hat1Δ* y *hat2Δ.* De los genes expresados en el doble mutante con respecto al silvestre durante crecimiento de forma levadura, 37 son de función desconocida; el resto tienen una función conocida o deducida por homología a partir de la secuencia correspondiente de *Saccharomyces cerevisiae*. El gen *HSP12* presenta un incremento significativo en la expresión en el doble mutante *hat1Δ, hat2Δ* con respecto al silvestre (con una relación mutante/silvestre de 5,06) dicho gen se encuentra reprimido en condiciones de inducción de filamentación (Lane *et al.*, 2001; Nantel *et al.*, 2002). *HSP12* es una proteína de choque térmico implicada

en la organización de la pared celular cuya transcripción se induce en una gran variedad de condiciones de estrés que incluyen: estrés osmótico, estrés oxidativo, estrés térmico, presencia de etanol, limitación de nutrientes por crecimiento en fuentes de carbono no fermentables así como la presencia agentes que afectan a la integridad de la pared celular (Karreman & Lindsey, 2005). Además se detectó la presencia de los genes *HSP31, HSP70, HSP78, HSP104* que codifican para proteínas de choque térmico. Otros genes sobreexpresados en la cepa *hat1Δ, hat2Δ* durante el crecimiento en forma de levadura es el gen que codifica la histona H4, *HHF22,* y dos genes que codifican para la histona H3, *HHT1* y *HHT21.* Ello parece indicar la existencia de mecanismos de activación de genes por represión de los genes H4 y H3. Cabe destacar que dentro de la subcategoría de regulación positiva de la transcripción aparecen sobreexpresadas 5 genes (*TLO1, TLO3, TLO11, TLO12* y *TLO16*) pertenecientes a una misma familia de genes telomericos. Se trata del grupo de probables activadores transcripcionales de *C. albicans* denominado *CTA2*. Las características de esta familia de genes apenas se han estudiado hasta el momento. Además se encuentra el grupo de subunidades de proteosoma (*PRE3, PRE4, PRE6, PRE7, PRE9, PUP2, RPN4* y *RPN12*).

Análisis del transcriptoma del doble mutante *hat1Δ, hat2Δ* durante la transición levadura-hifa

Hemos analizado el perfil transcripcional de la cepa MAH4 para profundizar en el estudio de la regulación de la expresión en esta cepa, prestando especial interés a los genes implicados en la formación de hifas. Para ello, se realizaron comparaciones entre los niveles de expresión genética de la transición levadura-hifa a los 15, 60 y 180 minutos. El resultado que obtuvimos fue que 787 genes alteraban significativamente su expresión, en al menos uno de los tres tiempos. De estos genes, 417 están subexpresados y 370 están sobreexpresados respecto a la cepa CAI4. 16 genes son comunes a los tres tiempos (6 genes sobr- y 10 genes subexpresados), 193 genes entre los 15 y 60 min (62 sobre- 131 sub-expresados) y16 genes entre los 60 y 180 min (10 sobre- y 6 sub-expresados) (Tabla 2.6). 272 genes (189 sobre- y 83 sub-expresados), 265 genes (97 genes sobre- y 168 sub-expresados) y 29 genes (10 sobre- y 19 sub-expresados) solo varian en un tiempo de inducción (15, 60 y 180 min respectivamenete). Considerando datos publicados por diversos autores sobre la modulacion de la expresión genica durante la transición levadura-hifa, se trató de determinar que genes implicados en diversos categoria se encontraban alterados en el mutante doble *hat1*Δ, *hat2*Δ durante la transición levadura-hifa.

