universität wien

DISSERTATION

Titel der Dissertation

Histone-modifier genes regulate morphogenesis of *Candida albicans*

angestrebter akademischer Grad

Doktor der Naturwissenschaften (Dr. rer. nat.)

Verfasser:	Denes Hnisz
Matrikel-Nummer:	0409564
Dissertationsgebiet (lt. Studienblatt)	Molekularbiologie
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Wien, am 7 Juli 2008

“The major problem, I think, is chromatin…”

(James D. Watson, 2003)

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1. SUMMARY

The human pathogen *Candida albicans* is able to undergo a reversible switch between two distinct cell types called *white* and *opaque*, which are considered different transcriptional states of cells harbouring identical genomes. *White* and *opaque* cells differ markedly in their gene expression profiles, cellular morphologies, virulence characteristics and mating abilities. To date, several transcription factors controlling *white-opaque* switching have been identified and have been recently mapped into a single genetic circuit. The transcriptional control explains some features of the process but is based on the assumption that switching occurs in a stochastic fashion; nevertheless it has been demonstrated that environmental cues and host environment are able to modulate the switching frequency. This thesis demonstrates that chromatin-modifying enzymes constitute an additional regulatory layer of switching. The work postulates that this layer may represent a possible strategy to integrate environmental stimuli to determine cell fate. Eight chromatin-modifying enzymes were identified as switching modulators. Using a genetic epistasis analysis the genes were mapped to signalling pathways, some of which overlay the transcriptional network. The analysis also reveals that the Set3/Hos2 histone deacetylase complex relies on the methylation status of lysine 4 on histone H3 for switching regulation. The observations indicate that a dual-layer regulatory network comprising of transcription factors and chromatin modifiers determines cell fate in *C. albicans*.

2. ZUSAMMENFASSUNG

Der Krankheitserreger *Candida albicans* ist in der Lage zwischen zwei verschiedene Zell-Typen – *White* und *Opaque* – zu wechseln, welche zwei transkriptionell unterschiedliche Zustände ein und des selben Genoms darstellen. Zellen des *White* und *Opaque*-Zustands unterscheiden sich in ihren Genexpressionsprofilen, zellulären Morphologien, Virulenz Charakteristiken und Paarungs-Vermögen. Bis heute wurden mehrere Faktoren identifiziert, welche den *White-Opaque*-Wechsel auf transkriptioneller Ebene kontrollieren. Diese Faktoren wurden kürzlich einem einzelnen genetischen Regelkreis zugeordnet. Diese transkriptionelle Kontrolle erklärt einige der mit diesem Prozess einhergehenden Charakteristiken, basiert jedoch auch auf der Annahme dass der *White-Opaque*-Wechsel ein stochastischer Prozess ist; andererseits können jedoch auch bestimmte Umwelteinflüsse die Häufigkeit dieses Wechsels beeinflussen. Die vorgelegte Doktorarbeit zeigt, dass Chromatin-modifizierende Enzyme eine weitere Ebene der Regulation des *White-Opaque* Übergangs darstellen und dass diese Ebene der Regulation eine mögliche Strategie zur Integration von Umwelteinflüssen auf Zelldeterminierung darstellt. Acht Chromatin-modifizierende Enzyme wurden als *White-Opaque*-Modulatoren identifiziert und konnten anhand von genetischen Epistasis-Analysen Signaltransduktionswegen zugeordnet werden, wobei einige mit der Ebene der transkriptionellen Kontrolle überlappen. Des weiteren konnte gezeigt werden, dass der Einfluss des Set3/Hos2 Histon-Deacetylierungs Komplex auf den *White-Opaque*-Wechsel vom Methylierungs-Status von Histon H3 an Lysin 4 abhängig ist. Diese Ergebnisse zeigen, dass die Zelldeterminierung in *Candida albicans* auf einem dualen regulatorischen Netzwerk aus Transkriptionsfaktoren sowie Chromatin-Modifizierern beruht.

3. INTRODUCTION

3.1. Brief overview of epigenetics research

3.1.1. Linneaus' Peloria

In 1742 Swedish naturalist and "the father of taxonomy" Carolus Linnaeus was presented a flower that looked like the common toadflax *(Linaria vulgaris)* in many features except that its flower showed radial symmetry instead of the common bilateral floral symmetry of *Linaria*. A vague and radical idea of this time, Linnaeus perceived the plant as a transformation of the common toadflax into a new species and named it *Peloria* (Greek for "monstrosity") (Linnaeus, 1744). Since Linnaeus' botanical classification relied on the structure of reproductive organs, *Peloria* was classified even in a different genus as *Linaria,* and the term Peloria (or peloric flower) was subsequently used to describe aberrations in floral symmetry in otherwise closely resembling plants ever since (Figure 1).

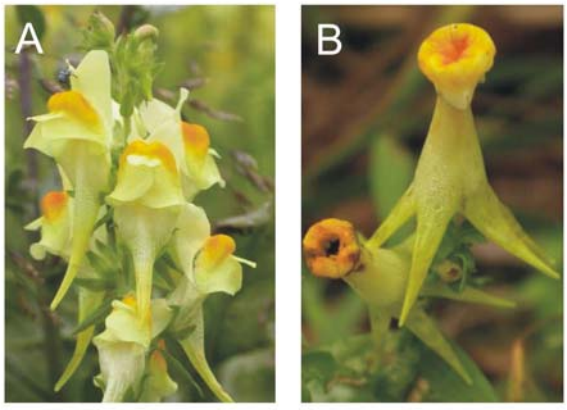

Figure 1. (A) Common and (B) peloric flowers of the common toadflax *Linaria vulgaris*. Photo by Emil Nilsson, Uppsala Näs Church, Upland

3.1.2. Mendel and the genetic era

The seminal work of Gregor Mendel (1822-1884) in the mid-nineteenth century opened up the genetic era. Some decades later, it also provided the foundation of the central tenet of molecular biology. Mendel, after conducting first ever genetic crossing experiments with sweet peas, postulated that the *phenotype* (the observed quality of an organism) is inherited by "factors" comprising of the *genotype* (the inherited genetic constitution of the individual). In the first half of the twentieth century, Mendel's factors were identified as DNA segments, or "genes", which carry the heritable information. The classic Mendelian view is reflected in the central dogma of molecular biology: that the genetic information is encoded by and inherited with the genes that drive expression of protein molecules performing cellular functions which

underlie the phenotype. Such an information flow is irreversible (Crick, 1958). Consequently, changes in the DNA sequence (=mutations) would be the underlying causes of alterations in phenotypes.

3.1.3. Modern epigenetics: variations to a theme

In the late twentieth century, it became evident that the base pairs of DNA are not the sole carriers of information inside an organism. Hence, certain phenotypes are not necessarily linked to changes in the DNA sequence (genotype). The term *epigenetics* was first used by Waddington to define "a branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being" (Waddington, 1942). Recently, though, Riggs and colleagues put forward a definition according to which epigenetics is the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained with changes in DNA sequence" (Russo et al., 1996). However, both definitions have limitations. Waddington only refers to the field of developmental biology of Metazoa, hence excluding unicellular species that also harbour mechanisms of epigenetic inheritance (discussed in Section 3.3.). By contrast, the definition put forward by Riggs and colleagues is exclusive, focusing on what epigenetics is not. Furthermore, the mechanisms stably altering gene function - that would fit Rigg's definition in theory - do not necessarily meet the criteria of being heritable in practice, as for instance in cases where they occur in terminally differentiated cells. Such constraints led Bird to propose a less restrictive definition of an epigenetic event, that is "the structural adaptation of chromosomal regions so as to register, signal and perpetuate activity states" (Bird, 2007).

3.1.4. Epilogue to a monster

Some 250 years after its discovery, it is now known that *Peloria* and *Linaria* are indeed the same species, and the "monstrous" shape of the latter is not caused by a gene mutation but a heritable silencing of a single gene without any alteration in the DNA sequence (Cubas et al., 1999). Thus, though originally falsely interpreted, Linnaeus' *Peloria* is probably the first documented case of epigenetic inheritance.

3.2. Mechanisms of epigenetic gene regulation

The following sections provide on overview of molecular mechanisms associated with epigenetic inheritance reflecting the definition of Bird. It is to note that the extent and depth of the descriptions is biased according to their relevance to the subsequent aim and results sections of this work.

3.2.1. DNA methylation

In 1986, while studying hereditary variations in somatic cell cultures, Jeggo and Holliday discovered that heritable changes in the expression of certain genes does not involve changes in the DNA sequence but is associated with the level of DNA methylation at the given locus. They proposed that: "epigenetic changes in gene activity based on DNA methylation should be referred to as epimutations to distinguish them from classical mutants which are due to changes in DNA base sequences" (Jeggo and Holliday, 1986).

22 years after Holliday's epimutation, DNA methylation is one of the best characterized modifications of chromatin. In mammals, almost all methylation occurs at the 5th carbon atom of a cytosine base (Figure 2). The methyltransfer-reaction is catalyzed either by *de novo* or maintenance DNA methyltransferases. Whereas the former act after DNA replication, the latter add methyl groups to hemimethylated DNA during replication (Groth et al., 2007). DNA methylation is essential both for normal murine development (Li et al., 1992) and for the survival of differentiated cells (Jackson-Grusby et al., 2001). In vertebrates, DNA methylation occurs almost exclusively at CpG islands (Bird, 2002) and correlates with transcriptional repression (Goll and Bestor, 2005).

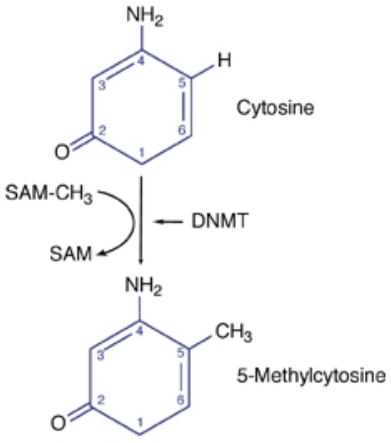

Figure 2. Creation of 5-methylcytosine. Image taken form Expert Rewievs in Molecular Medicine, 2002 Cambridge University Press. DNMT: DNA-methyltransferase, SAM: S-adenosyl methionine

Lower eukaryotes show either no or very little traces of DNA methylation. On the other hand, all three modifications (5-methylcytosine, N^4-methylcytosine and N^6-methyladenine) are found in various bacteria, where it is historically associated with restriction enzyme-based defence

mechanisms against foreign DNA such as transposons and viral DNAs (Noyer-Weidner and Trautner, 1992).

3.2.2. Histone modification

In eukaryotic cells, DNA is packaged in chromatin. The basic unit of chromatin is the nucleosome which comprises of an octamer of the four core histones: H2A, H2B, H3 and H4. 147 base pairs of DNA are wound around each nucleosome. A space of 20-80 base pairs of DNA separates individual nucleosomes. Polynucleosome fibers are folded in 30 nm filaments, which gives a compaction ratio of approximately 1:50. The fifth histone, H1 stabilizes the 30nm-fibres which are folded into higher order chromatin structures ultimately forming a chromosome (Felsenfeld and Groudine, 2003; Kornberg, 1977; Luger et al., 1997) (Figure 3).

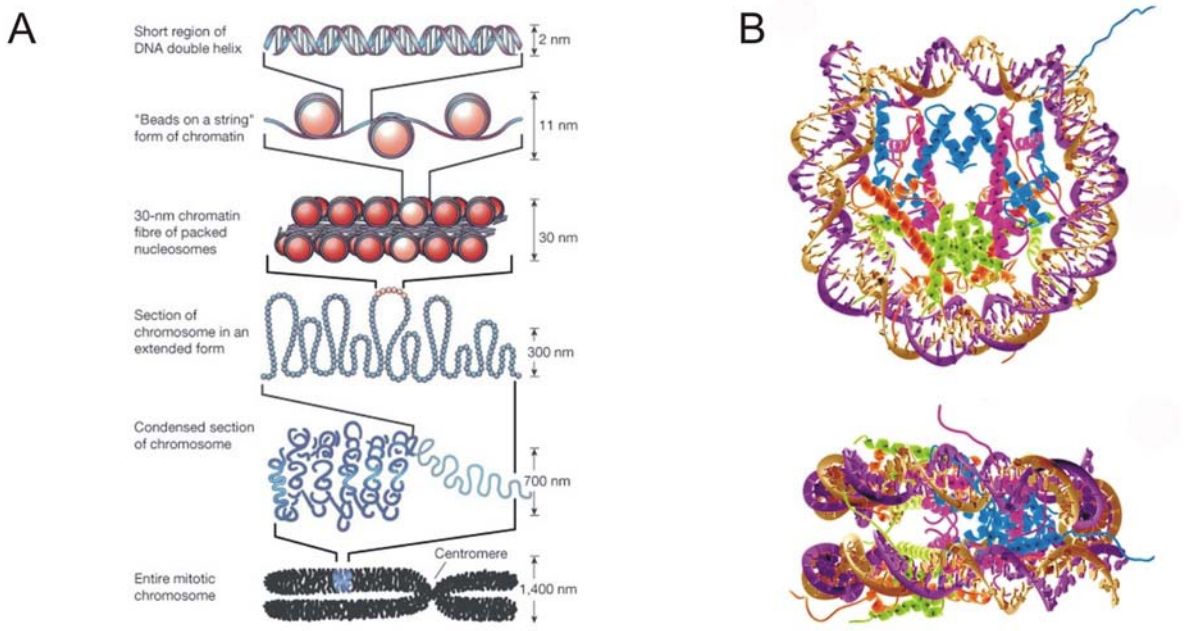

Figure 3. The structures of (A) eukaryotic chromatin and (B) the nucleosome.
(A) Architecture of eukaryotic chromosomes. Image adapted from (Felsenfeld and Groudine, 2003)
(B) 2.8Å X-ray diffraction images of a nucleosome in frontal and side views. 1.75 turns of superhelical DNA is wrapped around an octamer of the core histones. Blue: H3, purple: H4, Orange: H2A, Green: H2B. Note the protruding unstructured N-terminal tails. Image adapted from (Luger et al., 1997)

Histone proteins are highly conserved among eukaryotes. Histone equivalent proteins, as well as chromatin-like structures, have even been reported in Archaeabacteria (Sandman et al., 1998). Traditionally, histones were suggested to function in compacting DNA inside the nucleus; nevertheless, is has long been postulated that their association with DNA would influence transcription. Definitive evidence came in the 1980s, when two laboratories proved in elegant studies that the nucleosome is indeed an inhibitor of transcription initiation both *in vitro* and *in vivo* (Han and Grunstein, 1988; Lorch et al., 1987).

Interestingly, the N-terminal tails of core histones can be subjected to at least 8 types of known post-translational modifications. The modifications include acetylation, methylation, phosphorylation, sumoylation, ubiquitination, ADP-ribosylation, deimination and proline-isomerization (Table 1). To date, over 60 different residues have been identified to carry such modifications (Figure 4). Naturally, not all modifications appear on the same histone molecule at the same time. Rather, it is the dynamics and the combinations of the modifications that account for the functional outcomes, a hypothesis referred to as the "histone code" (Jenuwein and Allis, 2001). Histone modifications are suggested to have two types of mechanism of action: (1) regulating chromatin structure; and (2) regulating the recruitment of non-histone proteins, e.g. the transcription machinery, to certain chromosomal locations (Kouzarides, 2007).

Chromatin has two defined states: *euchromatin*, that is transcriptionally active, and *heterochromatin*, that remains condensed throughout the cell cycle and is transcriptionally silent. Centromeres and telomeres are trademark heterochromatic regions with crucial biological functions. For instance, proper segregation of chromosomes during mitosis requires heterochromatin formation at the centromeres (Pidoux and Allshire, 2005).

Chromatin Modifications	Residues Modified	Functions Regulated
Acetylation	**K**-ac	Transcription, Repair, Replication, Condensation
Methylation (lysines)	**K**-me1 **K**-me2 **K**-me3	Transcription, Repair
Methylation (arginines)	**R**-me1 **R**-me2a **R**-me2s	Transcription
Phosphorylation	**S**-ph **T**-ph	Transcription, Repair, Condensation
Ubiquitylation	**K**-ub	Transcription, Repair
Sumoylation	**K**-su	Transcription
ADP ribosylation	**E**-ar	Transcription
Deimination	**R > Cit**	Transcription
Proline Isomerization	**P-cis > P-trans**	Transcription

Table 1. Types of histone modifications, the list of involved residues and the functions the modifications are associated with. Adapted from (Kouzarides, 2007).

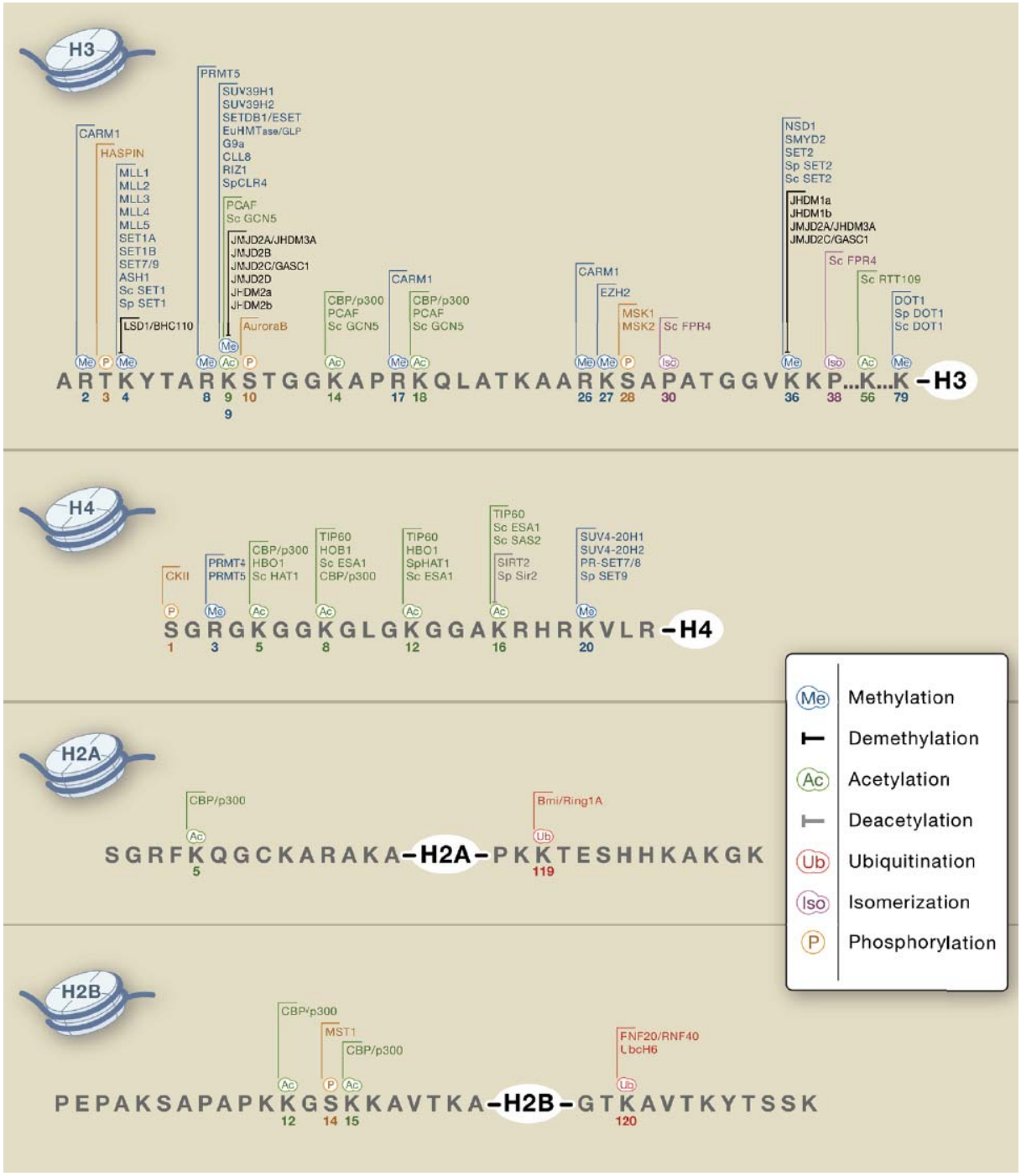

Figure 4. Various residues of the core histones can be subjected to modifications. Enzymes, whose specificity has been determined are also displayed. Image adapted from the SnapShot as appeared in the *Cell* 128 (802), February 23, 2007 issue.

There is a vast amount of knowledge generated in the past two decades about a large number of histone modifications as well as the genetic contexts they appear in. Therefore, the subsequent section aims to focus on only two of the best studied modifications that are most relevant to the study conducted in the frame of this Ph.D. thesis. For more detailed descriptions see (Kouzarides, 2007; Kurdistani and Grunstein, 2003; Martin and Zhang, 2005) and the references therein.

