Note

A Histone Deacetylation Inhibitor and Mutant Promote Colony-Type Switching of the Human Pathogen *Candida albicans*

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ABSTRACT

Most strains of *Candida albicans* undergo high frequency phenotypic switching. Strain WO-1 undergoes the white-opaque transition, which involves changes in colony and cellular morphology, gene expression, and virulence. We have hypothesized that the switch event involves heritable changes in chromatin structure. To test this hypothesis, we transiently exposed cells to the histone deacetylase inhibitor trichostatin-A (TSA). Treatment promoted a dramatic increase in the frequency of switching from white to opaque, but not opaque to white. Targeted deletion of *HDA1*, which encodes a deacetylase sensitive to TSA, had the same selective effect. These results support the model that the acetylation of histones plays a selective role in regulating the switching process.

MOST strains of the opportunistic yeast pathogen *Candida albicans* switch spontaneously, reversibly, and at high frequency (10^{-4} to 10^{-1}) between a number of general phenotypes distinguishable by colony morphology and, in some cases, cellular morphology (for reviews, see Soll 1992, 2001). In contrast to switching in other pathogens, switching in *C. albicans* is pleiotropic, affecting a variety of unrelated phenotypes, many of them putative virulence factors. The basic mechanism of phenotypic switching in *C. albicans* is unknown. In the white-opaque transition in strain WO-1 (Slutsky *et al.* 1987), which has evolved as a simple model system for investigating switching in *C. albicans*, cells switch between a white phase, characterized by white hemispherical colonies containing round budding cells with smooth cell walls, and an opaque phase, characterized by gray flat colonies containing large elongate cells with pimpled cell walls and a large cytoplasmic vacuole (Anderson and Soll 1987). The white-opaque transition affects antigenicity (Anderson *et al.* 1990), aspartyl proteinase secretion (Morrow *et al.* 1992; White *et al.* 1993; Hube *et al.* 1994), environmental constraints on the bud hypha transition (Anderson *et al.* 1989), drug susceptibility (Vargas *et al.* 2000), sensitivity to neutrophils and oxidants (Kolotila and Diamond 1990), and virulence in alternative animal models (Kvaal *et al.* 1997, 1999). The white-opaque transition also regulates expression of a variety of white and opaque phase-specific genes (Soll 2001).

The mechanism of cell-type switching is best understood in two nonpathogenic yeasts, the fission yeast *Schizosaccharomyces pombe* (Klar *et al.* 1998) and the budding yeast *Saccharomyces cerevisiae* (Herskowitz *et al.* 1992). Both switch between two mating types by genetic rearrangements at the locus that determines cell type. For example, *S. pombe* cells switch spontaneously between plus and minus types in ~45% of cell divisions by a transposition-substitution event in which a copy of either the *mat2-P* or *mat3-M* unexpressed "donor" locus is unidirectionally transferred to the expressed *mat1* locus. The *mat2* and *mat3* loci and the intervening ~11.0-kb region are repressed by several *trans*-acting factors (Grewal and Klar 1997; Klar *et al.* 1998). The *clr4* (Ivanova *et al.* 1998) and *swi6* (Lorentz *et al.* 1994) genes encode proteins containing chromo- and/or SET-domains, which are thought to be essential for organizing chromatin structure and which stably maintain specific states of gene expression through multiple rounds of cell divisions (Grewal and Klar 1996). Recent studies have suggested that silencing at the *mat* region occurs through a chromosomally borne epigenetic event that is duplicated along with the chromosome through mitotic and meiotic divisions (Grewal and Klar 1996; Nakayama *et al.* 2000). That silencing at the *mat2/3* interval occurs through organization of heterochromatin-like structure is further supported by the observation that *clr3* and *clr6* genes, which are essential for silencing,

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encode homologs of histone deacetylases (Grewal *et al.* 1998). Histone proteins are essential components of nucleosomes in eukaryotic chromosomes. A recent study showed that the repressed epigenetic state in the *mat2/3* region heritably changes to an expressed state by transient treatment of cells with the histone deacetylase inhibitor trichostatin-A (TSA; Grewal *et al.* 1998). Likewise, silencing of *HM* loci in *S. cerevisiae* is believed to involve assembly of a repressive chromatin structure (Holmes *et al.* 1996), but in this case TSA treatment does not relieve silencing (A. Klar, unpublished results).