Se detecta un número elevado tanto de genes sobreexpresados como subexpresados. Se encuentran genes cuya expresión diferencial se manifiesta principalmente a la 1 h, cuando las hifas/pseudohifas están formados. De acuerdo a las diferencias morfológicas entre ambas cepas, en este grupo se detecta la subexpresión de genes que codifican proteínas principalmente asociadas a la pared celular [CaAls1p, CaAls3p, CaAls4p (15 y 60 min), CaAls2p (15 min) y CaAls5p (60 min)] y genes implicados en transporte de azúcares *HGT6*, *HGT7* y *HGT8* (Fan *et al.*, 2002), genes implicados en la secreción y procesamiento de proteínas *SEC23* y *SEC26* (15 min), *SEC9, SEC11, SEC16, SEC24* y *SEC61* (60 min), *PMT1, PMT2* y *PMT4* (15 y 60 min) (Maglott *et al.*, 2007; Prill *et al.*, 2005; Timpel *et al.*, 1998) durante 15 y 60 min de transition levadura hifa. Probablemente este efecto se debe al mayor requerimiento de transporte de material hacia el ápice de las hifas, lugar donde se concentra la síntesis de pared celular durante el crecimiento polarizado (Mondesert *et al.*, 1997; Nantel *et al.*, 2002; Whiteway & Oberholzer, 2004). También se han detectado posibles genes implicados en la secreción y procesamiento de proteínas (*SEC16, SEC23, SEC26, PMT1, PMT2* y *PMT4*) sobreexpresados en el mutante *odc1Δ* durante 15 y 60 min de la transition levadura hifa (Rashki, Tesis doctoral 2009). La regulación de la expresión de genes de procesamiento de proteínas se ha asociado en diferentes trabajos (Cantero *et al.*, 2007). Entre los genes sobreexpresados, se observó 37 genes (15 min), 3 genes (60 min), 9 genes (15 y 60 min), 1 gene (60 y 180 min) y 2 genes (15, 60 y 180 min) relacionados con proteínas ribosomales. Se ha reportado que 63 genes son subexpresados durante el crecimiento en forma de levadura en el mutante simple *gcn5Δ* (Degano, Tesis doctoral en proceso). Además hemos observado que 12 genes que codifican para la misma categoría son subexpresados y 5 genes sobreexpresados en *hat2Δ* durante transición levadura-hifa, indicando la diferencia entre mutante doble y simple *hat2Δ*. Se ha demostrado que la deletion de los genes, *GCN5* y *HDA1*, conlleva la subexpresión un numero de genes que codifican proteínas ribosomales (Rashki, Tesis doctoral 2009). Hemos encontrado que 4 genes relacionados con transporte de drogas (*CDR1, CDR2, CDR4* y *QDR1*) y 14 genes implicados en factores de transcripción están subexpresados en el mutante doble. Otros grupos de genes subexpresados son: genes relacionados con pared celular y glicólisis. Además, se detectó genes sobreexpresados en categorías como respuesta a estrés y pared celular.

Interrupción del gen *SIN3* en la cepa RDG4 (*gcn5Δ*)

La interrupción de los dos alelos del gen *SIN3* en la cepa RDG4 (con el gen *GCN5* delecionado que había sido previamente construida en nuestro laboratorio) se llevó a cabo siguiendo la estrategia del "Ura-blaster". En la Fig. 3.1B se muestra la comprobación del genotipo de las cepas generadas mediante Southern. El ADN genómico fue digerido con *Nde*I y como sonda se utilizó la secuencia de 588 pb correspondiente a la región 3´ no codificante del gen *SIN3.* Como puede apreciarse en la Fig. 3.1, las cepas CAI4, RDG4 y los heterocigotos para la deleción del gen presentan la banda de 3.6 kb correspondiente al alelo silvestre de *SIN3.* Esta banda no aparece en las cepas homocigotas para la deleción de *SIN3* (ZAH4) 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 *gcn5Δ, sin3Δ* en el crecimiento exponencial

Mediante el seguimiento de la D.O alcanzada a lo largo del tiempo por los cultivos de las diferentes cepas, se detectó un retraso en el crecimiento del doble mutante *gcn5Δ, sin3Δ* en comparación con la cepa CAI4 en medio YEPD. Las curvas de crecimiento obtenidas para los mutantes ZAH4 (*gcn5Δ, sin3Δ*) y SFC4 (*sin3Δ*) fueron muy similares entre sí y, a su vez, diferentes a las cepas CAI4 y RDG4 (*gcn5Δ*). Estimamos que en este medio las cepas ZAH4 iniciaban la fase exponencial tras aproximadamente 18 horas de crecimiento (Fig. 3.2). Las células de la cepa doble delecionada son alargadas, lo mismo que hemos visto en las células de la cepa delecionada para el gen *SIN3,* y también gromada, lo que ha visto en las células de la cepa delecionada para el gen *GCN5.* La morfológia presenta defectos que son muy claros en ausencia de ambos genes (Fig. 3.3B).