3.2.2.1. Histone lysine methylation

So far, 6 different lysine residues of histones H3 and H4 have been reported to be subjected to methylation: H3K4, H3K9, H3K27, H3K36, H3K79 and H4K20 (see Figure 4). Lysines can be mono-, di- or trimethylated and the methylated forms have been associated both with transcriptional activation as well as repression. The methylation patterns of lysines are dynamic and the reactions are catalyzed by methyltransferases and demethylases. Out of all histone-modifying enzymes, methyltransferases are the most specific ones (Martin and Zhang, 2005).

H3K4 methylation

Methylated H3K4 is associated with actively transcribed genes in many organisms, yet different methylation statuses do not overlap. Trimethylation of H3K4 localizes to the promoter regions and the 5' end of coding regions, whereas dimethylation is distributed along the coding region of virtually all actively transcribed genes. The genome of *Saccharomyces cerevisiae* encodes one protein, Set1, responsible for all three methylation states of H3K4. By contrast, genomes of higher eukaryotes usually encode more, indicating a division of labour. The methylation process seems to be coupled to transcription, since Set1 was shown to physically associate with RNA polymerase II during the elongation process (Millar and Grunstein, 2006; Ng et al., 2003).

H3K9 methylation

The H3K9 methyltransferase Su(var)3-9 was one of the first histone-modifying enzymes identified in a *Drosophila* screen for genes suppressing the silencing of transgenes integrated at heterochromatic regions, a phenomenon termed *position effect variegation*. The role of H3K9 methylation in heterochromatin formation is conserved. For example, in fission yeast *Schizosaccharomyces pombe*, the Su(var)3-9 homolog Clr4 also mediates heterochromatin formation at the silent mating type loci (Martin and Zhang, 2005; Tschiersch et al., 1994).

H3K27 methylation

The methylation of H3K27 has also been linked to several silencing phenomena, like X-inactivation and genomic imprinting in mammals and polycomb-mediated gene silencing in *Drosophila* (Martin and Zhang, 2005).

H3K36 methylation

Similar to H3K4, methylation of H3K36 is associated with transcriptional activation, yet its precise function remains largely unknown. In *S. cerevisiae*, the enzyme Set2 catalyzes the methylation of H3K36, and like Set1, physically interacts with the RNA polymerase II complex (Krogan et al., 2003; Martin and Zhang, 2005).

H3K79 methylation

To date, H3K79 remains a largely underinvestigated modification. The *S. cerevisiae* H3K79-methyltransferase Dot1 prevents the spreading of histone deacyetylases into transcriptionally active chromosomal regions, thus linking H3K79 to transcriptional activation (Ng et al., 2002).

H4K20 methylation

In addition to methylated H3K9, methylated H4K20 is also a marker of heterochromatin in mammals. Furthermore, in mammals it is also linked to proper progression through the cell cycle, whereas in *S. pombe* the H4K20-methyltransferase Set9 was shown to function not in heterochromatin formation but in DNA break repair (Sanders et al., 2004; Schotta et al., 2004).

3.2.2.2. Histone lysine acetylation

Studies in the 1960s already demonstrated that increased acetylation of histones correlates with increased transcription. Notably, substitution of histones with positively charged peptides like polylysine in these experiments had the same effect (Allfrey et al., 1964). Contrary to methylation, acetylation neutralizes the positive charge of lysine, thereby altering the electrostatic relationship between nucleosomes and negatively charged DNA. The weakened electrostatic binding of nucleosomes thus loosens up chromatin, making DNA more accessible to the transcription machinery, as well as to other binding factors. This idea is supported by the finding that on a global scale histones display hyperacetylation at euchromatic (active) relative to heterochromatic (silent) regions, the latter being largely hypoacetylated. On the other hand, using *in vitro* reconstituted nucleosomes, it has also been demonstrated that histone-DNA interactions are not weakened by increased acetylation. Instead, it is proposed that acetylation interferes with the folding of higher order chromatin

structures to inhibit transcription. Taken together, it seems likely that acetylation affects chromatin state and DNA-based processes by various mechanisms (Kurdistani and Grunstein, 2003; Mutskov et al., 1998).

So far, more than 10 lysine residues of core histones have been shown to be subject to acetylation (Figure 4). Contrary to methylation, single residues are very scarcely associated with distinct functions. Acetylation and deacetylation is dynamic process and is catalyzed by histone acetyltransferases (HATs) and deacetylases (HDACs). In addition, HATs and HDACs have a lot broader substrate preference than the metyltransferases. Therefore, this section aims to provide an overview based on a functional categorization rather then a structural one.

Transcription

Acetylation is mostly associated with activation of transcription, whereas reversal of acetylation in most cases correlates with transcriptional repression. Numerous enzymes that catalyze histone acetylation and deacetylation have been identified as transcriptional co-regulators. Histone acetyltransferases (HATs) are grouped in three distinct families based on the sequences of their catalytical domains: these are the GNAT, MYST and CBP/p300 families. HATs display fairly broad substrate specificities and the substrates of various HAT complexes overlap (Table 2). Histone deacetylases (HDACs) are a lot more diverse set of enzymes, do not show much specificity for a particular acetylated residue and are grouped in three classes rather than families (Lee and Workman, 2007; Yang and Seto, 2008).

HAT complexes of the GNAT family									
SAGA (*Sc*)	**SLIK** (*Sc*)	**ADA** (*Sc*)	**HAT-A2** (*Sc*)	**SAGA** (*Dm*)	**ATAC** (*Dm*)	**PCAF** (*Hs*)	**STAGA** (*Hs*)	**TFTC** (*Hs*)	**HATB** (*Sc*)
Catalytic subunit									
Gcn5	Gcn5	Gcn5	Gcn5	GCN5	GCN5	PCAF	GCN5L	GCN5L	Hat1
Histones modified									
H2B/ H3/H4	H2B/ H3/H4	H3	H3	H3	H3/H4	H3/H4	H3/H4	H3/H4	H2A/H4

HAT complexes of the MYST family							
NuA4 (*Sc*)	**Pic. NuA4** (*Sc*)	**NuA3** (*Sc*)	**SAS** (*Sc*)	**TIP60** (*Dm/Hs*)	**HBO1** (*Hs*)	**MOZ/MORF** (*Hs*)	**MSL** (*Dm*)
Catalytic subunit							
Esa1	Esa1	Sas3	Sas2	TIP60	HBO1	MOZ/ MORF	MOF
Histones modified							
H2A/H4	H2A/H4	H3	H4	H2A/H4	H3/H4	H3	H4

Table 2. Members of the GNAT and MYST histone acetyltransferase families. Adapted from (Lee and Workman, 2007).

Both HATs and HDACs can be recruited to their target locations in a specific manner. For example, the transcriptional activator Gcn4 is able to recruit the HAT Gcn5 to the promoter of the *HIS3* gene in yeast (Kuo et al., 2000). The Rpd3 deacetylase (a class I HDAC) is recruited by the DNA-binding protein Ume6 to a specific DNA element of the *INO1* promoter to regulate inositol biosynthesis in *S. cerevisiae* (Kadosh and Struhl, 1997). In addition, Hda1 (a class II HDAC) is recruited by the transcriptional repressor Tup1 to its target promoters in yeast (Wu et al., 2001). On the other hand, HATs and HDACs can modify histones throughout the genome in a global and random manner, apparently independently of sequence-specific DNA-binding factors (Figure 5). Targeted (de)acetylation mostly occurs at promoter regions, whereas global (de)acetylation does not discriminate between promoters and coding regions.

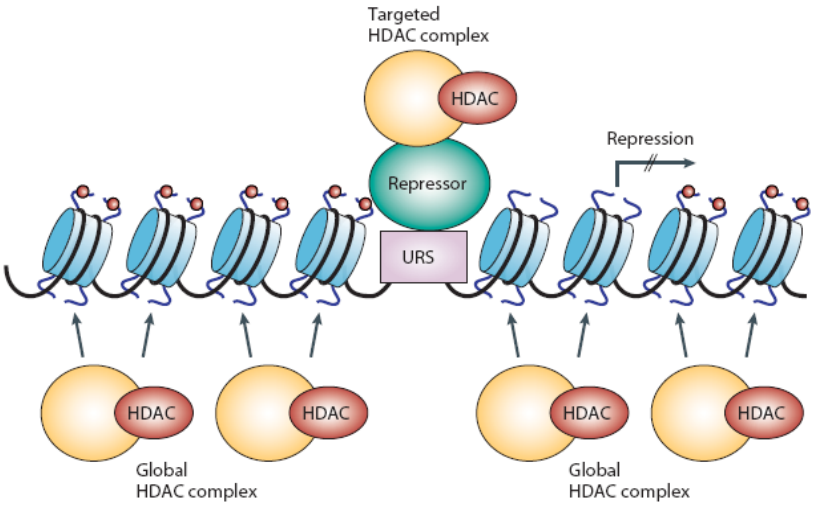

Figure 5. Model of site-specific and global targeting of HDACs. URS: Upstream Repressive Sequence. Image adapted from (Kurdistani and Grunstein, 2003)

One interesting example for a HDAC required for gene activity is *S. cerevisiae* Hos2 (a class II HDAC). Hos2 associates with the coding region of active genes without affecting the basal transcriptional level, but in the absence of Hos2 transcription takes place slower once the gene is activated (Wang et al., 2002).

Heterochromatin formation

HATs and HDACs not only function as transcriptional co-regulators. Acetylation and deacetylation may influence the recruitment of protein complexes to chromatin. For instance, in *S. cerevisiae* the silent mating-type loci are in the heterochromatin state by a silencing complex that includes Sir2 (an HDAC), Sir3 and Sir4. To establish heterochromatin, Sir4

recruits Sir3 and Sir2, but the interaction requires an intact H4 tail. Sir2 deacetlylates H4K16 on the neighbouring nucleosomes. Sir3 in turn binds to deacetylated H4K16, recruits more Sir4 which recruits more Sir2 to start the cycle again thus spreading the heterochromatin state. The boundaries of heterochromatin seem determined by largely unknown mechanisms depending on the activities of the HAT Sas2, which is catalytically active on H4K16 (Rusche et al., 2002; Suka et al., 2002).

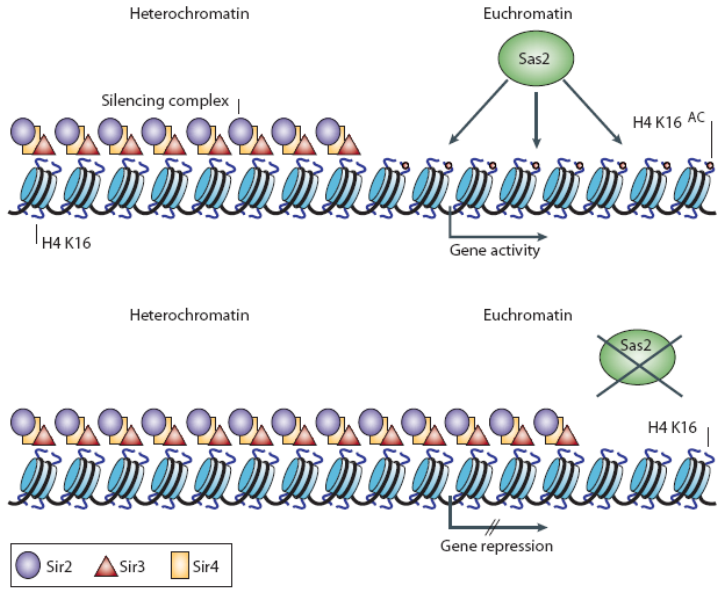

Figure 6. Heterochromatin formation at the *S. cerevisiae* silent mating type loci. The Sir silencing complex deacetylates H4K16 which is counteracted by the opposing HAT Sas2, thereby the border between the eu- and heterochromatic regions is determined in a dynamic fashion. Image adapted from (Kurdistani and Grunstein, 2003)

Replication

DNA replication in yeast and many other organisms starts at origins of replication. The origins start firing at various times during S-phase. Although the precise mechanisms when origins fire are unknown, the acetylation state of chromatin around the origin was demonstrated to influence its time of firing (Vogelauer et al., 2002). Likewise, in human cells the putative HAT Hbo1 was shown to associate with components of the replication machinery and replication timing shows correlation with histone acetylation (Iizuka et al., 2006).

DNA repair

Histone acetylation has been implicated in specific DNA repair pathways. For example, lysine acetylation of H4 and the HAT Hat1 were shown to be required for double stranded break repair (Qin and Parthun, 2006).

3.2.3. Noncoding RNA

Recently, compelling evidence emerged showing that noncoding RNAs regulate multiple epigenetic phenomena. Several noncoding RNA-dependent pathways have been investigated and were found to influence histone modifications. Such phenomena include centromeric silencing in certain fungi such as *Schizosaccharomyces pombe*, silencing of transposons and repetitive elements in plants, dosage compensation in *Drosophila* and mammals, and paramutations in maize and mice (Bernstein and Allis, 2005; Chandler, 2007; Zaratiegui et al., 2007).

3.3. Epigenetic phenomena in the microbial world

Although most of the molecular mechanisms underlying epigenetic inheritance were investigated in multicellular eukaryotes, the microbial world is also riddled with related phenomena. Phenotypic heterogeneity, i.e. marked phenotypic differences in a given microbial population has traditionally been attributed to the arousal of mutations. However, it has been recently appreciated that genetically homogenous microbial populations may also display a wide range of distinct phenotypes (Avery, 2006). Population level diversity in general is proposed to enhance the chances of survival in fluctuating environments and to enable colonization of new niches (Aertsen and Michiels, 2005; Booth, 2002; Stewart and Franklin, 2008). Such phenomena are especially important because of their clinical relevance, namely as subpopulations of microbial pathogens often display variable degrees of virulence and resistance to antibiotics depending on the actual host environment. For example, the clinical term "persistence" refers to a subpopulation a homogenous microbial population that is able to survive antibiotic treatment. Persistent cells, or persistence-type effects have been reported in a variety of pathogens, including *Staphylococcus aureus* (Massey et al., 2001), *Pseudomonas aeruginosa* (Drenkard and Ausubel, 2002), *Escherichia coli* (Balaban et al., 2004) and the malaria parasite *Plasmodium falciparum* (Ralph et al., 2005). Importantly, persistence is distinct from resistance, in which case antibiotic tolerance arises from mutations of DNA as described for instance in most cases of *Mycobacterium tuberculosis* infections (Sacchettini et al., 2008). Furthermore, a persistent phenotype is reversible as opposed to genomic mutations that are stably inherited. In addition to persistence, many phenotypes have been associated with microbial phenotypic heterogeneity, including variable expression of cell surface antigens, differences in cell motility or ability to sporulate (Table 3).

Phenotype	Comments	Underlying driver (and organisms investigated)
Variable expression of cell-surface antigens	Different epigenetic mechanisms operate in different systems	Epigenetic (*Plasmodium falciparum*, *Trypansoma brucei*, *Saccharomyces cerevisiae* and *Escherichia coli*)*
Phenotypic switching	White–opaque switching requires heterozygosity or hemizygosity at the mating-type locus. Histone deacetylases modulate switch frequency	Probably epigenetic (*Candida* spp.)
Antibiotic persistence	The proportion of persisters is influenced by the *hipBA* toxin-antitoxin module	Stochastic?‡ (*E. coli*)
Heterogeneous heat resistance	Variable expression of heat-shock-protein-encoding genes	Cell cycle and stochastic? (*S. cerevisiae* and *Salmonella enterica* serovar Typhimurium)
Heterogeneous DNA damage	Several mechanisms suggested	Ageing and ultradian rhythm? (*S. cerevisiae*) Stochastic? (*E. coli*)
Heterogeneous resistance to metals and pro-oxidants	Several deterministic mechanisms demonstrated	Cell cycle, ultradian rhythm and ageing (*S. cerevisiae*)
Bacterial motility and chemotaxis	Variable activity in *E. coli* of the CheR methyltransferase, a key upstream component of the chemotaxis signalling network	Stochastic? (*E. coli* and *Bacillus subtilis*)

Table 3. Microbial phenotypes associated with phenotypic heterogeneity. Adapted from (Avery, 2006)

The mechanisms underlying microbial phenotypic diversity can be both genetic and epigenetic and both seem mutually non-exclusive. Generally, genetic and epigenetic strategies differ in several aspects including responsiveness, stability and evolvability. The mutation rate of genetic changes varies on average around 10^{-6} to 10^{-8} per base pair per generation for most microbes (though the rate is not the same across all loci of the genome). Hence, generating alternative phenotypes by point mutations is considered to be a rather slow process. On the other hand, alteration between two states (switching) by epigenetic mechanisms allows for a more rapid phenotypic change. Most of the reported switching systems have indeed frequencies of 10^{-3} to 10^{-5} per generation, and in extreme cases can be as high as 10^{-1}. Furthermore, genetic changes can be stably inherited for many generations (approximately 10^{8}), whereas epigenetic changes are more frequently reversed. Timing is proposed to be a key feature. For instance, the malaria pathogen *Plasmodium falciparum* can switch between different states that have different antigenic properties. The switching rate is presumably fine-tuned, such that the time of exposure of the antigen is shorter than the time required for an effective immune response (Rando and Verstrepen, 2007).

Switching between alternating states is suggested to enhance the adaptability of the population to a wider range of environmental stimuli. There are two (extreme) types of phenotypic switching systems considered. *Responsive switching* occurs as a direct consequence of an environmental cue that is usually sensed by a signalling apparatus. *Stochastic switching* occurs spontaneously without direct sensing of the environment In the latter case varying phenotypes are generated at a random fashion, yet their abilities to adapt to different environmental conditions differ (Figure 7). Theoretical models postulate, that stochastic switching is favourable when the environment does not change frequently, whereas the cells prefer to maintain a signalling apparatus if their environment often fluctuates (Kaern et al., 2005; Kussell and Leibler, 2005). To date, there is still very little evidence linking theoretical models to experimental scenarios.

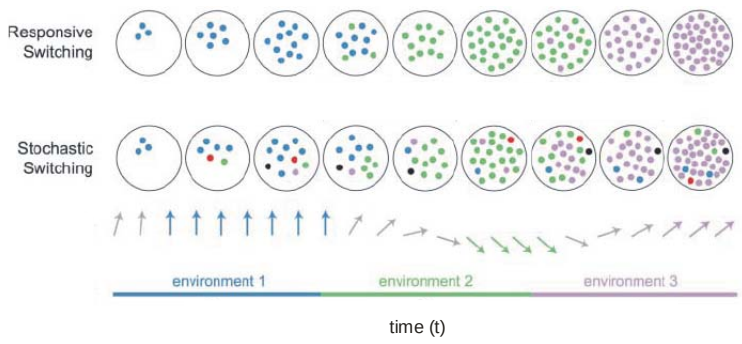

Figure 7. Responsive vs stochastic switching. Colours denote the different status of an environmental parameter as well as the phenotypes that favour that specific environmental status. The composition of the microbial population in both cases follows the changes of the environment albeit in a different fashion. Adapted from (Kussell and Leibler, 2005).

3.4. The genus *Candida* as opportunistic pathogens

Candida spp are commensal fungi found on mucosal surfaces and in the gastrointestinal tract of humans as well as a few another mammalian species. However, they can cause local mucosal infections and systemic candidaemia particularly when the immune system is weakened or when the competing microflora is eliminated. About 75% of all healthy women have at least one local vaginal infection in their lifetime; whereas patients with HIV infections, individuals going through chemotherapy or receiving antibiotic treatment are especially susceptible to develop *Candida* infections. In clinical practice, the most commonly encountered species of the genus is *C. albicans*, while other important pathogenic species include *C. glabrata*, *C. krusei*, *C. parapsilosis*, *C. dubliniensis* and *C. tropicalis*. In comparison to other pathogenic fungi, *C. albicans* ranks as the fourth most common hospital-acquired infection in the United States (Berman and Sudbery, 2002; Haynes, 2001). The high incidence of *Candida* and especially *C. albicans* infections has facilitated an extensive research of this genus.

3.5. *Candida albicans*

Candida albicans is a human fungal pathogen, perhaps first isolated by Robin from a case of thrush (yeast infection) in 1853 and named *Oidium albicans* (Robin, 1853) (Figure 8). The genus *Candida* was later created by Berkhout in 1923 for 9 species that she described as: "Few hyphae, prostrate, braking up into shorter or longer pieces. Conidia, arising by budding from the hyphae, or on top of each other, are small and hyaline" (Berkhout, 1923). Since the traditional classification of fungi relied on the sexual cycle and morphology of the spores, and *Candida spp* did not exhibit an apparent sexual cycle, the genus was grouped into the division of "Fungi Imperfecti." In the genomic era, where ribosomal RNA sequences became the basis of evolutionary classification (Blantz, 1987), the genus *Candida* – today comprising some 150 species - was moved to the Phylum Ascomycota, Classis Saccharomycetales, where the non-pathogenic baker's yeast *Saccharomyces cerevisiae* also belongs (Figure 9).