Efecto de la doble deleción *gcn5Δ, sin3Δ* en la filamentación en medios sólidos

El aspecto de las colonias de las cepas CAI4, RDG4, SFC4 y ZAH4 en los medios sólidos estudiados se muestra en la Fig. 3.4. El medio Spider es un inductor de miceliación en *C. albicans* (Liu *et al.*, 1994). En este medio las colonias de la cepa control (CAI4) presentan filamentos que emergen alrededor de las mismas. Los mutantes simples y el doble mutante presentan defectos de filamentación. También se ha analizado el comportamiento de las células de diferentes cepas creciendo embebidas dentro de una matriz de agar y en estas condiciones la cepa CAI4 tiene lugar una rápida producción de hifas (Brown *et al.*, 1999), lo que podría jugar un papel

importante durante la infección del tejido del hospedador (Giusani *et al*., 2002). Los resultados demuestran que el doble mutante *gcn5Δ, sin3Δ* al igual que mutante simple *gcn5Δ* es incapaz de producir hifas en esta condición de inducción. Siguiendo con la dinámica del estudio de la morfología de las colonias del doble mutante *gcn5Δ, sin3Δ* de *C. albicans*, se utilizó el medio SLADH. Este medio tiene bajo contenido en fuente de nitrógeno e induce crecimiento invasivo y pseudofilamentoso en *S. cerevisiae* y crecimiento en forma de hifas verdaderas en *C. albicans* (Csank *et al.,* 1998; Ernst, 2000). Las colonias del doble mutante forman colonias de aspecto rugoso y son incapaces de filamentar. En el medio Lee con NAcGlc, las colonias del doble mutante forman filamentos que penetran el agar. En la Fig. 3.4 se observa como los filamentos de la colonia del doble mutante están por debajo del agar y los de la cepa CAI4 están en la superficie. Por ultimo, en el medio Lee con suero al 4%, las colonias del doble mutante son incapaces de formar filamentos como ambos mutantes simples.

Efecto de la doble deleción *gcn5Δ, sin3Δ* en la transición dimórfica en medio líquido

La inducción de micelio se llevó a cabo en las cepas CAI4 y en el doble mutante *gcn5Δ, sin3Δ* de *C. albicans*. Para ello, después de un reposo metabólico a 4°C, las células se crecieron en medio Lee suplementado con uridina y suero al 4% a 37°C. La cepa silvestre forma hifas verdaderas a partir de un tubo germinativo que ya puede apreciarse claramente a los 60 min de inducción. En la cepa doble mutante *gcn5Δ, sin3Δ,* por el contrario, pueden visualizar se pseudohifas y agregados celulares (Fig. 3.5) al igual que en los mutantes simples.

Efecto de la deleción de los genes *GCN5* y *SIN3* en la formación de clamidosporas

La inducción de la formación de clamidosporas se llevó a cabo de forma análoga a la descrita en el análisis del mutante doble *hat1*Δ*, hat2*Δ. En el mutante doble *gcn5*Δ*, sin3*Δ, así como en los mutantes simples no se detectaron clamidosporas, ni siquiera después de un periodo de incubación prolongado (14 días) (Fig. 3.6). Sonneborn et al (1999a) describieron para el mutante *efg1* un fenotipo muy similar bajo las mismas condiciones de inducción de la formación de clamidosporas. Estas observaciones apoyan la hipótesis de la existencia de interacción entre Efg1p y Sin3p o Gcn5p expuesta en trabajos previos de nuestro grupo de investigación (Monterola, 2002; Tebarth *et al*., 2003). Como se ha indicado anteriormente, el requerimiento de los procesos de remodelación de la cromatina sobre la formación de clamidosporas se ha

hecho evidente por la observación de que una de las proteínas implicada en su desarrollo es CaIsw2p (Nobile *et al.*, 2003), cuyo homólogo de *S. cerevisiae* forma parte de un complejo remodelador de la cromatina, que actúa en paralelo con el complejo Rpd3p-Sin3p de *S. cerevisiae* (Fazzio *et al*., 2001). Otro gen cuya alteración causa defectos en la diferenciación de clamidosporas es *CaSCH9,* 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 (Nobile *et al.*, 2003). El mutante *sin3Δ* en *S. cerevisiae* presenta menor acumulación de glucógeno que la cepa silvestre (Wilson *et al.*, 2002), sería interesante por tanto determinar si la función de CaSin3p 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.