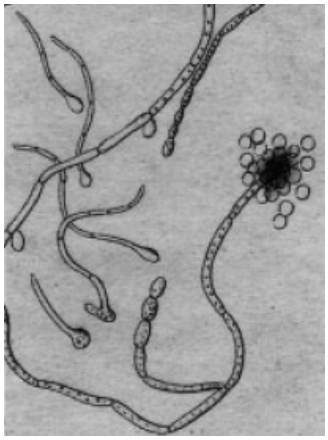

Figure 8. Robin's original drawing of *Oidium albicans*. Image adapted from (Barnett, 2004).

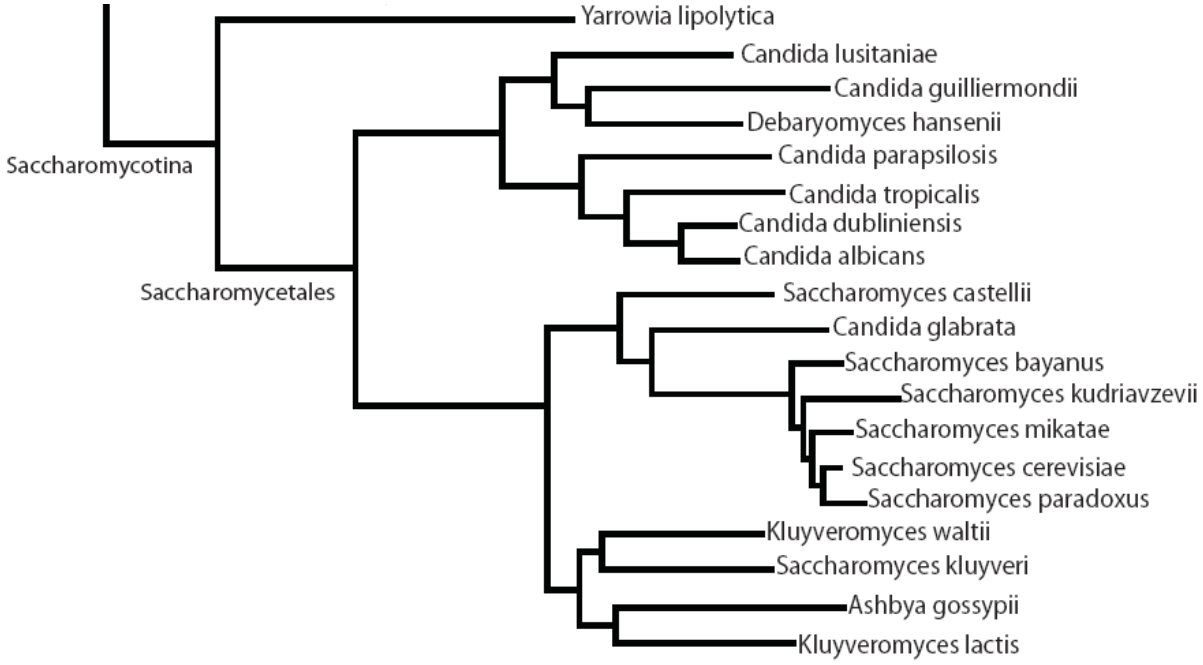

Figure 9. Simplified molecular phylogenetic tree of the Subphylum Saccharomycotina. Image adapted from (Fitzpatrick et al., 2006)

3.5.1. Discovery of mating in *C. albicans*

As mentioned above, the vast majority of *Candida* species do not have a described teleomorph (sexual generation) and are considered asexual. Interestingly, the molecular phylogeny classified the genus as having a close relationship with sexually reproducing species such as *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. Although *C. albicans* populations were indeed demonstrated to propagate clonally, traces of recombination were also found, which eventually culminated in the discovery of *C. albicans* mating both *in vitro* and *in vivo* in 2000 (Graser et al., 1996; Hull et al., 2000; Magee and Magee, 2000).

C. albicans and *S. cerevisiae* are related species separated by an evolutionary distance of several hundred million years. The sexual cycle of *S. cerevisiae* is well understood, the following section will thus focus on a comparison between the similarities and differences of the sexual cycles of these two species. More detailed comparisons are found in (Bennett and Johnson, 2005; Johnson, 2003) and the references therein.

S. cerevisiae has 16 chromosomes and its haploid genome contains roughly 5600 genes. *C. albicans* isolates are diploid harbouring 8 chromosomes and about 6500 genes. Approximately 4000 genes of the two species show strong homologies and presumably share the same or similar biochemical functions.

The mating behaviour in fungi is generally controlled by a single locus known as the mating-type (*MAT*) locus. In *S. cerevisiae* the *MAT* locus encodes DNA-binding factors, and has two alleles called "**a**" and "α." Haploid *MAT*a cells express a set of "**a**-specific" genes and mate as "**a**" cells, whereas haploid *MAT*α cells express a set of "α-specific" genes and mate as "α" cells. The "α" allele encodes two transcription factors, "α1" and "α2," the former promotes "α-mating", whereas the latter represses a set of "**a**-specific" genes. Cells are only able to mate with another cell of the opposite mating type. In the *MAT*a/α diploid mating products a heterodimer **a1**/α2

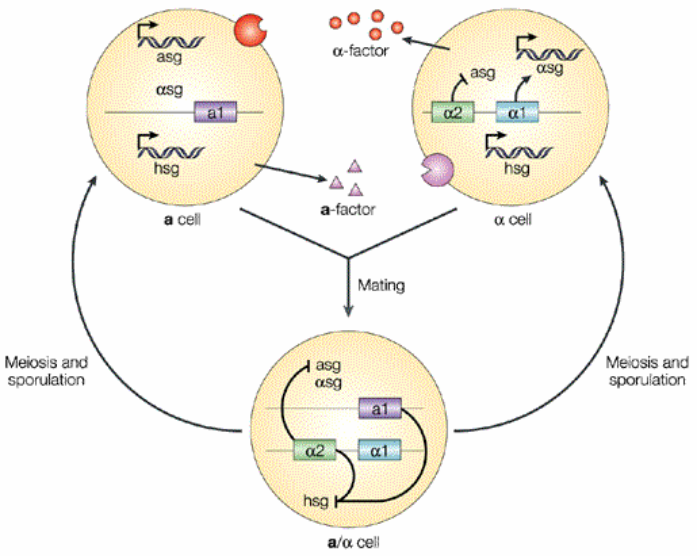

Figure 10. The life cycle of *S. cerevisiae*. Haploid **a** and α cells mate to form a diploid **a**/α progeny that returns to the haploid state through meiosis. asg: "**a**-specific" gene, αsg: "α-specific" gene, hsg: haploid-specific gene. Image adapted from (Johnson, 2003)

repressor, encoded in the "**a**" and "α" alleles, respectively, blocks mating. The diploid cells may, however, undergo meiosis to produce four haploid progeny, two of which with "**a**" and two with "α" mating types (Figure 10) (Johnson, 2003).

In 1999, the mating locus of *C. albicans* was discovered as a chromosomal region showing similarities to the *S. cerevisiae MAT* locus (Hull and Johnson, 1999). The locus was termed Mating-type like (*MTL*). The *C. albicans MTL* locus (9 kB) is much larger then the *MAT* locus of *S. cerevisiae* (0.7 kB), and the boundaries of the former are defined, so as to include three additional open reading frames (ORF) that showed allelic differences between the individual chromosomes of a diploid cell. These ORFs are: *PAP*, polyA polymerase; *PIK*, phospatidyl-inositol kinase; and *OBP*, a homolog of the human oxysterol binding proteins (Figure 11). Whereas the majority of *C. albicans* ORFs display an identity over 90% at the DNA level, *PAPa – PAPα, PIKa – PIKα* and *OBPa – OBPα* allelic pairs share an identity of about 60%. Another key difference is that the *MTL***a** allele of *C. albicans* encodes two transcription factors termed "**a1**" and "**a2**" rather than one as in the *MAT***a** locus of *S. cerevisiae*. Analysis of the *MTL* loci of the most common laboratory strains at that time revealed that they are all *MTL***a**/α heterozygotes, explaining why *C. albicans* mating could not be observed. Subsequently, it was indeed demonstrated that diploid *MTL***a**/**a** and *MTL*α/α are able to form tetraploid mating products in a murine model and on laboratory medium (Hull et al., 2000; Magee and Magee, 2000). However, the mating efficiency in both studies was extremely low, raising the question whether *C. albicans* mating indeed occurred *in vivo*.

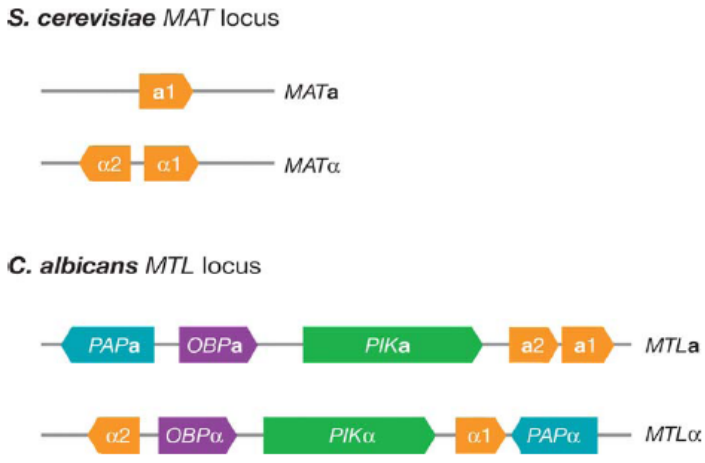

Figure 11. Comparison of the Mating type locus of *S. cerevisiae* and the Mating type-like locus of *C. albicans*. Image adapted from (Bennett and Johnson, 2005).

3.5.2. The *white-opaque* switch

*S. cerevisiae MAT***a** and *MAT*α cells are able to mate with haploid cells of the opposite mating type with a high efficiency. *C. albicans MTL***a**/**a** and *MTL*α/α cells, however, need to undergo a phenotypic transition called the *white-opaque* switch to become mating-competent.

White-opaque switching is an epigenetic phenomenon discovered in 1987. It represents the reversible alternation of *C. albicans* strains between two phenotypic states termed *white* and *opaque*. *White* phase cells have a round yeast shape and form dome-shaped white-coloured colonies on solid agar, whereas *opaque* cells display a longer, elongated shape and form flatter, opaque-looking colonies that can be stained red on media containing the dye Phloxin B (Figure 12) (Slutsky et al., 1987). The phases are heritable for many generations, and switching between two cell types is reversible, occurring at a frequency of roughly ~10^{-3}-10^{-4} (Rikkerink et al., 1988). Approximately 400 genes are differentially expressed in *white* and *opaque* cells, suggesting that a significant percentage (8%) of the genome is involved in switching (Lan et al., 2002). The two phases were first studied for their virulence properties in various *C. albicans*-host models. *Opaque* cells were found to be more potent colonizers of the skin while *white* cells were more virulent in a systemic murine infection model (Kvaal et al., 1999). Consequently, it was proposed that switching between the two phases might constitute a strategy to counteract or evade host defence mechanisms. *White-opaque* switching, nevertheless, was observed only in a limited number of clinical isolates, and vast majority of the isolates displayed only the predominant *white* phase; therefore the significance of *white-opaque* switching was not yet fully appreciated.

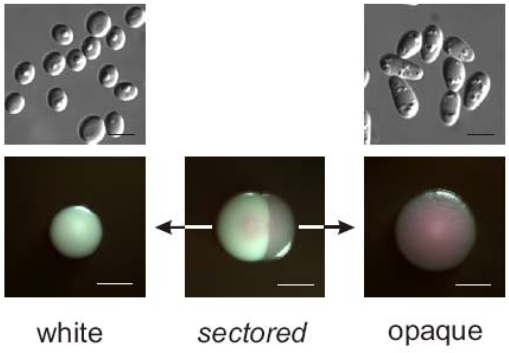

Figure 12. The *white* and *opaque* cell types of *C. albicans*. Scale bars correspond to 5µm (upper panel) and 2mm (lower panel).

3.5.3. *White-opaque* switching links the *MTL* locus to mating

Two years after the discovery of the *MTL* locus and sexual recombination in *C. albicans*, Miller and Johnson revealed a crucial connection between *white-opaque* switching and mating (Miller and Johnson, 2002). The key findings included that (1) the ability to undergo the *white*-to-*opaque* transition was controlled by the *MTL* locus. Engineered *MTL* hemizygous cells readily converted from the *white* to the *opaque* phase, whereas *MTL* heterozygotes remained locked in the *white* phase. (2) *Opaque* phase cells mated about 10^6 times more efficiently with *opaque* cells of the opposite mating type than *white* phase cells. These results

clarified the physiological role of *white-opaque* switching, namely, that it represents an additional step required for efficient mating in *C. albicans* as compared to *S. cerevisiae*. The results also explained prior studies that detected only low mating frequencies in *C. albicans*: as in these experiments *white* phase cultures were used. The former suggestion was confirmed by the analysis of a large set of *C. albicans* clinical isolates revealing that strains able to undergo the *white-opaque* transition are *MTL* homzoygotes, whereas *MTL* heterozygotes appeared to be locked in the *white* phase (Lockhart et al., 2002).

Opaque phase **a/a** and α/α *C. albicans* readily mate. The mating products may undergo karyogamy and form a tetraploid progeny, that harbours an *MTL***a**/**a**/α/α configuration displaying a *white* phase morphology (Miller and Johnson, 2002). The tetraploid cells appear stable, and there is no report of tetraploid cells undergoing meiosis, to date. However, on certain media, tetraploids were demonstrated to return to the diploid state through a loss of chromosomes. The chromosomes were shown to be lost in a random and concerted fashion, thus completing a parasexual life-cycle (Figure 13) (Bennett and Johnson, 2003). Such parasexual cycles were also observed in other fungal species belonging to the *Aspergillus* and *Penicillum* genera (Clutterbuck, 1992; Durand et al., 1993).

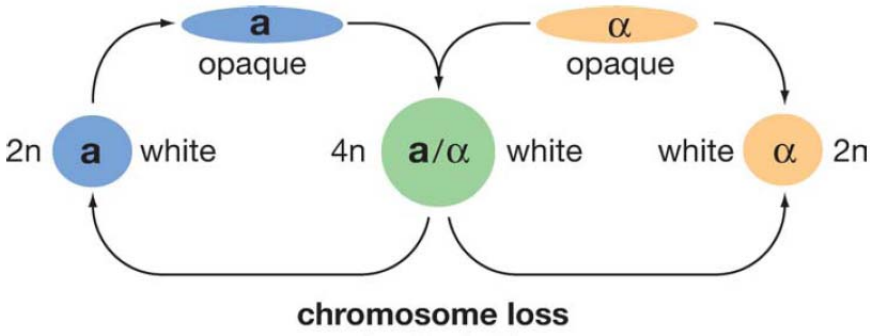

Figure 13. Life cycle of *C. albicans*. Diploid *MTL***a**/**a** and *MTL*α/α cells must undergo the white-to-opaque transition to become mating competent. Opaque cells are able to mate with opaque cells of the opposite mating type to form a tetraploid progeny. The mating products return to the diploid state through chromosome loss. Image adapted from (Bennett and Johnson, 2003)

The discovery of the inherent linkage between *white-opaque* switching and mating led to the hypothesis that switching evolved to limit mating to preferred host niches *in vivo*. The idea is supported by the findings that *white* cells are more virulent in a systemic model of infection, while *opaque* cells are better colonizers of the skin (Kvaal et al., 1999). Also, *white* cells are less sensitive to oxidative stress and killing by murine neutrophil granulocytes than *opaque* phase cells (Kolotila and Diamond, 1990). *Opaque* cells are stable at 25°C but convert *en*

masse to the *white* phase at 37°C *in vitro* (Rikkerink et al., 1988) which implied that *opaque* conversion and mating are likely to occur on the skin of the human host. Indeed, mating was demonstrated to occur at high frequencies on the skin of newborn mice (Lachke et al., 2003). Recently, it was also shown that anaerobic conditions stabilize the *opaque* phase even at 37°C (Dumitru et al., 2007), implying that mating could take place in anaerobic niches inside the human host, including the gastrointestinal tract.

On the other hand, the majority of *C. albicans* strains reproduce clonally in infection models and mating still appears as a rare process *in vivo*. Also, isolation of tetraploid strains (that appear stable in the laboratory) was not yet reported from human patients. Since the *white* and *opaque* cells interact differentially with the host (see above), some experts suggest that the *in vivo* function of mating and recombination is not the generation of variety. Rather, mating persisted because it is linked to *white-opaque* switching and it is in fact the *white-opaque* transition that has a significance for the survival of *C. albicans* in the human host (Magee and Magee, 2004). Such a rationale is supported by the finding that diploid strains are more virulent in a disseminated model of infection (Ibrahim et al., 2005), and that *Drosophila* S2 cells and murine macrophages differentially phagocytose *white* and *opaque* cells *in vitro* (Lohse and Johnson, 2008).

3.5.4. Regulation of *white-opaque* switching

The *white* and *opaque* phases are heritable and switching between phases is reversible. So far, no change in DNA sequence has been associated with it; in addition, early studies suggested that the regulation might involve a metastable change of the chromatin status of a master switch locus (Soll, 1997). The hypothesis was supported by two studies demonstrating that deletion of histone deacetylase genes *HDA1* and *RPD3* altered the frequency of *white-opaque* switching (Klar et al., 2001; Srikantha et al., 2001). The phases are thus considered as two transcriptional states of cells harbouring identical genomes. *White-opaque* switching, therefore, fulfils the requirements of an epigenetic phenomenon as defined by both Riggs and Bird (see section 3.1.3.) which is also supported by the function of Hda1 and Rpd3.

MTL *homozygosis*

The study by Miller and Johnson established that *MTL***a**/**a** and *MTL*α/α cells are able to switch from the *white* to the *opaque* phase, whereas *MTL***a**/α cells are locked in the *white* phase

(Miller and Johnson, 2002). However, most clinical isolates are *MTL***a**/α which apparently argues against the relevance of mating *in vivo*.

C. albicans cells have been reported to selectively lose one copy of chromosome 5 on sorbose medium *in vitro*. Chromosome 5 encodes at least 5 distant negative regulators of the *SOU1* gene, whose gene product is responsible for sorbose utilization; the loss of an entire chromosome is suggested to be a mechanism of eliminating all negative effects on *SOU1* expression elevating the level of Sou1. Notably, the *MTL* locus is also located on chromosome 5. Thus the monosomic derivatives are *MTL* hemizygotes and behave as either "**a**" or "α" cells. After replacing sorbose by glucose in the medium, the *MTL* hemizygous cells reduplicate chromosome 5 and thus become homozygotes for all genes on chromosome 5, including the *MTL* locus (Janbon et al., 1998; Kabir et al., 2005). Furthermore, mitotic recombination is also suggested to convert *MTL* heterozygotes to homozygotes. Interestingly, chromosome loss and reduplication seems to be a much more frequent event *in vitro* (Wu et al., 2005); however, Single Nucleotide Polymorphism (SNP) mapping revealed that *C. albicans* strains are able to undergo mitotic recombination events at significant frequencies when passaged through mice (Forche et al., 2005). Consequently, loss and reduplication of chromosome 5, as well as mitotic recombination are potential mechanisms to generate switching and mating competent *MTL* homozygotes *in vivo*.

Interestingly, *MTL* heterozygotes can also switch to the *opaque* phase. Pendrak et al have recently identified the essential *HBR* gene (Hemoglobin Response 1) encoding a positive regulator of *MTLα1* and *MTLα2* expression. *HBR1/hbr1* cells express *MTLα1* and *MTLα2* at undetectable levels, and appear phenotypically as "**a**" cells. Hence they can undergo both *white-opaque* switching and mating (Figure 14). The *HBR1* gene hence provides a paradigm that host stimuli are able to influence the expression of *MTL* genes, which again is a potential mechanism to generate switching and mating competent cells *in vivo* (Pendrak et al., 2004).