Efecto de la deleción de los genes *GCN5* y *SIN3* en la estructura de la pared celular

En el ensayo de sensibilidad a Zimoliasa se muestra en la Fig. 3.8. Como puede observarse, el doble mutante muestra un fenotipo igual al del mutante sencillo *sin3Δ*. Por último, decidimos analizar la sensibilidad a otros agentes que afectan a la pared celular: Cafeína, Calcofluor white, NaCl, SDS y Rojo congo. El doble mutante *gcn5Δ, sin3Δ* y el mutante sencillo *gcn5Δ* son sensibles a Higromicina a una concentración de 100 y 300 μg/ml. Como se puede observar en la Fig. 3.7, también son más sensibles a Cafeína, Itraconazol, Fluconazol, NaCl, KCl, $CaCL_2$ y Rojo congo. En la Fig. 3.9 se muestran los valores del colorante fijado por la cepa CAI4, RDG4, SFC4 y ZAH4 de *C. albicans*. Si tomamos como 100% la cantidad de colorante fijado por las células de la cepa CAI4, las células del doble mutante fijan un 73,44 %. El porcentaje de colorante fijado por las cepas SFC4 y RDG4 es de un 40,44% y 161,83% respectivamente. Además, podemos concluir que en términos absolutos el mutante simple *sin3Δ* y la cepa doble delecionada fijan menos colorante que el mutante *gcn5Δ* y la cepa CAI4. Estas diferencias se pueden atribuir a que el doble mutante y mutante simple *sin3Δ,* presentan una menor cantidad de fosfato en las manoproteínas presentes en la pared celular.

Virulencia

Hemos usado la cepa EVA1 obtenida tras la reconstrucción de uno de los locus *URA3* de la cepa SFC4 (*sin3Δ*) y ZAHR4 obtenida de la cepa ZAH4 (*gcn5Δ, sin3Δ*) para inocular por vía intraperitoneal en 10 ratones CD1 con 10^8 (data no muestrado) y 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.
Se muestra que no han muerto 80% de los ratones injectado con 5×10 de las celulas de los mutante simple *sin3Δ* y doble *gcn5Δ, sin3Δ* durante tiempo ensayadas (Fig. 3.10).

Análisis del perfil transcripcional

A la vista de los resultados obtenidos en la caracterización fenotípica de la cepa doble delecionado decidimos estudiar en detalle el patrón de transcripción de la cepa ZAH4 durante crecimiento de forma levadura y durante la transición levadura-hifa. Con el objetivo de averiguar si se detectaba una alteración en la expresión de los genes que codifican para proteínas que intervienen en la formación de hifa. Para ello, se realizaron comparaciones entre el perfil transcripcional de la transición levadura-hifa a los 15, 60 y 180 minutos. De esta manera observamos que 558 genes alteraban significativamente sus expresiones durante la fase de levadura y 1271 genes cambiaban significativamente su expresión en al menos uno de los tres tiempos indicados (Fig. 3.12B).

Análisis del perfil de transcripción del doble mutante *gcn5Δ, sin3Δ* durante la fase de levadura

Dentro del grupo de genes sobreexpresados durante crecemiento de forma de levadura, las categorías y subcategorías estadísticamente significativas son respuesta a estrés oxidativo, oxidación de ácidos grasos, replicación y síntesis de ADN, transporte de azúcares y obtención de energía (principalmente a través de TCA).
Entre los genes subexpresados, las categorías más significativas son: genes relacionados con pared celular, transporte, genes que codifican para proteínas ribosomales y factores de transcripción.
Dentro de los genes sobreexpresados, se detectaron genes relacionados con alteraciones morfológicas como *NUM11, NUM12, TUB1* y *TUB2*. Los genes *NUM11* y *TUB1* se han encontrado sobreexpresados en la cepa carente de *SIN3* (Martin, Tesis doctoral 2006). Los productos de estos genes participan en la biogénesis y función del