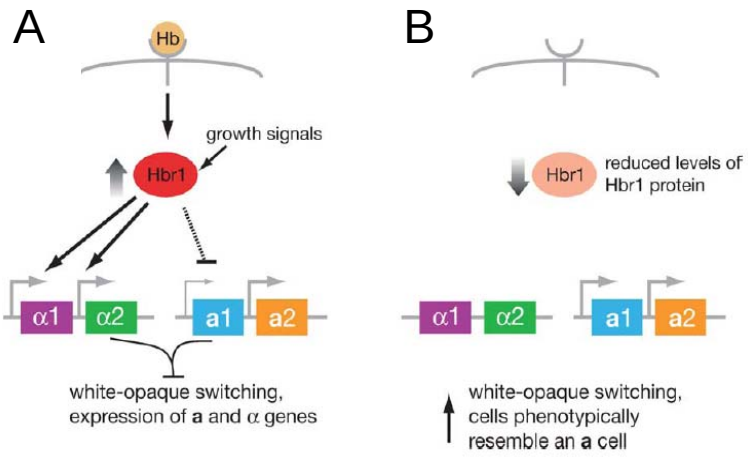

Figure 14. Hemoglobin regulates *MTLα* genes through Hbr1. (A) Hemoglobin stimulates Hbr1 which in turn positively regulates *MTLα* genes and represses *MTL*a1. Under these conditions, the **a1**α2 heterodimer blocks white-opaque switching and mating. (B) In the absence of hemoglobin Hbr1 level is reduced. Under such conditions *MTLα* genes are turned off and the cell behaves as an "**a**" cell. Image adapted from (Bennett and Johnson, 2005)

The master switch locus

Microarray analysis and chromatin immunoprecipitation experiments confirmed that in *MTL***a**/α cells an **a1**/α2 heterodimer encoded by the *MTL***a** and *MTL*α alleles, respectively (similar to the *S. cerevisiae* **a1**/α2 repressor), blocks expression of several genes including *WOR1* (White-Opaque Regulator 1), the master switch locus. Deletion of *WOR1* locks otherwise switching competent *MTL* homozygous cells in the *white* phase, while ectopic overexpression of *WOR1* drives *white* cells into the *opaque* phase. *WOR1* is expressed at low levels in *white* cells and at high levels in *opaque* cells. Stochastic (or bistable) expression of *WOR1* has been proposed to drive the conversion from *white* to *opaque* (Huang et al., 2006; Miller and Johnson, 2002; Srikantha et al., 2006; Zordan et al., 2006).

The transcriptional feedback model

According to the recent model, based on the analysis of *WOR1* as the master switch locus, the stochastic fluctuation of *WOR1* expression needs to reach an as yet undefined critical threshold to activate downstream effects to initiate the conversion to the *opaque* phase. Furthermore, Wor1 coordinates multiple positive feedback loops that ensure stable, high level expression of *WOR1*, which is proposed to account for the heritability of the *opaque* phase. So far, 3 different feedback loops converging to the *WOR1* locus have been identified (Figure 15).

The autocatalytic loop. Ectopic overexpression of *WOR1* was shown to increase the expression of the endogenous *WOR1* locus. Furthermore, Wor1 was demonstrated to bind at the intergenic region upstream of the *WOR1* ORF, suggesting that Wor1 forms a positive feed-forward loop to activate *WOR1* expression (Huang et al., 2006; Zordan et al., 2006; Zordan et al., 2007).

The co-factor loop. Wor1 also binds to the promoter region of its putative cofactor *WOR2*. *WOR2* is enriched in *opaque* phase cells and is required to maintain stable, high levels of Wor1. *wor2/wor2 white* cells are able to convert to *opaque* at an extremely low frequency and the *opaque* isolates convert readily back to *white*. Therefore, loss of *WOR2* compromises the heritability of the *opaque* phase (Zordan et al., 2007).

The CZF1-EFG1 branch. Efg1 is a transcriptional regulator enriched in *white* cells. It has been extensively studied for its role in many cellular processes, including filamentation, cell-wall composition and virulence (see also page 32). In *MTL* homozygote cells, deletion of *EFG1* converts *white* cells to *opaque*, whereas ectopic overexpression of *EFG1* in *opaque* cells converts them back to *white* (Sonneborn et al., 1999; Srikantha et al., 2000).

Furthermore, *EFG1* is proposed to repress *WOR2* (Zordan et al., 2007). Czf1 is a Zn-finger transcription factor that is enriched in *opaque* cells, and is a negative regulator of *EFG1*. Deletion of *CZF1* decreases the ability of *white* cells to convert to *opaque*, but has no influence on the heritability of the *opaque* phase. In addition, Wor1 binds directly to the upstream intergenic regions of both *CZF1* and *EFG1*. As proposed, Wor1 downregulates *EFG1* both directly and indirectly through the activation of *CZF1*, a repressor of *EFG1*. The decrease of Efg1 level in turn relieves the repressive effect on *WOR2*, thus resulting in a net positive effect on *WOR1* expression (Zordan et al., 2007).

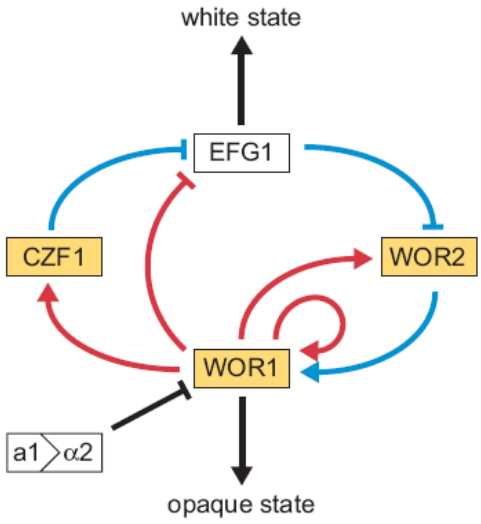

Figure 15. The transcriptional feedback model of white-opaque switching regulation. White boxes denote white-enriched, yellow boxes denote opaque-enriched factors. Red lines are based on chromatin-IP experiments, blue lines on genetic epistasis. Image from (Zordan et al., 2007).

3.5.5. Additional morphogenetic phases of *C. albicans*

Besides the *white-opaque* transition, *Candida albicans* is able switch between other phenotypically distinct growth modes, including unicellular yeast and multicellular filamentous (hyphae and pseudohyphae) forms. Yeast cells reproduce by budding; the elongated cells of pseudohyphae stay attached after cell division; whereas hypha formation is a highly polarized growth mode involving the formation of a germ tube. The polarity of growth is thought to be governed by a vesicle-rich structure, termed Spitzenkörper (Figure 16). The ability to switch between various morphologies is considered to represent a major virulence factor of the species (as well as other filamenting fungi) because several mutations that compromise the ability of *C. albicans* to change growth modes also abrogate the virulence of the strain (Gow et al., 2002; Liu, 2001; Lo et al., 1997; Whiteway and Bachewich, 2007). It is important to note that not every species of the genus are able to display all of the morphologies. *C. glabrata*, for instance has never been reported to form

germ tubes; nevertheless, it is an emerging clinically relevant pathogen (Haynes, 2001). Furthermore, since the mycelia of higher mushrooms are composed of filemantous hyphae, *C. albicans* has been used as a significant model to elucidate the genetic mechanisms underlying this growth mode.

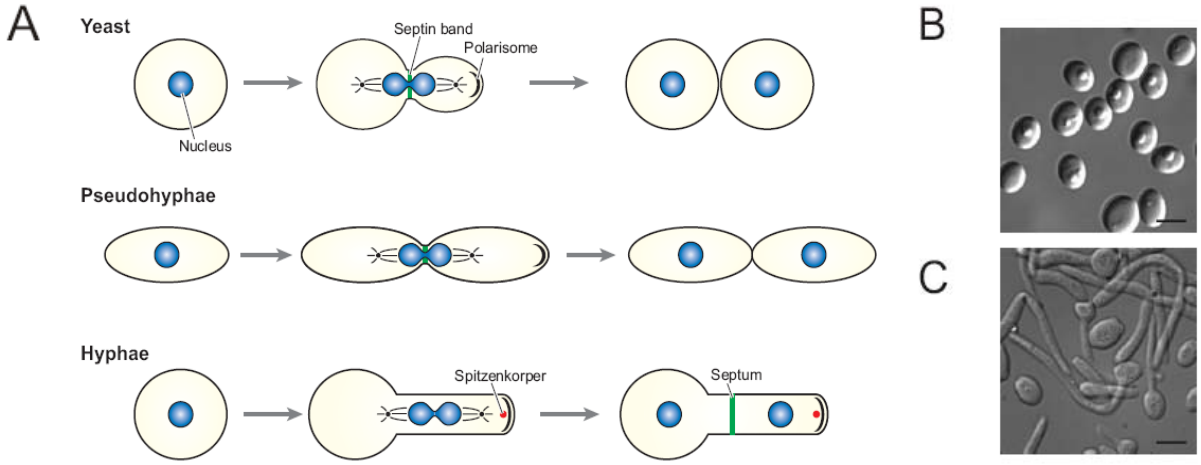

Figure 16. (A) Schematic representation of the yeast, pseudohyphal and hyphal growth modes of *C. albicans*. Image adapted from (Whiteway and Bachewich, 2007). (B) Yeast and (C) hypha phase *C. albicans* cells. Scale bars correspond to 5µm.

The various growth modes can be triggered by environmental cues, and the signals are transmitted through conserved signalling pathways (and perhaps unknown ones) to the nucleus where they activate the genes of the adequate transcriptional programs. Generally, high temperature, high concentrations of glucose or amino acids and contact with cells of the host immune system are all able to trigger filament formation both on solid and in liquid medium *in vitro*. In the following paragraphs, some of the most important signalling pathways regulating filamentation are discussed (Figure 17). For more details see reviews (Liu, 2001; Sudbery et al., 2004; Whiteway and Bachewich, 2007) and the references therein.

Mitogen-activated protein kinase (MAPK) pathway

The highly conserved MAPK pathway is involved in both the response to mating pheromone and filamentation in *C. albicans*. The pathway consists of a kinase cascade whose downstream activation target is the Cph1 transcription factor. Mutants of the members of this pathway are defective in filament formation under a limited number of hypha-inducing conditions in vitro, thus the pathway is considered to be a positive regulator of filamentation (Liu, 2001).

cAMP-dependent protein kinase pathway

Characteristic members of this pathway include the adenylate-cyclase Cyr1, the protein kinase A-homologs Tpk1 and Tpk2 and the transcription factor Efg1 (see also page 29). Mutations of the pathway abolish the ability of the strain to form filaments under almost all known filament-inducing condition; however, depletion of Efg1 enhances filamentation under embedded conditions (Liu, 2001; Lo et al., 1997).

The Tup1 repressor

Tup1 is a transcriptional repressor that is a negative regulator of filament formation. Deletion of *TUP1* results in constitutive filamentous growth (mainly pseudohyphae) under filament non-inducing conditions. Tup1 is suggested to act together with DNA-binding co-repressors such as Nrg1 and Rfg1 (Braun and Johnson, 1997; Kadosh and Johnson, 2001; Murad et al., 2001).

Other pathways

In addition to the ones discussed above, several other signalling pathways and transcription factors have been identified to influence filament formation, including the pH-responsive Rim101 pathway, Czf1, Tec1 and the Rbf1 repressor. Disruption of these transcriptional pathways has only minor effects and results in specific filamentation phenotypes that are not discussed within the scope of this thesis. For details see reviews (Liu, 2001; Sudbery et al., 2004; Whiteway and Bachewich, 2007) and the references therein.

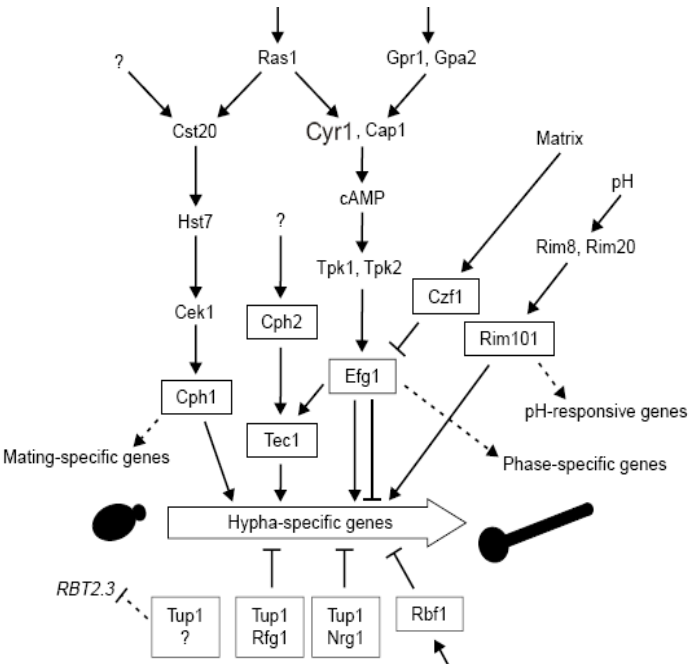

Figure 17. Signalling pathways regulating filamentation of *C. albicans*. Boxes mark transcription factors, arrows indicate positive, bars indicate negative regulatory effects, respectively. Image adapted from (Liu, 2001).

4. AIM OF THE THESIS

It is well established now that *white-opaque* switching is a unique developmental process of *Candida albicans* that links virulence, morphogenesis and mating. *White* and *opaque* cells contain identical genomes, and the two phases are considered as distinct transcriptional states. *White* and *opaque* cells differ markedly in their gene expression profiles, cellular morphologies, virulence characteristics and mating abilities. The transition between the two phases is reversible and occurs at a lower frequency than standard mutation rates.

White-opaque switching is proposed to be stochastic; nevertheless, environmental cues such as a temperature shift, oxygen concentration and host environment alter switching frequencies. Furthermore, the two cell types interact differentially with host factors, arguing that *C. albicans* cells harbour a sensing apparatus that determines cell fate rather than switching stochastically *in vivo*.

To date, as many as 6 transcription factors controlling *white-opaque* switching have been identified. These genes were recently mapped into a single genetic circuit. It has also been postulated that altered chromatin state at key loci could regulate switching, which was supported by the finding that deletion of two globally acting histone deacetylases also alters the frequencies of switching. Although the transcriptional model explains many features of *white-opaque* switching including the *in vitro* observed frequencies and the heritability of the phases, it is still based on the assumption that switching is a stochastic process.

The aim of the thesis was to identify novel genes in switching regulation and elucidate their mechanisms of action. Candidate genes were selected based on two notions that (1) cells must be able to integrate multiple stimuli to determine cell fate rather than switching merely in a random fashion and; (2) although *white-opaque* switching is unique to *C. albicans*, parts of the regulatory mechanism could be conserved. As an intersection of the two rationales, a large set of histone-modifying-enzyme mutants were generated and analysed with genetic, microbiological and biochemical approaches to unravel new regulatory principles underlying morphogenetic switching.

5. MATERIALS AND METHODS

This section features methods not described in Experimental Procedures section of the subsequent manuscript-format Results and Discussion chapter. All the additional methods used during the work are described on page 50.

Genomic DNA isolation

C. albicans genomic DNA was isolated as described (Sambrook and Russell, 2001) with modifications. Cultures were grown in 5ml YPD overnight; cells were harvested by centrifugation and resuspended in DNA extraction buffer (2% Triton X 100, 1% SDS, 100mM NaCl, 10 mM Tris pH 8.0, 1mM EDTA). Glass beads (250-600 µm, Sigma) were added and cells were broken by vortexing. DNA was subsequently isolated by multiple rounds of Phenol:Choloroform:Isoamylalcohol (Fluka) extractions.

Southern blot analysis

Southern blotting was performed as previously described (Sambrook and Russell, 2001) with modifications. 10 µg of genomic DNA was digested overnight and separated on 0.8% (wt/vol) agarose gels. DNA was blotted onto a Hybond N+ nylon membrane (Amersham) and fixed by UV cross-linking. Probes were internally labelled with ^{32}P-dCTP using the Prime It II, Random Primer Labelling Kit (Stratagene) and hybridized overnight at 65^{o}C. Autoradiography was performed at -70^{o}C using CL-Exposure films (Kodak).

6. RESULTS AND DISCUSSION

6.1. Transcriptional loops meet chromatin: a dual-layer network controls white-opaque switching in *Candida albicans*

Hnisz D., Schwarzmüller T., Kuchler K. (2008) *(Submitted for publication)*

This section is shown in a manuscript format including the following sections: Title Page, Summary, Introduction, Results, Discussion, Experimental Procedures, Acknowledgements, References, Figure and Table Legends, Figures and Tables, Supplementary Material and Supplementary References.

Transcriptional loops meet chromatin: a dual-layer network controls white-opaque switching in *Candida albicans*

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Summary

The human pathogen *Candida albicans* is able to undergo a reversible switch between two distinct cell types called white and opaque which are considered as different transcriptional states of cells harboring identical genomes. The present model of switching regulation includes the bistable expression of a master switch gene that is controlled by multiple transcriptional feedback loops. Here we show that chromatin-modifying enzymes constitute an additional regulatory layer of switching. We identified eight chromatin modifiers as switching modulators and with an epistasis analysis mapped them in signaling pathways, some of which overlay the known transcriptional network. The analysis identified the conserved Set3/Hos2 histone deacetylase complex as a key regulator that relies on the methylation status of lysine 4 on histone H3 in switching modulation. Our observations indicate that chromatin modifications may serve as a means to integrate environmental stimuli by the underlying transcriptional circuits to determine cell fate in *C. albicans*.

Introduction

Individual cells in a genetically homogenous microbial culture may display different phenotypic characteristics. Such cell-to-cell variability is suggested to enhance the ability of the microbial population to adapt to a wide range of environmental stimuli, which in the case of pathogens may represent a strategy to evade host defenses (Avery, 2006). The fungal pathogen *Candida albicans* displays a remarkable spectrum of heritable morphogenetic variation which is considered to be a major factor in the transition from a harmless commensal to a pathogen of its human host (Whiteway and Bachewich, 2007). An intriguing and unique ability of *C. albicans* is to form two distinct cell types: the so-called white and opaque phases. White and opaque cells contain the same genome, yet they differ in cellular morphology, colony shape, gene expression profile and virulence properties. In addition, white cells are unable to mate, whereas opaque cells are mating-competent (Bennett and Johnson, 2005).

White-opaque switching is an epigenetic phenomenon that was described some 20 years ago (Slutsky et al., 1987); however, the underlying molecular mechanisms have been only recently investigated. The white and opaque phases are heritable for many generations and switching between the two phases is reversible, occurring at a frequency of one per ~10^3-10^4 cell divisions (Rikkerink et al., 1988). The regulation of switching is believed to be transcriptional, and several involved transcription factors have been identified. *C. albicans* is diploid and harbours a mating-type-like locus (*MTL*) holding two alleles, "**a**" and "α". Hence, the possible *MTL* configurations include **a**/**a,** α/α and **a**/α (Hull and Johnson, 1999). A heterodimer **a**/α repressor encoded in the **a** and α alleles, respectively, blocks *MTL* heterozygous cells in the white phase (Miller and Johnson, 2002) by repressing *WOR1*, an opaque-promoting factor (Zordan et al., 2006). *MTL* homozygous cells lack the **a**/α repressor, and are permissive to switching. In **a**/**a** or α/α white cells *WOR1* is expressed at a very low level, and high-level expression of *WOR1* is required for the conversion to the opaque phase (Huang et al., 2006; Zordan et al., 2006). Conversely, Efg1 is enriched in white cells and is required for maintenance of the white phase (Sonneborn et al., 1999; Srikantha et al., 2000). According to a recent model, a stochastic increase in Wor1 levels drives the transition from the white to the opaque phase. Furthermore, Wor1 (1) autoregulates its own expression; (2) facilitates the expression its co-factor *WOR2*; and (3) represses *EFG1* both directly and indirectly through driving expression of *CZF1*, a repressor of *EFG1*. Since *EFG1* is proposed to be a repressor of *WOR2*, *WOR1* thus coordinates three positive feedback loops to ensure

high Wor1 levels, explaining the heritability of the opaque phase (Zordan et al., 2007). In addition, histone deacetylases Hda1 and Rpd3 have also been implicated in the regulation of white-opaque switching (Klar et al., 2001; Srikantha et al., 2001) but their precise role has not been elucidated.