citoesqueleto afectando de este modo a la organización intracelular. Mutaciones en los genes homólogos de *S. cerevisiae*, *ScNUM1* y *ScTUB1,* causan defectos en la correcta migración del núcleo (Heil-Chapdelaine *et al.*, 2000; Schatz *et al.*, 1988). En el caso de *ScTUB1,* se ha descrito que su sobreexpresión supone un bloqueo temporal del ciclo celular, durante el cual continúa el crecimiento causando un incremento del tamaño de las células (Weinstein & Solomon, 1990). Las células mutantes doble como *sin3Δ* son más alargadas que las silvestres, la sobreexpresión de genes del citoesqueleto que participan en organización celular y polaridad podría contribuir a este fenotipo. Se ha detectado la sobreexpresion de un número significativo de genes relacionados con el metabolismo oxidativo como *GRX3, SOD2* y *TRX1*. Con el fin de averiguar si la expresión diferencial de estos genes tenía consecuencias biológicas se analizó la sensibilidad de la cepa doble delecionada a un agente oxidante, el peróxido de hidrógeno. A partir de células recogidas en fase exponencial de crecimiento se preparó una suspensión de 10^7 células/ml de cada cepa. Tras diferentes concentraciones a H_2O_2, se recogieron muestras de 5 μl y se depositaron sobre placas de YEPD. En concordancia con los resultados de los microarrays, se observó que el mutante doble como mutante simple *sin3*Δ presentaba mayor resistencia a la acción del peróxido de hidrógeno (H_2O_2) que la cepa parental, CAI4.

Análisis del transcriptoma del doble mutante *gcn5Δ, sin3Δ* durante la transición levadura-hifa

El mismo estudio de microarrays se llevó a cabo durante la transición levadura-hifas. Este análisis mostró 1271 genes alterados con 277 genes de función desconocida. 528, 891 y 146 genes expresados diferencialmente en cada uno de los tiempos analizados (Fig 3.12). 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 que la expresión de 1271 genes (690 sobre- y 581 sub-expresados) está afectada en el mutante doble *gcn5Δ, sin3Δ*. 43 genes son comunes a los tres tiempos ensayados (10 sobre- y 33 sub-expresados), 156 genes en los 15 y 60 min (102 sobre- y 54 sub-expresados) y 47 genes en 60 y 180 min. (9 sobre- y 38 sub-expresados). 324 genes (227 sobre- y 97 sub-expresados), 645 genes (315 sobre- y 330 sub-expresados) y 51 genes (24 sobre- y 27 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 que genes implicados en morfogénesis se encontraban alterados en el mutante doble *gcn5Δ, sin3Δ*. Los 43 genes comunes a los tres tiempos se muestran en la Tabla 3.4. De acuerdo a las morfología del mutante doble durante transición

levadura-hifa, en este grupo se detecta la subexpresión de genes que codifican proteínas principalmente asociadas a la pared celular que habían sido previamente reconocidos como hifa-específicos: *ECE1* (Birse *et al.*, 1993), *RBT1* (Braun *et al.*, 2000), *HWP1* (Sharkey *et al.*, 1999), *ALS1* y *ALS3*. Tal y como se muestra en la Tabla 3.4 de material suplementario, 3 genes que codifican las histonas H2, H3 y H4 (*HHF22, HHT1* y *HHT21*) están subexpresados en el 60 min de la transición analizado. La subexpresión de estos genes podría estar relacionada en este mutante con el retraso de crecimiento que muestra esta cepa respecto a la cepa parental, como se ha sugerido en otros mutantes donde estos genes se encuentran subexpresados y muestran crecimiento más lento (Bensen *et al.*, 2002). Se ha detectado la regulación de un número de genes relacionados con la pared celular incluidos: *PGA1* (Castillo *et al.*, 2006; De Groot *et al.*, 2003), *PGA49* (De Groot *et al.*, 2003), *CWH41* (Mora-Montes *et al.*, 2007), *ECM1* (Harcus *et al.*, 2004), *FGR12* (Uhl *et al.*, 2003), *FGR41* (De Groot *et al.*, 2003), *IFF5* (De Groot *et al.*, 2003) sobreexpresados y *KRE1* (Castillo *et al.*, 2006), *PGA6, PGA45, PGA53, PGA62*, *PGA63, ECM21, ECM33* (Martinez-Lopez *et al.*, 2008), *FGR6* y *PHR1* subexpresados en 60 minuto de inducción (Fig. 3.14B). Hemos detectado la sobreexpresion de un número de los genes relacionado con proteínas ribosomales (*NOC4, NOP2, NOP10, RPB8, RPC10, RPC31, RPC82, RPL25, RPL26A, RPL27A, RPL35, RRP1, RRP3, RRP9, RRS1, RSA2* y *SIK1*) y la subexpresion de *RPL2, RPL7A, RPL13, RPL14, RPL15A, RPL18, RPL30, RPN2, RPP2A, RPP2B, RPS4A, RPS16A, RPS17B* y *RPS20* genes durante 60 minutos de incubación. No hemos encontrados genes relacionados en este categoría en otros tiempos de inducción (15 y 180 min). Después de 60 minutos de inducción se han observado la subexpresión de genes que codifican proteínas ribosomales en el mutante simple *sin3Δ*. Este comportamiento, subexpresión de genes implicados en procesamiento de ARN seguido de subexpresión de genes que codifican proteínas ribosomales, se corresponde con el descrito en la respuesta a diferentes tipos de estrés en *S. cerevisiae* (Boorsma *et al.*, 2005; Gasch *et al.*, 2000; Gasch *et al.*, 2004). Parece que la subexpresión de estos genes podría estar relacionada con la sobreexpresión de genes de respuesta a estrés oxidativo y sobre todo de la activación de la respuesta a carencia de aminoácidos. Se ha demostrado que la inducción de *ScGCN4* conlleva la subexpresión de genes que codifican proteínas ribosomales (Natarajan *et al.*, 2001). 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 *SIN3*.

Conclusiones

1. Se ha llevado a cabo la deleción del gen *CaHAT1* en la cepa RDH4 (*hat2Δ*). La ausencia de ambas proteínas tiene un efecto en morfogénesis, ya que el doble mutante *hat1Δ, hat2Δ* presenta defectos similares a los del mutante sencillo *hat1Δ*: morfología de pseudohifa cuando crecen a 28°C, defectos de filamentación y formación de pseudohifas en lugar de hifas en medios líquidos en condiciones de inducción.

2. Se ha observado que los mutantes *hat1Δ, hat2Δ* y *hat1Δ* muestran sensibilidad a NaCl, Rojo congo, Calcoflour white y Cafeína. Tanto el mutante doble como el mutante simple *hat1Δ,* también mostraron sensibilidad moderada frente a compuestos como Fluconazol, Itraconazol, Higromicina y Anfotericina B.

3. Se observó que las proteínas Hat1p y Hat2p se localizan en el núcleo en *C. albicans*. Además, se observó que en el mutante *hat1Δ*, la proteína Hat2p, no se localiza en el núcleo. La localización de la proteína Hat2p es dependiente de la proteína Hat1p. Estos resultados son contrarios a los observados en *S. cerevisiae.*

4. Se ha comparado el transcriptoma del mutante simple *hat2Δ* con el de la cepa parental CAI4, observándose que: en fase exponencial de crecimiento a 28°C, en la cepa delecionada se encuentra alterada la expresión de 109 genes, considerando un factor de variación ≥ 1.5 veces. Entre estos genes, aparecen subexpresados genes implicados en respiración, ensamblaje de histonas y subexpresados genes implicados en la ruta de glicólisis, TCA, biosíntesis de ribosomas, pared celular y respuesta a estrés. Durante la transición dimórfica, aparece alterada la expresión de 429 genes. Las principales categorías asociadas a sobreexpresión son: metabolismo de carbohidratos (glicólisis), pared celular, transporte de glucosa, genes relacionados con la familia de la telomerasa y proteínas ribosomales. Entre los genes subexpresados se encuentran principalmente genes que codifican proteínas relacionadas con la maquinaria de transcripción (factores de trascripción), el procesamiento de rARN, respiración, pared celular y biosíntesis de ergosterol.

5. Se ha comparado el transcriptoma del mutante doble *hat1Δ*, *hat2Δ* con el de la cepa parental CAI4, observándose que: en fase exponencial de crecimiento a 28°C, en la cepa delecionada se encuentra alterada la expresión de 219 genes, considerando un factor de variación ≥1.5 veces. Entre estos genes, aparecen sobrexpresados genes implicados en pared celular, respuesta a estrés, genes relacionados con proteosoma, 5 genes relacionados con la familia de la telomerasa. Tres genes de esta familia son comunes entre el mutante doble y *hat1Δ* lo que sugería que Hat1p interviene en el silenciamiento telomérico. Entre estos genes, aparecen subexpresados genes implicados en ruta de glicólisis, TCA y transportadores.