In this work, we provide evidence that a complex dual-layer network comprising of transcriptional regulators and chromatin-modifying enzymes determines the cellular identity in *C. albicans*. Our results experimentally verify previous suggestions that cellular shape and phase-specific genes are regulated at different branching points of the transcriptional circuit, and that the genetic information affecting phase commitment converges at the *WOR1* locus. Next, we identify eight genes (*SET1*, *RPD31*, *SET3*, *HOS2*, *HST1*, *HST2*, *PHO13* and *NAT4*), encoding orthologues of histone-modifying enzymes in *Saccharomyces cerevisiae*, as novel modulators of white-opaque switching in *C. albicans*. An epistasis analysis maps various histone-modifiers into the transcriptional circuit. We also show that the Set3/Hos2 histone deacetylase complex is a key regulator of *WOR1* expression and conversion to the opaque phase. Finally, we provide genetic evidence that Set3/Hos2 function requires the methylation of histone H3 on lysine 4. We propose a comprehensive model according to which chromatin modifiers constitute a layer of regulation imposed on the transcriptional circuits driving phase changes. This additional layer offers a possible mechanism to integrate environmental stimuli, contrary to the current models that explain switching as a stochastic process. Moreover, we postulate that the dependence of the Set3/Hos2 complex on H3K4 methylation at certain loci may be an evolutionary conserved mechanism among other eukaryotic taxa.

Results

WOR1 acts downstream of *EFG1* in phase commitment, while *EFG1* acts downstream of *WOR1* in morphology determination

The white and opaque cell types of *C. albicans* are distinguished on four criteria. (1) Cellular morphology: white cells have a round shape; opaque cells are larger and elongated (Slutsky et al., 1987). (2) Colony appearance: white cells form white, dome-shaped colonies on solid agar, while opaque cells form larger, flattened colonies that are stained pink on media containing Phloxin B (Slutsky et al., 1987). (3) Gene expression profile: about 400 genes are regulated differentially in the two phases (Lan et al., 2002). For diagnostic purposes, white-specific genes *WH11* (Srikantha and Soll, 1993) and *EFG1* (Sonneborn et al., 1999), as well as opaque-specific genes *OP4* (Morrow et al., 1993) and *SAP1* (Morrow et al., 1992) are commonly used. (4) Mating competence: white cells are mating incompetent, whereas opaque cells can mate with opaque cells of the opposite mating type (Miller and Johnson, 2002).

Previous studies established the role of *WOR1* as the master regulator of the opaque phase. Deletion of *WOR1* locks **a**/**a** or α/α cells in the white phase, whereas ectopic overexpression of *WOR1* results in the conversion to the opaque phase (Huang et al., 2006; Zordan et al., 2006). On the other hand, *MTL* homozygous *efg1*Δ/Δ cells predominantly occur in the opaque phase, while ectopic *EFG1* expression converts opaque cells to the white phase (Sonneborn et al., 1999). Recently, *EFG1* was suggested to promote the white phase by repressing *WOR2*, a co-factor of *WOR1* (Zordan et al., 2007). In addition, *EFG1* has been postulated to act downstream of the switch event to regulate cellular morphology (Srikantha et al., 2000).

In order to experimentally verify the latter two suggestions, we created an *efg1*Δ/Δ *wor1*Δ/Δ double mutant in an *MTL***a**/**a** background. The **a**/**a** *efg1*Δ/Δ *wor1*Δ/Δ mutant displayed an elongated cell shape, albeit shorter than wild type opaque cells, similar to the rare **a**/**a** *efg1*Δ/Δ white cells as well as **a**/α *efg1*Δ/Δ cells. In addition, the **a**/**a** *efg1*Δ/Δ *wor1*Δ/Δ mutant formed large, flattened colonies appearing light pink on Phloxin B agar, intermediate to the white and pink color of wild type white and opaque cells, respectively, while the color of **a**/**a** *efg1*Δ/Δ white isolates and **a**/α *efg1*Δ/Δ cells was white on Phloxin B plates (Figure 1A). We inspected over 2000 colonies and all of them displayed the described morphology.

Next, we found that the *efg1*Δ/Δ *wor1*Δ/Δ double mutant expressed the white-specific transcript *WH11* similar to wild type white and *wor1*Δ/Δ cells (the latter being locked in the white phase). Conversely, opaque-specific transcripts *OP4* and *SAP1* were virtually

undetectable. **a**/α *efg1*Δ/Δ cells also showed a white-phase expression profile (Figure 1B). This data again suggests that in switching-permissive cells, loss of *EFG1* results in opaque formation due to the upregulation of *WOR1*, which we directly confirmed by immunoblotting (Figure 1C). As expected, in **a**/α *efg1*Δ/Δ cells the **a**/α repressor still inhibits *WOR1* expression, thus locking cells in a white-like phase (Figure 1A, 1B and 1C). Therefore, *EFG1* indeed promotes the white phase by repressing *WOR1* either directly or indirectly.

We also tested the mating ability of the **a**/**a** *efg1*Δ/Δ *wor1*Δ/Δ double mutant, and found that its mating competence was as negligible as that of wild type white cells (Figure 1D). Therefore, **a**/**a** *efg1*Δ/Δ *wor1*Δ/Δ cells are functionally white, express white-specific genes; yet they show an elongated morphology distinguishable of wild type white cells. These data demonstrate that *WOR1* acts downstream of *EFG1* in phase commitment, while *EFG1* acts downstream of *WOR1* in morphology determination.

Histone-modifier genes are modulators of white-opaque switching

Although transcription factors were identified in the regulation of white-opaque switching, neither a rearrangement in DNA sequences nor any modification of chromatin has been associated with it. Two studies, however, showed that deletion of histone deacetylase genes *HDA1* and *RPD3* modify the frequencies of switching (Klar et al., 2001; Srikantha et al., 2001). Therefore, we decided to analyze the contribution of histone-modifying enzymes to phase transitions on a comprehensive scale. We analyzed the genome of related fungal species *Saccharomyces cerevisiae* (www.yeastgenome.org) to identify open reading frames (ORFs) encoding putative histone modifiers (acetyltransferases, deacetylases, methyltransferases and dephosphorylases) either as regulatory or catalytic subunits of larger protein complexes. Out of some 90 genes, we selected those encoding catalytic subunits, yielding a total of 23 genes (including one additional ORF: *SET3*), which were subjected to BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST) against the *C. albicans* genome to identify potential orthologues. This analysis revealed that many *S. cerevisiae* genes are highly conserved in *C. albicans*. Subsequently, we attempted to construct homozygous deletion mutants of the listed ORFs in an *MTL***a**/**a** *C. albicans* strain. Out of 23 candidates, we successfully created deletion strains of 18 genes. The identified ORFs, their predicted functions, the BLAST E-values and whether a deletion mutant was created are listed in Supplementary Table 1.

Next, we analyzed the effect of gene deletions on the frequency of the white to opaque transition using quantitative switching assays. Briefly, pure white cultures were plated on Phloxin B plates, and the frequency of pure opaque colonies or colonies containing at least

one opaque sector growing on the plates was scored (as monitored by colony morphology and microscopy). The knock-out mutants showing significant alterations relative to the background control strain are listed in Table 1. The *C. albicans* genes whose deletion increased the formation of opaque colonies or sectors included *SET1*, a H3K4 methyltransferase (Raman et al., 2006; Roguev et al., 2001) required for gene silencing at telomeres and rDNA sequences in *S. cerevisiae* (Nislow et al., 1997); *HDA1*, a histone deacetylase (Carmen et al., 1996) acting as a global repressor of transcription in *S. cerevisiae* (Rundlett et al., 1996); *HDA1* served as a control in our gene set, since its loss was already shown to increase the frequency of opaque formation in *C. albicans* (Klar et al., 2001); and *RPD31*, one of the two orthologues of the *S. cerevisia*e *RPD3*, a principal histone deacetylase involved in transcriptional repression in *S. cerevisiae* (Rundlett et al., 1996). The genome of *C. albicans* encodes two potential orthologues of *RPD3* designated *RPD3* and *RPD31* (Supplementary Table 1). Interestingly, deletion of *RPD3* has a similar effect on the white to opaque switching frequency (Srikantha et al., 2001). The genes whose deletion significantly decreased opaque conversion relative to wild type included *SET3*, an essential component of the Set3 histone deacetylase complex involved in the suppression of meiotic genes in *S. cerevisiae* (Pijnappel et al., 2001); *HOS2*, a histone deacetylase and subunit of the Set3 complex (Pijnappel et al., 2001) required for gene activity in *S. cerevisiae* (Wang et al., 2002); *HST2*, a histone deacetylase similar to *SIR2* (Landry et al., 2000) required for centromeric and rDNA silencing in *S. cerevisiae* (Durand-Dubief et al., 2007); and *NAT4*, an acetyltransferase involved in acetylation of histones H4 and H2A (Song et al., 2003).

Furthermore, we analyzed the effect of the deletions on the heritability of the opaque phase in quantitative switching assays. In these assays, opaque phase cultures were plated on Phloxin B agar, and the arising frequency of pure white colonies and colonies containing at least one white sector was scored (Table 1). Genes whose deletion increased the heritability of the opaque phase (i.e. displaying a lower frequency of conversion to white than wild type) included *HST1*, a histone deacetylase, a non-essential subunit of the Set3 complex (Pijnappel et al., 2001), as well as an essential subunit of the Sum1/Rfm1/Hst1 complex, which functions as a repressor of sporulation-specific genes in *S. cerevisiae* (Xie et al., 1999). Genes whose deletion destabilized the opaque phase (i.e the deletion mutant showed a higher frequency of conversion to white than wild type) included *PHO13*, a phosphatase dephosporylating H2A *in vitro* (Tuleva et al., 1998) and implicated in carbohydrate metabolism in *S. cerevisiae* (Van Vleet et al., 2007); and *NAT4* (see above).

Unexpectedly, the loss of either *SET3* or *HOS2* resulted in a unique phenotype: the opaque colonies of the *set3*Δ/Δ and *hos2*Δ/Δ mutants displayed filamentous growth (data not shown). We assayed the opaque to white switching frequencies of these filamenting mutants, and found an increase in the conversion to the white phase compared to wild type cultures (Table 1); however, this data should only be considered as an approximation, because (1) the filaments could not be reliably fragmented into individual colony forming units with our method (see Experimental Procedures), and (2), the deletion of *SET3* or *HOS2* likely had pleiotropic effects influencing both white-opaque switching and filamentation, i.e. two distinct transcriptional programs, whose putative cross-talk would inherently impact the scoring method.

We then created a second independent deletion mutant of all genes showing an effect on switching and repeated the quantitative switching assays in both directions. In all cases, the second independent deletion strains qualitatively reproduced the phenotype of the first deletion strains (data not shown). As summarized in Figure 2A these results show that histone-modifying enzymes of various classes provide a substantial input to white-opaque switching in multiple ways.

Histone modifiers act upstream of *WOR1*

Where and how do the histone modifiers act to modulate phase conversion? Previous work has already demonstrated that the formation of mating-competent opaque cells requires Wor1 (see above). Indeed, an immunoblot analysis failed to detect Wor1 in any of the investigated white phase single mutant cultures. By contrast, opaque phase mutant cells expressed Wor1 in levels comparable to those of wild type opaque strains (Figure 2B). Furthermore, white and opaque phase single deletion mutants exhibited mating competence comparable to wild type white and opaque strains, respectively (Figure 2C). These results suggest that these histone modifiers act either upstream or at the level of *WOR1*.

To address whether phase-enrichment of the genes accounts for their effects on phase changes, we performed quantitative RT-PCR to compare expression levels of the switching modulators in the white and opaque phases (Figure 2D). We failed to detect a significant difference of transcript levels between the two phases, neither in our **a**/**a** background strain (Figure 2D) nor in an independent **a**/**a** clinical isolate, L26 (data not shown). These results suggest that the activities rather then expression levels of histone modifiers modulate the outcome of the transcriptional circuit(s), which converge(s) at the master switch locus *WOR1*.

Epistasis of *SET3, HOS2, HST2, NAT4* and *EFG1* reveals multiple pathways

Interestingly, deletion of *SET3*, *HOS2*, *HST2* and *NAT4* reduced the switching frequency about 5-10 fold from white to opaque; although the genes have different molecular functions (see above). Altered chromatin state can influence the accessibility of DNA to non-histone proteins e.g. the transcription machinery, or transcription factors can recruit chromatin-modifying enzymes to facilitate their activities (Kouzarides, 2007). *C. albicans* Efg1 is a basic helix-loop-helix protein displaying DNA-binding activity *in vitro* (Stoldt et al., 1997). To elucidate whether *SET3*, *HOS2*, *HST2* or *NAT4* modulate white to opaque switching in concert with *EFG1*, we constructed *efg1*Δ/Δ *hos2*Δ/Δ, *efg1*Δ/Δ *set3*Δ/Δ, *efg1*Δ/Δ *hst2*Δ/Δ and *efg1*Δ/Δ *nat4*Δ/Δ double mutants, and compared their switching frequencies to those of the corresponding single mutants (Tables 1 and 2). The phases were verified by colony morphology, microscopy (data not shown) and quantitative mating assays (Supplementary Table 5). The results were as follows: *efg1*Δ/Δ: 97.7%, *set3*Δ/Δ: 1.8%, *hos2*Δ/Δ: 1.5%, *hst2*Δ/Δ: 0.4% and *nat4*Δ/Δ: 1.4%, whereas for the double mutants: *efg1*Δ/Δ *set3*Δ/Δ: 3.0%, *efg1*Δ/Δ *hos2*Δ/Δ: 3.6%, *efg1*Δ/Δ *hst2*Δ/Δ: 87.9%, and *efg1*Δ/Δ *nat4*Δ/Δ: 89.2% (Tables 1 and 2). Deletion of *EFG1* is thus dominant over the deletion of either *HST2* or *NAT4* while *HOS2* or *SET3* deletion qualitatively suppresses the loss of *EFG1*. In other words, although the net repression of *EFG1* on *WOR1* expression is relieved, high-level expression of *WOR1* still requires both *SET3* and *HOS2*. On the other hand, *HST2* and *NAT4* are likely to exert their effect in a transcriptional loop converging at the *WOR1* locus either at the level of *EFG1* or upstream of it. The switching frequencies from opaque to white also showed similar epistatic relationships (right panel, Table 2). To verify that *HOS2* and *SET3* indeed act in an independent pathway of either *NAT4* or *HST2,* we tested the switching frequencies of *efg1*Δ/Δ *hst2*Δ/Δ *hos2*Δ/Δ and *efg1*Δ/Δ *nat4*Δ/Δ *set3*Δ/Δ triple mutants in both switching directions. As predicted, deletion of *NAT4* in an *efg1*Δ/Δ *set3*Δ/Δ mutant, and deletion of *HST2* in an *efg1*Δ/Δ *hos2*Δ/Δ mutant had no significant effect on switching frequencies when compared to the respective double deletion strains (Table 2). In summary, these data reveal at least two independent regulatory pathways affecting the transcriptional loops controlling morphogenetic switching.

Loss of *SET3* or *HOS2* suppresses deletion of *RPD31* or *HDA1*

The *S. cerevisiae* orthologues of Hos2, Hda1 and Rpd31 are histone deacetylases catalytically active on multiple acetylated lysine residues of core histones (Pijnappel et al., 2001; Suka et al., 2001; Wu et al., 2001). Set3 is an integral subunit of the Set3/Hos2

deacetylase complex (Pijnappel et al., 2001). To address whether there is a division of labor between deacetylase complexes in the regulation of *C. albicans* white-opaque switching, we created a series of double deletion strains and compared their switching frequencies to those of single deletion mutants.

As shown in Tables 1 and 3, deletion of *SET3* is dominant over the deletion of *HDA1* and *RPD31,* whereas deletion of *HOS2* is dominant over deletion of *RPD31*. The phases were verified by colony morphology, microscopy (data not shown) and quantitative mating assays (Supplementary Table 6). These results support the notion that Set3/Hos2 in *C. albicans* acts as a complex functioning as a downstream regulator of white-opaque switching.

Deletion of *SET3* or *HOS2* is suppressed by loss of H3K4 methylation

Where and how is the Set3/Hos2 complex recruited? Inspection of the CaSet3 primary sequence revealed two characteristic domains: a SET and a PHD (Plant HomeoDomain) domain (Figure 3A). This domain architecture is conserved among several genes implicated in epigenetic regulation, including *ASH1* and *Thrithorax* in *Drosophila* (Stassen et al., 1995; Tripoulas et al., 1996). The SET domains exert two functions: methyltransferase activity acting on histones (Rea et al., 2000) or other non-histone substrates; or they may serve as protein-protein interaction surfaces (Rozenblatt-Rosen et al., 1998). The PHD finger is a specialized methyl-lysine binding domain found in various proteins "reading" histone marks (Shi et al., 2006). Recently, the purified PHD finger of ScSet3 was shown to preferentially bind trimethylated H3K4 (Shi et al., 2007). Notably, *CaSET1* was identified as the only *C. albicans* methyltransferase modifying H3K4, and its deletion results in complete loss of H3K4 methylation (Raman et al., 2006) (data not shown).

To address whether the Set3/Hos2 complex requires H3K4 methylation in the regulation of white-opaque switching in *C. albicans*, we compared the switching frequencies of *set1*Δ/Δ *set3*Δ/Δ and *set1*Δ/Δ *hos2*Δ/Δ double mutants with that of the respective single deletion strains (Tables 1 and 4). Strikingly, deletion of *SET1* almost completely suppressed the loss of *SET3* or *HOS2* (white to opaque switching frequencies: *set1*Δ/Δ: 19.5%, *hos2*Δ/Δ: 1.5%, *set3*Δ/Δ: 1.8%, *set1*Δ/Δ *set3*Δ/Δ 10.5%, *set1*Δ/Δ *hos2*Δ/Δ 12.8%). To verify the rescue effect, we performed an epistasis analysis of *SET1* and *HOS2* in a genetic background where the transcriptional feedback mediated by *EFG1* towards the *WOR1* locus is disrupted. Therefore, we compared the white to opaque switching frequencies of *efg1*Δ/Δ, *efg1*Δ/Δ *set1*Δ/Δ, *efg1*Δ/Δ *hos2*Δ/Δ and *efg1*Δ/Δ *set1*Δ/Δ *hos2*Δ/Δ mutants (Tables 1, 2 and 4). White *efg1*Δ/Δ cells converted at high frequencies to opaque (97.7%, Table 2). Notably, *efg1*Δ/Δ *set1*Δ/Δ

cells almost exclusively existed in the opaque phase, as we failed to isolate single white phase colonies of this mutant. *efg1*Δ/Δ *hos2*Δ/Δ white cells convert at low frequencies to opaque (3.6%, Table 2). As expected, *efg1*Δ/Δ *set1*Δ/Δ *hos2*Δ/Δ white cells also readily convert to the opaque phase (80.6%, Table 4), while the opaque phase appears as stable as in *efg1*Δ/Δ and *efg1*Δ/Δ *set1*Δ/Δ opaque isolates (Tables 2 and 4). The phases were verified by colony morphology, microscopy (data not shown) and quantitative mating assays (Supplementary Table 7). These results demonstrate that deletion of *SET1* and loss of H3K4 methylation suppresses the deletion of *SET3* or *HOS2*.

Discussion

A model of white-opaque switching in *C. albicans* including two regulatory layers

In this study, we used the *C. albicans* white-opaque switching system to analyze the mechanisms of heritable phenotypic variation in a unicellular organism. The white and opaque cell types of *C. albicans* represent different transcriptional states of cells containing identical genomes. Switching is considered to provide distinct cell variants with different capabilities to adapt to various host niches and/or host defenses *in vivo*.

The current models explain the regulation of switching by a transcription circuitry amplifying the stochastic changes of expression of one master regulator gene, *WOR1*. In this study we used a reverse genetic approach to decipher the role of chromatin-modifying enzymes in white-opaque switching. Based on our data, we propose a dual-layer network model for the regulation of switching (Figure 3B).

Transcriptional layer of regulation

The architecture of the transcriptional circuit is very similar to the one proposed by Zordan et al: in *MTL* heterozygous cells, *WOR1* is repressed by the **a**/α repressor. In *MTL* homozygous white cells, Wor1 level is low because *EFG1* represses *WOR2*, a putative cofactor of *WOR1*. Once the expression level of Wor1 reaches a threshold, cells convert to the opaque phase. Opaque cells express Wor1 at a high level as a result of multiple positive feedback loops mediated by Wor1: (1) on the *WOR1* locus; (2) through the activation of *WOR2*; (3) through the repression of *EFG1* directly and indirectly by activating *CZF1*, a repressor of Efg1. In this model, *EFG1* and *WOR1* promote the white and opaque phases, respectively (Zordan et al., 2007). However, we found that cells lacking both *EFG1* and *WOR1* not only fail to express opaque-specific genes *OP4* and *SAP1* but also express the white-specific *WH11* transcript as do wild type white cells (Figure 1B). Wor1 in wild type opaque cells thus represses certain white-specific genes such as *WH11*, irrespective of repressing *EFG1*. This argues that wild type white cells are in the white phase primarily because they lack Wor1 and not because they express Efg1. Furthermore, we could also demonstrate that *EFG1* regulates cellular morphology downstream of *WOR1* (Figure 1A) arguing that wild type opaque cells display an elongated cell shape primarily because they express Efg1 at a lower level than the round shaped wild type white cells (Figure 1B), as postulated previously (Srikantha et al., 2000).

Chromatin-level layer of regulation

In addition, we found that numerous chromatin-modifying enzymes can modulate white-opaque switching. The genes can be grouped in functional categories based on the direction(s) of transition they influence, which suggests that chromatin modifications interfere with the underlying transcriptional network at multiple branching points. Interestingly, the phenotype of chromatin-modifier deletions in some cases correlated with the phenotype of transcription factor deletions, which suggests that they function at the same branch of the transcriptional circuit. For example, deletion of *HST2* results in a 10-fold decrease in the white to opaque switching frequency, has no influence on the opaque to white switching frequency and is suppressed by the loss of *EFG1* (Tables 1 and 2), while all of these effects are phenocopied by the deletion of *CZF1* (Zordan et al., 2007), arguing that *HST2* has an impact on transcriptional regulation at the *CZF1*-branch. This input could be, for instance, exerted either at the *CZF1* locus or through the *CZF1*-dependent repression of *EFG1*. In addition, loss of *NAT4* promotes the white phase, which is suppressed by the deletion of *EFG1* (Tables 1 and 2), suggesting that *NAT4* mediates transcriptional information converging at the *EFG1* locus, independent of *CZF1*. The possibility that *NAT4* modulates binding of Wor1 at the *EFG1* promoter is plausible but requires further experimental confirmation. Based on a similar logic, since loss of either *HOS2* or *SET3* promotes the white phase and their deletions suppress the loss of *EFG1*, *HOS2* and *SET3* map to a pathway that functions downstream of *EFG1* and upstream of *WOR1*, possibly at the *WOR1* or the *WOR2* locus, for instance.

Set3 and Hos2 function as a complex in *C. albicans*

In this study, we provide four lines of genetic evidence that Set3 and Hos2 act as a complex in *C. albicans*. (1) The phenotype of single deletions is identical; (2) loss of either *HOS2* or *SET3* is dominant over the loss of *EFG1*; (3) loss of either *HOS2* or *SET3* is dominant over the loss of *RPD31*; and (4) the deletion of *SET1* suppresses the loss of either *HOS2* or *SET3*. Hence, the situation in *C. albicans* appears similar to the Set3/Hos2 complex in *S. cerevisiae*, where deletion of either *SET3* or *HOS2* prevents assembly of the functional deacetylase complex *in vivo* (Pijnappel et al., 2001). Likewise, a similar architecture seems to be conserved in the mammalian HDAC3/SMRT complex (Guenther et al., 2000).

The observation that loss of H3K4 methylation suppresses the disruption of the complex suggests that proper localization of Set3/Hos2 requires an interaction of the PHD finger of Set3 with a methylated H3K4 residue. This notion is supported by the finding that the purified PHD domain of ScSet3 specifically binds trimethylated H3K4 *in vitro* (Shi et al., 2007). It is

important to note that loss of *SET1* failed to revert the opaque filamentation phenotype of the *hos2*Δ/Δ and *set3*Δ/Δ mutants, which suggests that Set3/Hos2 localization depends on H3K4 methylation only at specific loci.

Buffering bistability with dual control

So far, the models describing *C. albicans* white-opaque switching are based on the assumption that the process is stochastic; however, several lines of evidence argue that the transition is responsive to environmental stimuli. For instance, high temperature causes opaque cells to convert en masse to the white phase (Rikkerink et al., 1988) while anaerobic conditions stabilize opaque cells even at elevated temperatures *in vitro* (Dumitru et al., 2007). Theoretical models support that for microbial populations stochastic switching is favorable when the environment only frequently changes, and more uncertain the environment is, the more beneficial it is to a maintain a signaling apparatus that enables the cells to actively respond to changes (Kussell and Leibler, 2005). The human host, in our belief, provides a fluctuating enough environment for *C. albicans* to have developed a cell fate decision machinery that is able to process and probably also to store information in order to adapt in a favorable fashion. Opaque phase *C. albicans* cells, for instance, are better colonizers of the skin while white phase cells are more prevalent in a bloodstream infection (Kvaal et al., 1999). Furthermore, opaque cells are more susceptible to killing by neutrophils than white cells (Kolotila and Diamond, 1990), whereas macrophages preferentially phagocytose white cells over opaque cells (Lohse and Johnson, 2008). The regulation of chromatin status hence the accessibility of a given locus to regulatory factors offers a layer of regulation to integrate the signals determining cell fate. This relay function of chromatin is further supported by the finding, that in opaque cells Wor1 can be immunoprecipitated from the promoters of many genes whose expression does not change during the white-opaque switch under laboratory conditions (Zordan et al., 2007).

Finally, we conclude that *C. albicans* during its co-evolution with the human host has developed an elaborate system of signal integration comprising of two layers: a transcriptional level which coordinates the response and a chromatin-level layer that may have a relay function at key loci integrating the stimuli affecting cellular identity. Moreover, such an architecture including specific transcription factors and chromatin modifiers is reminiscent of the cell-fate decision machinery of higher eukaryotes which makes *C. albicans* an attractive alternative model system to dissect chromatin dynamics and enzyme recruitment.

Experimental Procedures

Media and growth conditions

Rich medium (YPD) and complete synthetic medium (SD) was prepared as previously described (Kaiser et al., 1994). Modified Lee´s medium was prepared as described (Bedell and Soll, 1979). Cultures were routinely grown at 25°C unless indicated otherwise.

Strain construction

The complete list of strains, primers and plasmids used in this study are listed in Supplementary Tables 2, 3 and 4, respectively. All strains were derived from SN152 (Noble and Johnson, 2005) a leucine, histidine, arginine auxotrophic derivative of clinical isolate SC5314 (Gillum et al., 1984). The **a**/α SN152 was cultured on sorbose medium (Janbon et al., 1998) to construct the **a**/**a** strain DHCA202. *MTL* homozygosis was verified by PCR and Southern blot analyses (data not shown). Single gene deletions (*SET1*, *SET2*, *HDA1*, *SAS2*, *RPD31*, *SET3*, *HOS2*, *HST1*, *SIR2*, *HST2*, *ELP3*, *PHO13*, *PHO8*, *DOT1*, *HOS1*, *HPA2*, *HOS3* and *WOR1*) were created as described in (Noble and Johnson, 2005).

Multiple gene deletion mutants, as well as the *efg1*Δ/Δ in the DHCA202 and SC5314 backgrounds were created using the "SAT1-flipping" method (Reuss et al., 2004). *EFG1* was deleted in the *wor1*Δ/Δ, *hos2*Δ/Δ, *set3*Δ/Δ, *hst2*Δ/Δ, *nat4*Δ/Δ and *set1*Δ/Δ strains to create the double deletions strains *efg1*Δ/Δ *wor1*Δ/Δ, *efg1*Δ/Δ *hos2*Δ/Δ, *efg1*Δ/Δ *set3*Δ/Δ, *efg1*Δ/Δ *hst2*Δ/Δ, *efg1*Δ/Δ *nat4*Δ/Δ and *efg1*Δ/Δ *set1*Δ/Δ, respectively. To obtain the *hda1*Δ/Δ *set3*Δ/Δ, *rpd31*Δ/Δ *set3*Δ/Δ, *rpd31*Δ/Δ *hos2*Δ/Δ, *hst2*Δ/Δ *hos2*Δ/Δ, *nat4*Δ/Δ *set3*Δ/Δ, *set1*Δ/Δ *hos2*Δ/Δ and *set1*Δ/Δ *set3*Δ/Δ double deletion strains *SET3* and *HOS2* were deleted in the single deletion *hda1*Δ/Δ, *rpd31*Δ/Δ, *hst2*Δ/Δ, *nat4*Δ/Δ or *set1*Δ/Δ strains. Triple deletion strains *efg1*Δ/Δ *hst2*Δ/Δ *hos2*Δ/Δ, *efg1*Δ/Δ *nat4*Δ/Δ *set3*Δ/Δ and *efg1*Δ/Δ *set1*Δ/Δ *hos2*Δ/Δ were created by the disruption of *EFG1* in the *hst2*Δ/Δ *hos2*Δ/Δ, *nat4*Δ/Δ *set3*Δ/Δ and *set1*Δ/Δ *hos2*Δ/Δ double deletion strains, respectively. Except for single gene deletions that did not display any phenotypes (Supplementary Table 2), at least two independent homozygous deletion strains were created derived from independent heterozygote isolates. Transformation was performed via electroporation as described (Reuss et al., 2004). Genomic integration events were verified with PCR and Southern blot analyses (data not shown).

The mating tester strains DHCA210 (*MTL*α/α) and DHCA209 (*MTL***a**/**a**) were created in the SC5314 background using the sorbose selection method and the subsequent disruption of the *ADE2* gene using the "SAT1-flipping" strategy (see Supplementary Tables 2 and 3).

Microscopy

Colony morphology was analyzed using a Discovery V12 Stereoscope (Zeiss) equipped with an Axiocam MR5 camera (Zeiss). Microscopic analysis was performed with using an Axioplan 2 microscope (Zeiss) equipped with a Spot Pursuit camera (Sony). Images were analyzed with the Axiovision 4.1 software (Zeiss).

White-opaque switching assays

Quantitative switching assays were performed as previously described (Miller and Johnson, 2002) with modifications. Briefly, white strains were streaked from frozen stocks on YPD plates and grown at 30°C for 2 days. Single colonies were then restreaked onto modified Lee´s medium (Tables 1 and 2) or SD medium (Tables 3 and 4) and grown at 25°C for 5 days. Single colonies were picked and resuspended in sterile H_2O, checked by microscopy and spread onto modified Lee´s plates (Tables 1 and 2) or SD plates (Tables 3 and 4) containing 5 µg/ml Phloxin B. Formation of opaque colonies or sectors was scored after 7 days. The opaque to white switching assays were performed using pure opaque colonies obtained in the white to opaque switching assays. The frequency of white colonies or colonies containing at least one white sector was scored after 7 days. For each strain, at least 3 independent experiments were carried out. The data listed in Tables 1, 2, 3 and 4 were obtained using one deletion strain of the genotype. For each genotype at least two independent deletion strains were created. The analysis of independent deletion mutants showed qualitatively similar results (data not shown).

Quantitative mating assays

Quantitative mating assays were performed essentially as described (Miller and Johnson, 2002) with modifications. Pure white and opaque cultures were isolated on plates as described above. Strains were grown in liquid medium at 25°C until an OD_{600} 1-3. $3 \cdot 10^7$ cells of each mating partner were mixed, and deposited on sterile Whatman filter paper placed onto a YPD plate supplemented with 100 µg/ml adenine, and incubated at 25°C for 18 hours. Cells were washed off the filter, resuspended in 10 ml sterile H_2O and were dispersed by vortex-mixing. Serial dilutions were plated on double-selective (-arginine -adenine) SD plates to select for the prototrophic conjugants, and on single selective (-arginine or –adenine) SD plates to score the single parent population plus conjugants. The mating frequencies were calculated as the ratio of conjugants and the limiting parent plus conjugants.

RNA isolation and quantitative RT-PCR

Cultures were grown in modified Lee´s medium until OD_{600} 1-3 and harvested by centrifugation. Pellets were washed with sterile H_2O, frozen in liquid nitrogen and mechanically pulverized in a sterile porcelain mortar in the frozen state. RNA was extracted using TRI reagent (Molecular Research Center). About 1-5 µg of total RNA was reverse-transcribed with the First Strand cDNA synthesis kit (Fermentas). cDNA amplification was monitored quantitatively by SYBR Green incorporation in a Realplex Mastercycler (Eppendorf).

Immunoblotting

Cultures were grown in liquid medium until OD 1-3 and cells were harvested by centrifugation. Cell pellets were resuspended in 0.25M NaOH and 1% ß-mercaptoethanol, and incubated on ice for 10 min. Proteins were precipitated by the addition of 5.8 V/V% Trichloroacetic-acid (TCA) for 10 min on ice, centrifuged and resuspended in SDS sample buffer. Total protein extracts derived from 0.5 OD of the starting cultures were separated by SDS/PAGE and analyzed by Western blotting. The C-terminal anti-Wor1 antibody has been previously described (Zordan et al., 2006). Loading controls were visualized using a monoclonal anti-tubulin antibody (Sigma).

Acknowledgements

We thank all laboratory members for helpful discussions; W. Glaser for assistance with the bioinformatics and statistical analyses; N. Landstetter for comments on the manuscript. We are indebted to Alexander Johnson for providing fungal strains, plasmids and the anti-Wor1 antibody; J. Morschhäuser for critical reading of the manuscript and for the pSFS2A plasmid; S. Rupp for the strain HLC67; D.R. Soll for the strain L26. This work was supported by a grant from the Christian Doppler Society to KK. D.H. was supported through the Vienna Biocenter PhD Programme WK001, and T.S. in part by the EraNet Pathogenomics project FunPath (FWF-API-0125).

Figure and Table Legends

Figure 1. *WOR1* acts downstream of *EFG1* in phase commitment, while *EFG1* acts downstream of *WOR1* in morphology determination

(A) Colony and cellular morphologies on modified Lee´s medium containing 5 µg/ml Phloxin B. Scale bars correspond to 5 µm (upper panel) and 2 mm (lower panel).

(B) qRT-PCR analysis of phase-specific mRNA transcripts. *WH11*, *EFG1* (white-specific) and *OP4*, *SAP1* (opaque-specific) transcript levels were normalized to the transcript level of *PAT1* (Zordan et al., 2006). qRT-PCR reactions were performed in triplicates and cDNA isolated from two independent cultures were analyzed. Data are shown as mean +/- SD.

(C) Immunoblot analysis confirms that *WOR1* can be repressed by *EFG1* and the **a**/α repressor. Tubulin indicates equivalent loading.

(D) *WOR1* is required for mating. Quantitative mating assays were performed with an opaque phase *MTL*α/α tester strain. At least two independent experiments per genotype were performed yielding qualitatively similar results. Values are shown of one representative experiment. [#]: tested with both an **a**/**a** and an α/α tester strain.

Figure 2. Histone modifiers act upstream of *WOR1*

(A) Functional categories of single gene deletions on white-opaque switching.

(B) Immunoblot analysis demonstrates that Wor1 is expressed in a similar pattern in wild type and mutant white (W) and opaque (O) cultures. Tubulin indicates equivalent loading.

(C) Mating competence is differentially regulated in single mutant cells similar to wild type. Quantitative mating assays were performed with an opaque phase *MTL*α/α tester strain. At least two independent experiments per genotype were performed giving qualitatively similar results. Values are shown of one representative experiment.

(D) Transcript levels of histone modifiers are phase-independent. qRT-PCR was performed in triplicates and cDNA isolated from two independent cultures were analyzed. Transcript levels are normalized to *PAT1*. Data are shown as mean +/- SD.

Figure 3. The Set3/Hos2 complex is a key regulator of white-opaque switching

(A) The PHD finger of CaSET3. The amino acid sequence was aligned to the PHD fingers of ScSet3, ScPho23, ScYng1, ScCti6 and MmIng2 that were shown to bind H3K4me3 specifically *in vitro* (Shi et al., 2006; Shi et al., 2007). Colors indicate homologous residues.

Arrowheads highlight the residues of the characteristic Cys_4-His-Cys_3 Zn^{2+} coordination motif.

(B) Dual-layer model of the regulation of white-opaque switching in *C. albicans*. The dotted gray circle denotes the transcriptional circuit as described (Zordan et al., 2007). White and opaque enriched regulators are shown in white and gray, respectively. Colored elements represent histone-modifying pathways modulating the output of the transcriptional circuit.

Table 1. Histone-modifier genes are modulators of white-opaque switching

Quantitative white to opaque (left panel) and opaque to white (right panel) switching assays were performed with multiple homozygous deletion mutants. The percentages represent the fraction of colonies that showed an alteration of the original phenotype. The gene deletions were constructed in the wild type **a**/**a** background strain (second row). As expected, wild type **a**/α strains are locked in the white phase. Data is displayed as a mean +/- SD as well as the total number of colonies scored in 3 independent experiments carried out with the same strain. *: $P < 0.05$ and **: $P < 0.005$ relative to wild type (Student's t-test), §: informative value (see text).

Table 2. Epistasis analysis of *SET3, HOS2, NAT4, HST2* and *EFG1*

Quantitative white to opaque (left panel) and opaque to white (right panel) switching assays were performed with multiple homozygous deletion mutants. The percentages represent the fraction of colonies that showed an alteration of the original phenotype. All strains are *MTL***a**/**a** strains. Data is displayed as a mean +/- SD as well as the total number of colonies scored in 3 independent experiments carried out with the same strain.

Table 3. Loss of *SET3* or *HOS2* suppresses deletion of *HDA1* or *RPD31*

Quantitative white to opaque (left panel) and opaque to white (right panel) switching assays were performed with multiple homozygous deletion mutants. The percentages represent the fraction of colonies that showed an alteration of the original phenotype. All strains are *MTL***a**/**a** strains. The opaque to white switching frequencies were not scored because of the opaque-specific filamentation phenotype caused by the loss of *SET3* or *HOS2* (see text). Data is displayed as a mean +/- SD as well as the total number of colonies scored in 3 independent experiments carried out with the same strain. NA: not assayed.

Table 4. Deletion of *SET3* or *HOS2* is suppressed by the loss of H3K4 methylation

Quantitative white to opaque (left panel) and opaque to white (right panel) switching assays were performed with multiple homozygous deletion mutants. The percentages represent the fraction of colonies that showed an alteration of the original phenotype. All strains are *MTL***a**/**a** strains. Data is displayed as a mean +/- SD as well as the total number of colonies scored in 3 independent experiments carried out with the same strain. NA: not assayed.

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Figures and Tables

Figure 1.

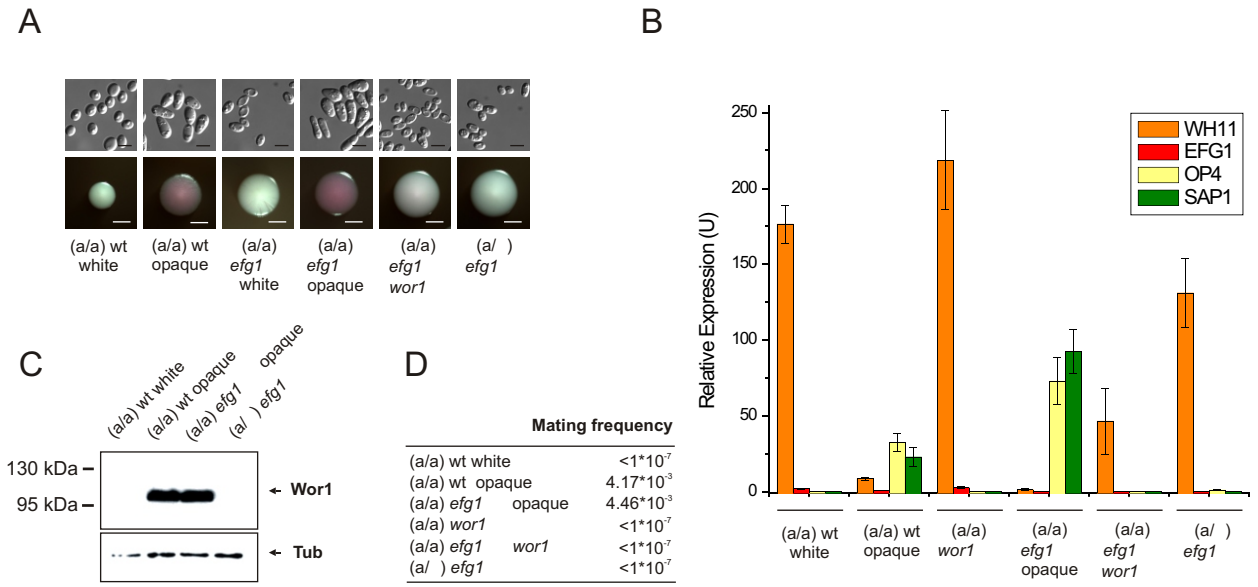
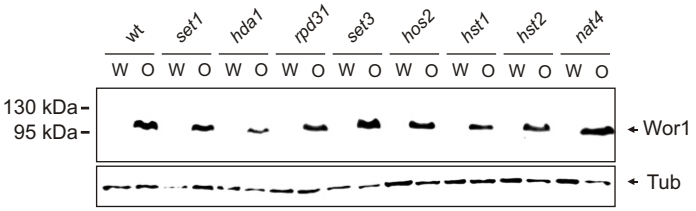


Figure 2.