Durante la transición dimórfica, aparece alterada la expresión de 794 genes. Las principales categorías asociadas a sobreexpresión son respuesta a estrés, procesamiento de rARN y síntesis de proteínas, pared celular y 6 genes relacionados con el complejo del proteosoma (*NOP1, NOP2, NOP4, NOP5, NOP14* y *NOP15*). Se ha detectado la subexpresión secuencial de genes relacionados con pared celular, glicólisis, ruta de secreción (*SEC9, SEC11, SEC16, SEC23, SEC24, SEC26* y *SEC61*), resistencia a drogas (*CDR1, CDR2, CDR4* y *QDR1)* y 14 genes relacionados con factores de transcripción.

6. Se ha llevado a cabo la deleción del gen *CaSIN3* en la cepa RDG4 (*gcn5Δ*). La ausencia de ambas proteínas tiene un efecto intermedio en morfogénesis: pseudohifa y morfología alargada de las levaduras cuando crecen a 28°C, defectos de filamentación y formación de pseudohifas en lugar de hifas en medios líquidos de inducción e incapacidad para formar clamidosporas.

7. Se comprobó que el mutante simple *sin3Δ* y doble *gcn5*Δ*, sin3*Δ afectan a la virulencia de *C. albicans* en un modelo de infección sistémica de ratón.

8. Se ha observado que tanto el mutante *gcn5Δ, sin3Δ* como el mutante simple *gcn5Δ* muestran sensibilidad a los compuestos como NaCl, Rojo congo, KCl, $CaCl_2$, Higromicina y Cafeína.

9. La comparación del perfil transcripcional completo del mutante doble *gcn5Δ, sin3Δ* con el de la cepa parental CAI4 indica que:

- En fase exponencial de crecimiento a 28°C la deleción de *GCN5* y *SIN3* altera la expresión 558 genes que entre ellos 294 genes sobre- y 264 genes subexpresados. Entre ellos se identificaron sobreexpresados varios genes relacionados con síntesis y replicación de ADN, genes que codifican para factores transcripcionales, genes relacionados con estructura celular como *TUP1, NUM11*, pared celular y ciclo de TCA. Las principales categorías asociadas a sobreexpresión son pared celular (*CWH41, ECM1, FGR12, FGR41, IFF5, PGA1* y *PGA49*) (60 min), 17 genes que codifican proteínas ribosomales y genes relacionados con factores de transcripción, incluyendo el gen que codifica el activador transcripcional Gcn4p. Se ha detectado la subexpresión secuencial de genes relacionados con filamentación, genes que codifican factores transcripción y proteínas relacionadas con pared celular, genes que codifican proteínas ribosomales, genes relacionados con biosíntesis de vitaminas, ruta de glicólisis y además genes que codifican para proteínas estructurales de las hifas. La subexpresión de genes que codifican para proteínas ribosomales y otros factores relacionados con funciones generales de crecimiento, reflejan una respuesta en el mutante *gcn5Δ, sin3Δ* al retraso en el crecimiento.

10. Durante la transición dimórfica, aparece alterada la expresión de 1271 genes.Las principales categorías asociadas a sobreexpresión son metabolismo de aminoácidos,

pared celular, factores de transcripción incluyendo el gen que pared celular, factores de transcripción incluyendo el gen que codifica el activador transcripcional Gcn4p.

11. Se ha observado que el nivel de expresión de los genes relacionados con pared celular como *ALS1, ALS3, MNT1, PGA53, PGA54, PGA45, HYR1, ECM33, TOS1, PMT1* y *SUN41,* fue disminuyendo a la largo de transición levadura-hifa en los mutantes simples *gcn5Δ, sin3Δ* y en el mutante doble *gcn5Δ, sin3Δ*, lo que sugiere que estos genes están regulados por separado por ambos genes.

12. Se ha observado que el nivel de expresión de los genes relacionados con pared celular como *PHR1, YWP1, PGA62* y *KRE1* aumentan en el mutante simple *gcn5Δ* y disminuyendo a la largo de transición levadura-hifa en los mutantes *sin3Δ* y doble indicando que estos genes se regulan independientemente por ambos genes.

13. Se ha observado que el nivel de expresión de los genes relacionados con metabolismo de carbohidrate como *TPI1, TPS2, PGK1, TDH3, OSM1, GSY1, FBA1, ADH5, PGI1* y *PDC11* fue disminuyendo durante 60 y 180 de de transición levadura-hifa en el mutante simple *gcn5Δ* y doble *gcn5Δ, sin3Δ*.

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