A

white ⇨ opaque	⇧	⇩	Ø
⇧ white		*hos2* *set3* *nat4*	*pho13*
⇩ opaque			*hst1*
Ø	*set1* *rpd31* *hda1*	*hst2*	

B



C

Strain	Mating frequency White	Opaque
wt	$<1*10^{-7}$	$3.92*10^{-3}$
set1	$<1*10^{-7}$	$1.89*10^{-3}$
hda1	$<1*10^{-7}$	$1.04*10^{-3}$
rpd31	$<1*10^{-7}$	$1.29*10^{-3}$
set3	$<1*10^{-7}$	$3.88*10^{-3}$
hos2	$<1*10^{-7}$	$1.41*10^{-3}$
hst1	$<1*10^{-7}$	$2.55*10^{-3}$
hst2	$<1*10^{-7}$	$2.76*10^{-3}$
pho13	$<1*10^{-7}$	$2.86*10^{-3}$
nat4	$<1*10^{-7}$	$2.59*10^{-3}$

D

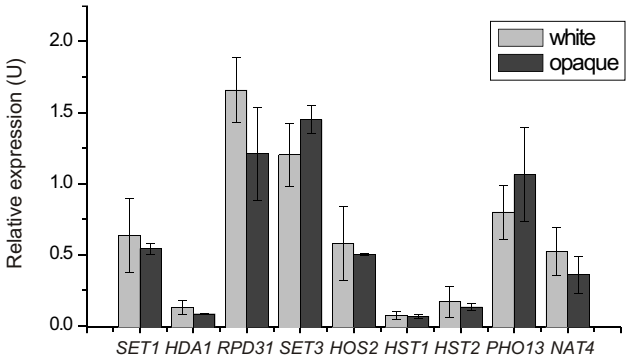


Figure 3.

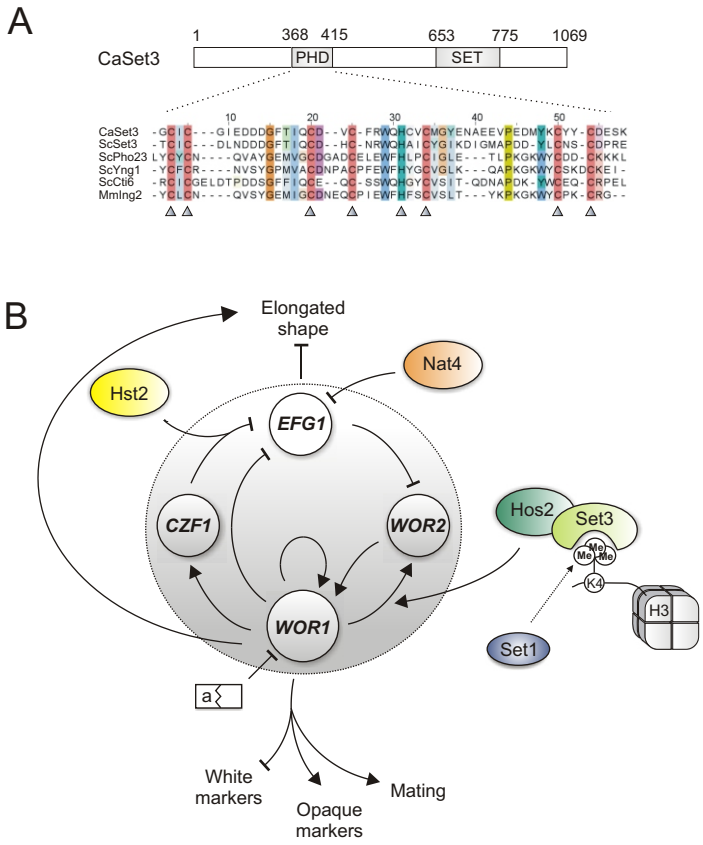

A
1
368
415
653
775
1069
CaSet3
PHD
SET
10
20
30
40
50
CaSet3
ScSet3
ScPho23
ScYng1
ScCti6
Mmlng2
B
Elongated shape
Nat4
Hst2
EFG1
CZF1
WOR2
WOR1
Hos2
Set3
Me
Me
K4
H3
Set1
a
White markers
Opaque markers
Mating

Table 1.

	White → Opaque		Opaque → White	
Strain	**Switch (%)**	**(n)**	**Switch (%)**	**(n)**
wt (**a**/α)	0 ± 0	1808	-	-
wt (**a**/**a**)	11.3 ± 1.9	1113	10.2 ± 1.1	1089
*set1*Δ/Δ	19.5 ± 4.5*	863	10.0 ± 4.5	1886
*hda1*Δ/Δ	30.8 ± 13.2*	2328	10.8 ± 5.4	1320
*rpd31*Δ/Δ	32.2 ± 0.7**	1289	12.5 ± 1.4	800
*set3*Δ/Δ	1.8 ± 0.3**	1352	27.6 ± 2.4$^{** \S}$	743
*hos2*Δ/Δ	1.5 ± 0.2**	1539	23.4 ± 2.2$^{* \S}$	1495
*hst2*Δ/Δ	0.4 ± 0.4**	1994	19.0 ± 14.0	1272
*nat4*Δ/Δ	1.4 ± 0.6**	2006	34.9 ± 13.7*	1171
*hst1*Δ/Δ	14.6 ± 4.8	2038	3.8 ± 0.4**	1270
*pho13*Δ/Δ	10.5 ± 1.1	807	51.1 ± 7.9**	1033

Table 2.

	White → Opaque		Opaque → White	
Strain	**Switch (%)**	**(n)**	**Switch (%)**	**(n)**
wt	11.3 ± 1.9	1113	10.2 ± 1.1	1089
*efg1*Δ/Δ	97.7 ± 1.0	1110	0.6 ± 0.8	1576
*efg1*Δ/Δ *hst2*Δ/Δ	87.9 ± 20.1	940	1.9 ± 2.6	1969
*efg1*Δ/Δ *nat4*Δ/Δ	89.2 ± 10.7	812	0.3 ± 0.6	1568
*efg1*Δ/Δ *set3*Δ/Δ	3.0 ± 1.6	2685	12.9 ± 6.7	1299
*efg1*Δ/Δ *hos2*Δ/Δ	3.6 ± 1.3	1148	5.2 ± 6.3	1061
*efg1*Δ/Δ *hst2*Δ/Δ *hos2*Δ/Δ	5.6 ± 3.6	1117	5.5 ± 3.7	599
*efg1*Δ/Δ *nat4*Δ/Δ *set3*Δ/Δ	2.7 ± 1.3	1322	10.4 ± 6.9	906

Table 3.

	White → Opaque		Opaque → White	
Strain	**Switch (%)**	**(n)**	**Switch (%)**	**(n)**
wt	11.6 ± 4.5	560	21.8 ± 9.5	609
*hda1*Δ/Δ *set3*Δ/Δ	1.4 ± 0.8	1295	NA	-
*rpd31*Δ/Δ *set3*Δ/Δ	2.1 ± 0.9	1409	NA	-
*rpd31*Δ/Δ *hos2*Δ/Δ	0.7 ± 0.3	1479	NA	-

Table 4.

	White → Opaque		Opaque → White	
Strain	**Switch (%)**	**(n)**	**Switch (%)**	**(n)**
wt	11.6 ± 4.5	560	21.8 ± 9.5	609
*set1*Δ/Δ *hos2*Δ/Δ	12.8 ± 2.7	1303	NA	-
*set1*Δ/Δ *set3*Δ/Δ	10.5 ± 4.4	1108	NA	-
*efg1*Δ/Δ *set1*Δ/Δ	NA	-	0.2 ± 0.3	1636
*efg1*Δ/Δ *set1*Δ/Δ *hos2*Δ/Δ	80.6 ± 7.5	1318	1.4 ± 1.5	1730

Supplementary Material

Supplementary Table 1. Open Reading Frames (ORFs) identified as putative switching modulators

Function	*S. cerevisiae* gene name	*C. albicans* ORF	Protein length	Protein BLAST E-value**	*C.albicans* Deletion(s) created	*S. cerevisiae* Reference	*C.albicans* Reference
HMT (KMT)§	*SET1(KMT2)*§	19.6009	1040	$4 \cdot 10^{-101}$	+ +	(Roguev et al., 2001)	(Raman et al., 2006)
	SET2(KMT3)§	19.1755	844	$1 \cdot 10^{-115}$	+		
	DOT1(KMT4)§	19.7402	1343	$2 \cdot 10^{-56}$	+		
HDPH	*PHO8*	19.4736	551	$<10^{-150}$	+		
	PHO13	19.4444	308	$5 \cdot 10^{-78}$	+ +	(Tuleva et al., 1998)	
HAT (KAT)§	*GCN5(KAT2)*§	19.705	449	$<10^{-150}$			
	SAS2(KAT8)§	19.2087	352	$2 \cdot 10^{-60}$	+		
	HAT1(KAT1)§	19.779	413	$7 \cdot 10^{-82}$			
	SAS3(KAT6)§	19.2540	805	$8 \cdot 10^{-98}$			
	ELP3(KAT9)§	19.7387	547	$<10^{-150}$	+		
	NAT4	19.4664	226	$3 \cdot 10^{-13}$	+ +	(Song et al., 2003)	
	SPT10	19.2361	469	$3 \cdot 10^{-60}$	+		
	HPA2	19.6323	192	$5 \cdot 10^{-32}$	+		
HDAC	*HDA1*	19.2606	833	$<10^{-150}$	+ +	(Carmen et al., 1996)	(Klar et al., 2001)
	RPD3	19.6801 *(RPD3)*	577	$<10^{-150}$		(Rundlett et al., 1996)	(Srikantha et al., 2001)
	RPD3	19.2834 *(RPD31)*	480	$<10^{-150}$	+ +		
	SIR2	19.1992	515	$4 \cdot 10^{-103}$	+		
	HST1	19.4761	657	$5 \cdot 10^{-105}$	+ +	(Xie et al., 1999)	
	HST2	19.2580	331	$2 \cdot 10^{-72}$	+ +	(Landry et al., 2000)	
	HOS1	19.4411	436	$2 \cdot 10^{-56}$	+		
	HOS2	19.5377	454	$<10^{-150}$	+ +	(Pijnappel et al., 2001)	
	HOS3	19.2772	713	$<10^{-150}$	+		
Additional*	*SET3*	19.7221	1069	$2 \cdot 10^{-47}$	+ +	(Pijnappel et al., 2001)	

HMT: histone methyltransferase, HDPH: histone dephosphorlyase, HAT: histone acetyltransferase, HDAC: histone deacetylase, KMT: K-Methyltransferase, KAT: K-Acetyltransferase

* HDAC complex subunit

** Performed at http://www.ncbi.nlm.nih.gov/blast/Blast.cgi using the algorithm described in (Altschul et al., 1990)

§ Name according to the nomenclature described in (Allis et al., 2007)

Supplementary Table S2. Strains used in this study

Description	Name	Mating Type	Parent	Genotype	Reference	Figure/Table
wt	SC5314	a/α	Clinical isolate		(Gillum et al., 1984)	
wt	SN152	a/α	SC5314	*arg4*Δ/*arg4*Δ *his1*Δ/*his1*Δ *leu2*Δ/*leu2*Δ *ura3*Δ::*λimm*^{434}/*ura3*Δ::*λimm*^{434} *IRO1*/*iro1*Δ::*λimm*^{434}	(Noble and Johnson, 2005)	Figure 1
wt	DHCA202	a/a	SN152	*arg4*Δ/*arg4*Δ *his1*Δ/*his1*Δ *leu2*Δ/*leu2*Δ *ura3*Δ::*λimm*^{434}/*ura3*Δ::*λimm*^{434} *IRO1*/*iro1*Δ::*λimm*^{434}	This study	Figure 1 Table 1
*efg1*Δ/Δ	DHCA216	a/α	SC5314	*efg1*Δ::FRT/*efg1*Δ::FRT	This study	Figure 1
*efg1*Δ/Δ	HLC67	a/a	SC5314	*ura3*Δ::*λimm*^{434}/*ura3*Δ::*λimm*^{434} *efg1*Δ::*hisG*/*efg1*Δ::*hisG*	(Lo et al., 1997)	Figure 1C
*efg1*Δ/Δ	DHCA212 DHCA214	a/a	DHCA202	*efg1*Δ::FRT/*efg1*Δ::FRT	This study	Figure 1, Table 3
*wor1*Δ/Δ	DHCA218 DHCA220	a/a	DHCA202	*wor1*Δ::*C.d.HIS1*/*wor1*Δ::*C.m.LEU2*	This study (Zordan et al., 2006)	Figure 1
*efg1*Δ/Δ*wor1*Δ/Δ	DHCA222 DHCA224	a/a	DHCA202	*efg1*Δ::FRT/*efg1*Δ::FRT *wor1*Δ::*C.d.HIS1*/*wor1*Δ::*C.m.LEU2*	This study	Figure 1
*set1*Δ/Δ	DHCA226 DHCA228	a/a	DHCA202	*set1*Δ::*C.d.HIS1*/*set1*Δ::*C.m.LEU2*	This study	Table 1, Figure 2
*hda1*Δ/Δ	DHCA232 DHCA234	a/a	DHCA202	*hda1*Δ::*C.d.HIS1*/*hda1*Δ::*C.m.LEU2*	This study	Table 1, Figure 2
*rpd31*Δ/Δ	DHCA238 DHCA240	a/a	DHCA202	*rpd31*Δ::*C.d.HIS1*/*rpd31*Δ::*C.m.LEU2*	This study	Table 1, Figure 2
*set3*Δ/Δ	DHCA242 DHCA244	a/a	DHCA202	*set3*Δ::*C.d.HIS1*/*set3*Δ::*C.m.LEU2*	This study	Table 1, Figure 2
*hos2*Δ/Δ	DHCA246 DHCA248	a/a	DHCA202	*hos2*Δ::*C.d.HIS1*/*hos2*Δ::*C.m.LEU2*	This study	Table 1, Figure 2
*hst1*Δ/Δ	DHCA250 DHCA252	a/a	DHCA202	*hst1*Δ::*C.d.HIS1*/*hst1*Δ::*C.m.LEU2*	This study	Table 1, Figure 2
*hst2*Δ/Δ	DHCA256 DHCA258	a/a	DHCA202	*hst2*Δ::*C.d.HIS1*/*hst2*Δ::*C.m.LEU2*	This study	Table 1, Figure 2

pho13Δ/Δ	DHCA262 DHCA264	a/a	DHCA202	*pho13Δ::C.d.HIS1/pho13Δ::C.m.LEU2*	This study	Table 1, Figure 2
nat4Δ/Δ	DHCA268 DHCA270	a/a	DHCA202	*nat4Δ::C.d.HIS1/nat4Δ::C.m.LEU2*	This study	Table 1, Figure 2
efg1Δ/Δ hos2Δ/Δ	DHCA302 DHCA304	a/a	DHCA202	*hos2Δ::C.d.HIS1/hos2Δ::C.m.LEU2* *efg1Δ*::FRT/*efg1Δ*::FRT	This study	Table 2
efg1Δ/Δ set3Δ/Δ	DHCA298 DHCA300	a/a	DHCA202	*set3Δ::C.d.HIS1/set3Δ::C.m.LEU2* *efg1Δ*::FRT/*efg1Δ*::FRT	This study	Table 2
efg1Δ/Δ hst2Δ/Δ	DHCA306 DHCA308	a/a	DHCA202	*hst2Δ::C.d.HIS1/hst2Δ::C.m.LEU2* *efg1Δ*::FRT/*efg1Δ*::FRT	This study	Table 2
efg1Δ/Δ nat4Δ/Δ	DHCA310 DHCA312	a/a	DHCA202	*nat4Δ::C.d.HIS1/nat4Δ::C.m.LEU2* *efg1Δ*::FRT/*efg1Δ*::FRT	This study	Table 2
efg1Δ/Δ hst2Δ/Δ hos2Δ/Δ	DHCA318 DHCA320	a/a	DHCA202	*hst2Δ::C.d.HIS1/hst2Δ::C.m.LEU2* *hos2Δ*::FRT/*hos2Δ*::FRT *efg1Δ*::FRT/*efg1Δ*::FRT	This study	Table 2
efg1Δ/Δ nat4Δ/Δ set3Δ/Δ	DHCA322 DHCA324	a/a	DHCA202	*nat4Δ::C.d.HIS1/nat4Δ::C.m.LEU2* *set3Δ*::FRT/*set3Δ*::FRT *efg1Δ*::FRT/*efg1Δ*::FRT	This study	Table 2
rpd31Δ/Δ hos2Δ/Δ	DHCA290 DHCA292	a/a	DHCA202	*rpd31Δ::C.d.HIS1/rpd31Δ::C.m.LEU2* *hos2Δ*::FRT/*hos2Δ*::FRT	This study	Table 3
hda1Δ/Δ set3Δ/Δ	DHCA282 DHCA284	a/a	DHCA202	*hda1Δ::C.d.HIS1/hda1Δ::C.m.LEU2* *set3Δ*::FRT/*set3Δ*::FRT	This study	Table 3
rpd31Δ/Δ set3Δ/Δ	DHCA286 DHCA288	a/a	DHCA202	*rpd31Δ::C.d.HIS1/rpd31Δ::C.m.LEU2* *set3Δ*::FRT/*set3Δ*::FRT	This study	Table 3
set1Δ/Δ hos2Δ/Δ	DHCA326 DHCA328	a/a	DHCA202	*set1Δ::C.d.HIS1/set1Δ::C.m.LEU2* *hos2Δ*::FRT/*hos2Δ*::FRT	This study	Table 4
set1Δ/Δ set3Δ/Δ	DHCA330 DHCA332	a/a	DHCA202	*set1Δ::C.d.HIS1/set1Δ::C.m.LEU2* *set3Δ*::FRT/*set3Δ*::FRT	This study	Table 4
efg1Δ/Δ set1Δ/Δ	DHCA334 DHCA336	a/a	DHCA202	*set1Δ::C.d.HIS1/set1Δ::C.m.LEU2* *efg1Δ*:FRT/*efg1Δ*:FRT	This study	Table 4
efg1Δ/Δ set1Δ/Δ hos2Δ/Δ	DHCA338 DHCA340	a/a	DHCA202	*set1Δ::C.d.HIS1/set1Δ::C.m.LEU2* *hos2Δ*::FRT/*hos2Δ*::FRT *efg1Δ*::FRT/*efg1Δ*::FRT	This study	Table 4
Maa (mating test strain)	DHCA209	a/a	SC5314	*ade2Δ*::FRT/*ade2Δ*::FRT	This study	Figure 1C
Mαα (mating test strain)	DHCA210	α/α	SC5314	*ade2Δ*::FRT/*ade2Δ*::FRT	This study	Figures 1C, 2B
Additional						
set2Δ/Δ	DHCA230	a/a	DHCA202	*set2Δ::C.d.HIS1/set2Δ::C.m.LEU2*	This study	
sir2Δ/Δ	DHCA254	a/a	DHCA202	*sir2Δ::C.d.HIS1/sir2Δ::C.m.LEU2*	This study	
elp3Δ/Δ	DHCA260	a/a	DHCA202	*elp3Δ::C.d.HIS1/elp3Δ::C.m.LEU2*	This study	
pho8Δ/Δ	DHCA266	a/a	DHCA202	*pho8Δ::C.d.HIS1/pho8Δ::C.m.LEU2*	This study	
dot1Δ/Δ	DHCA272	a/a	DHCA202	*dot1Δ::C.d.HIS1/dot1Δ::C.m.LEU2*	This study	

hos1Δ/Δ	DHCA274	a/a	DHCA202	*hos1Δ::C.d.HIS1/hos1Δ::C.m.LEU2*	This study	
hos3Δ/Δ	DHCA280	a/a	DHCA202	*hos3Δ::C.d.HIS1/hos3Δ::C.m.LEU2*	This study	
spt10Δ/Δ	DHCA276	a/a	DHCA202	*spt10Δ::C.d.HIS1/spt10Δ::C.m.LEU2*	This study	
hpa2Δ/Δ	DHCA278	a/a	DHCA202	*hpa2Δ::C.d.HIS1/hpa2Δ::C.m.LEU2*	This study	

Supplementary Table 3. Primers used in this study

Name	Sequence (5´- 3´)*	Reference
Gene deletion constructs based on fusion PCR strategy (Noble and Johnson, 2005)		
5C_CA6009	CGGTTTTCACTTAACTGCTGC	
53_CA6009	cacggcgcgcctagcagcggCATAGCAAGGTGTATGTTTGATTAC	
35_CA6009	gtcagcggccgcatccctgcTAATTGCATAAACGTGTGATAAATC	
33_CA6009	TTAGTAGTAGGCGAATAGACACAGAC	
55_CA1755	GATCTTGAAGAAGTAATGTTTCCTG	
53_CA1755	cacggcgcgcctagcagcggCATGATTTGTTAAATGAGTGTTTATC	
35_CA1755	gtcagcggccgcatccctgcTAGATATTTATTTTGTTCATACAAGAAAAA	
33_CA1755	GTTTCTGCTGAAGAAGCCG	
55_CA2606	GTGTGAGTAGAGTTCACAACAAGTG	
53_CA2606	cacggcgcgcctagcagcggCATTCTTAAAAAGGAAAATGATAATAG	
35_CA2606	gtcagcggccgcatccctgcTGATTCGAGTAGAAACAACAACAAC	
33_CA2606	CAACACTTTCCAAACACTCTCAG	
55_CA2087	AAGGCAAGAAGAGAATCTTTGG	
53_CA2087	cacggcgcgcctagcagcggCATGCATGAACTTTTATGATATTAGATG	
35_CA2087	gtcagcggccgcatccctgcTGAATTAGTTACATACTTTTTTTTTTAAA	
33_CA2087	ACATGGAATACGTTGACTGGG	
55_CA6801	AATAACAGTTACTGTCACCGCC	
53_CA6801	cacggcgcgcctagcagcggCATGGTGGACGAGTTTGGTTG	
35_CA6801	gtcagcggccgcatccctgcTAAAAGTTCATAAATAAAAGGATATTTAGATTG	
33_CA6801	TCCGAAGGGAATTTAATTGG	
55_CA7221	ATTGTACGAAGAAGCGGAGC	
53_CA7221	cacggcgcgcctagcagcggCATTAAAATAAACACTTATAAAGACTACTATC	
35_CA7221	gtcagcggccgcatccctgcTAGTTTTTTGTTTAGAGTTTGTATATTG	
33_CA7221	CAAAAGGACAATCAATTGGATG	
55_CA5377	CAGAATCTTGACCTGTGATTCC	
53_CA5377	cacggcgcgcctagcagcggCATTTATATTAACTACTTTTCTCCTATGG	
35_CA5377	gtcagcggccgcatccctgcTAGTTTGTCTTGATACACATATACATATATATA TA	
33_CA5377	GAAAAATGGATGCCAAGTTG	
55_CA4761	CACCCTTTCCTCTATTCTTTGC	
53_CA4761	cacggcgcgcctagcagcggCATTATTGATTGTTATTTGTTATTAGTAATAAT TG	
35_CA4761	gtcagcggccgcatccctgcTGAAGAGCCAAAACAAGATAAAG	
33_CA4761	TATTGCTGCTTATAATGTATAGGGAG	
55_CA1992	ATCACAACAGTAGGGCATCAAC	
53_CA1992	cacggcgcgcctagcagcggCATTACTAGAGGATTTCTCTCAAATAAC	
35_CA1992	gtcagcggccgcatccctgcTGATTAAATTAATATTGGTGTCTTTAATG	
33_CA1992	TCAAGATCAACAATATGTGGTGG	
55_CA2580	CATTCGAGCTAAACGAAGCTC	
53_CA2580	cacggcgcgcctagcagcggCATGGTTTAAAGTTATAGTCTGTTGTAAG	
35_CA2580	gtcagcggccgcatccctgcTTAAACTGTCTCAACAAAAATATAATATAC	
33_CA2580	GCCTTCATAAAACGTTCTCTCAC	
55_CA7387	AAGTCTGACATTACACCAATATAAGG	
53_CA7387	cacggcgcgcctagcagcggCATTATGTAGTGTGTTTATTAGGAGAAG	
35_CA7387	gtcagcggccgcatccctgcTAAGACTTGATACTTACAGATTTGTATATG	
33_CA7387	CTATTGTTGATGGTGAACCACC	
55_CA4444	AGATAAAGAGTTGTTCTCTCAGTTCC	
53_CA4444	cacggcgcgcctagcagcggCATTAGAAACGGAATGTATTGGG	
35_CA4444	gtcagcggccgcatccctgcTAGAAACATATGCCTTGCTATTTTT	
33_CA4444	TGCAAGCAAAACTAAGAACGTC	
55_CA4736	CCTATCTTTACACTACTGTTCACCG	
53_CA4736	cacggcgcgcctagcagcggCATGATGGAATATGTAGCAATAAAAAG	
35_CA4736	gtcagcggccgcatccctgcTAAGTGTGTATGTACATATACCAAGAAC	
33_CA4736	ACAATGAAGCAAGTGAATAACG	

55_CA4664	TTTATTGGTTCTCAATGGCAG	
53_CA4664	cacggcgcgcctagcagcggCATTGTTGTTTGTGTTGGCA	
35_CA4664	gtcagcggccgcatccctgcTAAGTATTTGTGTAGAACATTCTTCCC	
33_CA4664	CAACAAATGGGTAGCAAGGTC	
55_CA7402	CGCTCCTAATTTCTCCAAGC	
53_CA7402	cacggcgcgcctagcagcggCATTATTAGTATTTGTCAAGTGCACAC	
35_CA7402	gtcagcggccgcatccctgcTAAAATGTTTTATAGCTAAGCTGTACC	
33_CA7402	AAATATGGCTTATCGACGGAG	
55_CA4411	TGCCAGTAAACGAGGTTACG	
53_CA4411	cacggcgcgcctagcagcggCATTAATCTATTAACAATAATACAAACCAG	
35_CA4411	gtcagcggccgcatccctgcTAAAATTATATATCTACCACTAAACTGTTTAAG	
33_CA4411	TTGTTACTGGAAAGGGACCC	
55_CA2361	TCTTCATCGTAATACGACGGTC	
53_CA2361	cacggcgcgcctagcagcggCATGATCTATTTAAAGATTGTAAGGAAT	
35_CA2361	gtcagcggccgcatccctgcTAGTTAAAAAGATCACACACTTACTGTTG	
33_CA2361	TGATTTCGATCAAAGAGCTGC	
55_CA6323	TGTTATGTATGAATGCACCGAG	
53_CA6323	cacggcgcgcctagcagcggCATTGACCCCTGGCTCCG	
35_CA6323	gtcagcggccgcatccctgcTGACCAATGTACACGACCTATG	
33_CA6323	TGAATCTTCAATTCATCGGC	
55_CA2772	CAATGGAAATATTGTTACAGATGG	
53_CA2772	cacggcgcgcctagcagcggCATTATAAAAAACTTCCCCAAGTAA	
35_CA2772	gtcagcggccgcatccctgcTAGAGTATATTAGAACATGAAATGTATATATATAAG	
33_CA2772	AACAACTACAACAACTACTACTACTCCAC	
55_CA4884	GCCTTTCCTGTATTGGTATTGG	
53_CA4884	cacggcgcgcctagcagcggCATTGCTTAATATTGAATTGAATTATAC	
35_CA4884	gtcagcggccgcatccctgcTAGTTGAATTAATACGGTGATTCTG	
33_CA4884	GCAATATTACAATTCCCTTCATG	
M5	ccgctgctaggcgcgccgtgACCAGTGTGATGGATATCTGC	(Noble and Johnson, 2005)
M3	gcagggatgcggccgctgacAGCTCGGATCCACTAGTAACG	(Noble and Johnson, 2005)
Gene deletion constructs based on the SAT1-flipping strategy (Reuss et al., 2004)		
CaHOS2_55	gctagggccctagccgatcgCAGAATCTTGACCTGTGATTCC	
CaHOS2_53	gtcgctcgagcgacCATTTATATTAACTACTTTTCTCCTATGG	
CaHOS2_35	gctaccgcggtagcTAGTTTGTCTTGATACACATATACATATATATATA	
CaHOS2_33	gtcggagctccgaccgatcgGAAAAATGGATGCCAAGTTG	
CaSET3_55	gctagggccctagccgatcgATTGTACGAAGAAGCGGAGC	
CaSET3_53	gtcgctcgagcgacCATTAAAATAAACACTTATAAAGACTACTATC	
CaSET3_35	gctaccgcggtagcTAGTTTTTTGTTTAGAGTTTGTATATTG	
CaSET3_33	gtcggagctccgaccgatcgCAAAAGGACAATCAATTGGATG	
CaEFG1_55	gctagggccctagccgatcgAGAGACAAGCAAACAAACGACC	
CaEFG1_53	gtcgctcgagcgacCATTAATATGGGTTATATTCTTGGTAGTC	
CaEFG1_35	gctaccgcggtagcTGACTCAAGGTTCAGTTCACCC	
CaEFG1_33	gtcggagctccgaccgatcgCACCTACACGCCACAAACTATC	
CaADE2_55	gctagggccctagccgatcgCATAATTTCCTAAGCTAGTCGTGATGG	
CaADE2_53	gtcgctcgagcgacCATTATGTGTAGTGCTTGTATATGCGTG	
CaADE2_35	gctaccgcggtagcGATATCAATAGCATATTAAACAAGTAAACG	
CaADE2_33	gtcggagctccgaccgatcgAGAATCAATGCTATTGAGGGGTTAG	
Quantitative Real-time PCR		
RT5_WH11	CAGAACAATTCAAGGATAAGGTTACTG	(Zordan et al., 2006)
RT3_WH11	TTGGAGTCACCAAAAATAGCATCAG	(Zordan et al., 2006)

RT5_EFG1	CATCACAACCAGGTTCTACAACCAAT	(Zordan et al., 2006)
RT3_EFG1	CTACTATTAGCAGCACCACCC	(Zordan et al., 2006)
RT5_SAP1	TTCAACAAGATGTTGCTCAAG	(Zordan et al., 2006)
RT3_SAP1	GTTGACCGTTAGCGTAGCTC	(Zordan et al., 2006)
RT5_OP4	CCTCAAAAGCTGCTACCTC	(Zordan et al., 2006)
RT3_OP4	GTATCAACAGTTGGAGTAGAAGTAG	(Zordan et al., 2006)
RT5_PAT1	TTATCGGAATGGTCCTCGTG	(Zordan et al., 2006)
RT3_PAT1	CCAGAAGAACCATCATCAAC	(Zordan et al., 2006)
RT5_CA6009	TGGTCTTGGAAGAGCAAGAACAG	
RT3_CA6009	CTCAACACATCGGATTCAGAACC	
RT5_CA2606	CATTCCAACTTCCGATCCCAG	
RT3_CA2606	CCAATCCACCATTGATTCATCC	
RT5_CA6801	TCCAATGATGGTAGTTGGTGGAG	
RT3_CA6801	TCGGAGCCTCCCTTTGTGTC	
NRT5_CA7221	TACCTTGGGAATGGGATGTGG	
NRT3_CA7221	CCGTTGATTCAACCTTTCCTCC	
RT5_CA5377	TTCCCAGGTACAGGGTCAGTTG	
RT3_CA5377	GATTGACATCATTCAATACGCTCG	
RT5_CA4761	GATGACGATGACGAGGAAGAGG	
RT3_CA4761	GATGAGGTAAATCCCAAGATTCACC	
RT5_CA4444	TGCGACGGTGTCTTATGGTTG	
RT3_CA4444	ATCAGACCCACCAACAGTGGTG	
RT5_CA4664	TCGAAGCATCTCACAATATGGATG	
RT3_CA4664	CAGTTGTCCTAATTTCTGGCCTTG	

* Lower case and upper case letters denote exogenous and endogenous sequences, respectively.

Supplementary Table 4. Plasmids used in this study

Name	Parent	Target locus	Reference
pSFS2A	-	-	(Reuss et al., 2004)
pDH102	pSFS2A	*HOS2*	This study
pDH104	pSFS2A	*SET3*	This study
pDH106	pSFS2A	*EFG1*	This study
pDH108	pSFS2A	*ADE2*	This study

Supplementary Table 5. Mating frequencies

	Mating frequency	
Strain	**White**	**Opaque**
wt	$< 1 \cdot 10^{-7}$	$4.23 \cdot 10^{-3}$
*efg1*Δ/Δ	NA	$4.39 \cdot 10^{-3}$
*efg1*Δ/Δ *hst2*Δ/Δ	NA	$4.72 \cdot 10^{-3}$
*efg1*Δ/Δ *nat4*Δ/Δ	NA	$3.57 \cdot 10^{-3}$
*efg1*Δ/Δ *set3*Δ/Δ	$< 1 \cdot 10^{-7}$	$1.21 \cdot 10^{-3}$
*efg1*Δ/Δ *hos2*Δ/Δ	$< 1 \cdot 10^{-7}$	$3.79 \cdot 10^{-3}$
*efg1*Δ/Δ *hst2*Δ/Δ *hos2*Δ/Δ	$< 1 \cdot 10^{-7}$	$9.09 \cdot 10^{-4}$
*efg1*Δ/Δ *nat4*Δ/Δ *set3*Δ/Δ	$< 1 \cdot 10^{-7}$	$1.50 \cdot 10^{-3}$

Supplementary Table 6. Mating frequencies

	Mating frequency	
Strain	**White**	**Opaque**
wt	$< 1 \cdot 10^{-7}$	$4.23 \cdot 10^{-3}$
*hda1*Δ/Δ *set3*Δ/Δ	$< 1 \cdot 10^{-7}$	$5.67 \cdot 10^{-3}$
*rpd31*Δ/Δ *set3*Δ/Δ	$< 1 \cdot 10^{-7}$	$5.68 \cdot 10^{-3}$
*rpd31*Δ/Δ *hos2*Δ/Δ	$< 1 \cdot 10^{-7}$	$2.52 \cdot 10^{-3}$

Supplementary Table 7. Mating frequencies

	Mating frequency	
Strain	**White**	**Opaque**
wt	$< 1 \cdot 10^{-7}$	$4.23 \cdot 10^{-3}$
*set1*Δ/Δ *hos2*Δ/Δ	$< 1 \cdot 10^{-7}$	$1.13 \cdot 10^{-3}$
*set1*Δ/Δ *set3*Δ/Δ	$< 1 \cdot 10^{-7}$	$1.92 \cdot 10^{-3}$
*efg1*Δ/Δ *set1*Δ/Δ	NA	$2.41 \cdot 10^{-3}$
*efg1*Δ/Δ *set1*Δ/Δ *hos2*Δ/Δ	NA	$3.44 \cdot 10^{-4}$

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6.2. Future prospects

The analysis presented in this work doubled the number of known regulators of *white-opaque* switching in *Candida albicans*. Importantly, we functionally mapped some of the newly identified regulators into the transcriptional circuit of the previously studied ones. In addition, the data raised several important possibilities for future studies that are discussed in the following section.

6.2.1. Histone-modifying enzymes regulate filamentation

As described above (page 40), mutations of several histone modifiers influence the yeast-filament transition. Since mutations that compromise the ability of *C. albicans* to change growth modes also abrogates the virulence of the strain (Gow et al., 2002; Lo et al., 1997), characterization of such mutants is important both for practical and theoretical considerations. The data presented above already revealed that the histone-modifier genes *SET3* and *HOS2*, encoding two essential subunits of the Set3/Hos2 histone deacetylase complex display several morphogenetic phenotypes. Disruption of either *HOS2* or *SET3* results in constitutive and de-regulated filament formation under non-inducing conditions *in vitro*. Furthermore, *opaque* phase *set3*Δ/Δ and *hos2*Δ/Δ cells also display filamentous growth on defined media where *set3*Δ/Δ and *hos2*Δ/Δ white cells and both phases of wild type strains grow as yeasts. These observations indicate that the Set3/Hos2 complex is a repressor of the filamentous growth program. Importantly, it may represent an integrator of multiple stimuli such as temperature and developmental phase (Figure 18A and 18B).

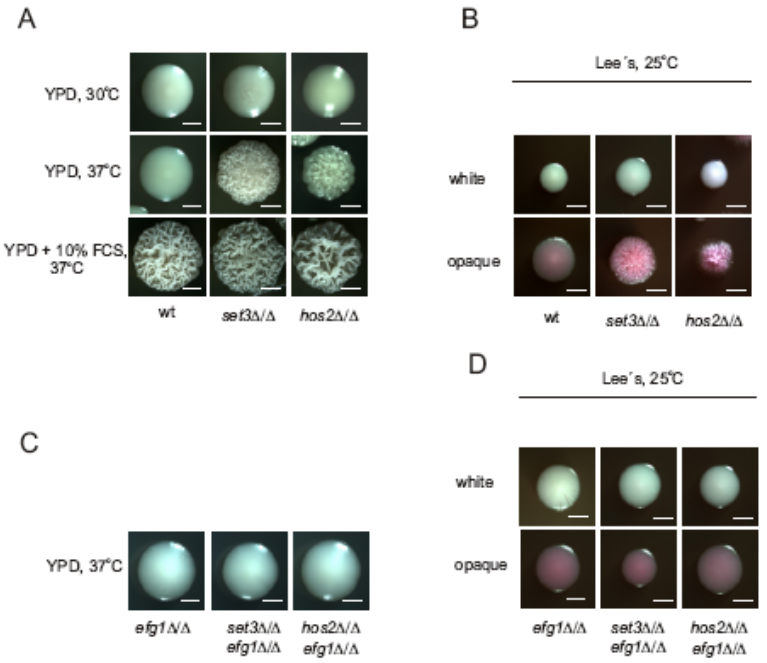

Figure 18. (A) and (B) *SET3* and *HOS2* are negative regulators of the filamentous growth program. (C) and (D) Deletion of *SET3* or *HOS2* is suppressed by the loss of *EFG1* in filament formation. Scale bars correspond to 2mm. FCS: Foetal calf serum.

As already mentioned above, Efg1 is a transcription factor that has been extensively studied (pages 29, 32, 43). Efg1 is not only involved in the regulation of *white-opaque* switching but is also a key pleiotropic regulator of filamentation. Efg1 is activated by the PKC-cAMP signalling pathway and is a major activator of hyphal growth. Deletion of *EFG1* severely impairs the ability of *C. albicans* to grow as filaments in liquid and on solid media; however, it promotes filamentation under embedded conditions (Stoldt et al., 1997). Data presented above demonstrated that deletion of *HOS2* or *SET3* suppresses the loss of *EFG1* in the *white-opaque* switching system (page 43). On the other hand, phenotypic analysis of *efg1*Δ/Δ *hos2*Δ/Δ and *efg1*Δ/Δ *set3*Δ/Δ mutants revealed an opposite epistatic relationship concerning the regulation of the yeast-hypha transition (Figure 18).

Taken together, these findings suggest that there is a strong link between the yeast-hypha and *white-opaque* growth programs; however, the outline of the genetic interactions between the individual genes raises many questions that need to be addressed by more precise biochemical approaches. Indeed, the Tup1 repressor of *S. cerevisiae* was shown to physically interact with class I histone deacetylases (where Hos2 belongs, see page 15) and in *C. albicans* Tup1 is proposed to act independently of Efg1 (Braun and Johnson, 2000; Watson et al., 2000; Wu et al., 2001). Therefore, the utilization of the Set3/Hos2 complex by the Tup1 repressor at specific loci (also dependent on H3K4 methylation) is likely to be an evolutionary conserved molecular mechanism.

In addition, *SET3* and *HOS2* as well as others out of the 8 novel identified switching modulator genes are likely to represent virulence factors of *C. albicans*. One of them, *SET1*, has indeed been demonstrated to contribute to the pathogenesis of invasive candidiasis (Raman et al., 2006).

6.2.2. Bridging genetic interactions with biochemical function

The presented data identified many genetic interactions between transcription factors and chromatin modifying enzymes based on functional and phenotypic analyses. Histone-modifying enzymes and their respective genes were identified by a bioinformatics approach, revealing a high conservation of orthologous sequences across fungal species (page 66). However, the question remained open whether the biochemical functions are also conserved. Moreover, the mechanisms driving recruitment of histone-modifying enzymes to specific genomic locations is at present still a major enigma in the field of chromatin biology.

The data presented in this Ph.D. thesis indicates that *C. albicans* is a suitable model to study chromatin-based epigenetic mechanisms and offers a possibility to perform comparative biochemical studies, rather than relying on *S. cerevisiae* as a sole fungal model system. In particular, the opposite epistatic relationship between *EFG1* and Set3/Hos2 as well as between *SET1* and Set3/Hos2 in the regulation of *white-opaque* switching and filamentation may represent a new, intriguing model system to study recruitment of chromatin modifying enzymes and integration of multiple signals at key loci determining functional outcomes.

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8. ACKNOWLEDGEMENTS

I would like to thank Karl Kuchler for his support and the opportunity to work in his lab; all the present and former members of the Kaku group for a nice working environment.

I am particularly indebted to Stefan Ameres for the translation of the Summary.

Last but not least, I wish to point out that completion of this thesis was less the results of personal efforts rather than the motivation and support by several people. Since the originality and elegance of the work is rather limited, I hereby wish to forgo naming them, so that they remain protected should anybody want to hold them accountable…

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