On the basis of the mechanism of mating-type silencing in *S. pombe* and *S. cerevisiae*, we and others (this article; Soll 1992, 2001; Perez-Martin *et al.* 1999) have entertained the hypothesis that a heritable change in chromatin structure at key loci represents the basic switch mechanism in *C. albicans*. To date, it was not possible to test this hypothesis genetically because of the inability of *C. albicans* to undergo meiosis. However, the finding that TSA treatment changed the epigenetic imprint in *S. pombe* (Grewal *et al.* 1998) provided us with a possible pharmacological test of the specific hypothesis that the level of acetylation of histones is involved in switching, which could then be confirmed by a mutational analysis.

Trichostatin-A selectively promotes switching in the white-to-opaque direction: Treatment of white phase cells during growth on plates containing Lee's medium with TSA stimulated the white-to-opaque transition. In three separate preparations, treatment of white phase cells that were >99.9% pure with TSA for 48 hr resulted in 39, 68, and 56% opaque phase colonies, and 9, 9, and 5% white phase colonies with opaque phase sectors (Table 1, Figure 1). Treatment of white phase cells with DMSO, in which the TSA was dissolved, or water as controls resulted in 0% opaque phase colonies (Table 1). Treatment of three separate preparations of opaque phase cells with TSA for 48 hr had no effect on the proportion of white colonies. In TSA-, DMSO-, and H_2O-treated control preparations, the proportion of white phase colonies was <0.2%.

In Figure 1, A and B, representative images of control colonies formed by white or opaque phase cells treated with water or DMSO are presented. Cells were plated on agar containing phloxine-B, which preferentially stains opaque phase colonies red (Anderson and Soll 1987). Both the white phase and opaque phase phenotypes were homogeneous. In Figure 1, C and D, representative images are presented of colonies formed by white and opaque phase cells treated with TSA for 48 hr. The high incidence of opaque phase colonies was apparent in the TSA-treated white phase cell preparations (Figure 1C), while no effect on switching was apparent in the TSA-treated opaque phase preparation (Figure 1D). The selective effect of TSA on white phase cells was also evident in the treated cell cultures prior to plating. While the majority of cells of untreated white phase cultures exhibited the round white phase phenotype (Figure 2A), roughly a third of the cells of white phase cultures treated with TSA for 48 hr exhibited the unique elongate opaque phase phenotype (Figure 2B). To be sure that TSA-stimulated switching indeed generated opaque phase cells exhibiting the unique signature opaque phase phenotype, cells were analyzed by scanning electron microscopy. TSA-stimulated opaque phase cells exhibited the signature elongate morphology and wall pimples of opaque phase cells (Anderson *et al.* 1990) and were indistinguishable from opaque phase cells formed at lower frequencies in untreated cultures (data not shown).

TABLE 1

The effect of TSA on the white-to-opaque transition

Preparation	Total no. of Colonies	White phase colonies (%)	Opaque phase colonies (%)	White phase colonies with opaque sector (%)
H_2O	500	500 (100)	0 (0)	0 (0)
	500	500 (100)	0 (0)	0 (0)
	500	500 (100)	0 (0)	0 (0)
DMSO	500	500 (100)	0 (0)	0 (0)
	500	500 (100)	0 (0)	0 (0)
	500	500 (100)	0 (0)	0 (0)
TSA	255	133 (52)	100 (39)	22 (9)
	201	47 (23)	137 (68)	17 (9)
	200	78 (39)	111 (56)	11 (5)

Cells from three independent white colonies were mixed with 3 μl of H_2O, DMSO, or DMSO containing 10 μg/μl of TSA; spotted on agar medium; and allowed to grow at 25° for 48 hr. The cell preparation was then replated on agar containing phloxine B and the phenotypes of single colonies were assessed after 5 days of growth at 25°. Similar experiments with opaque phase cells revealed no effect on the opaque-to-white transition, so those data were not included.

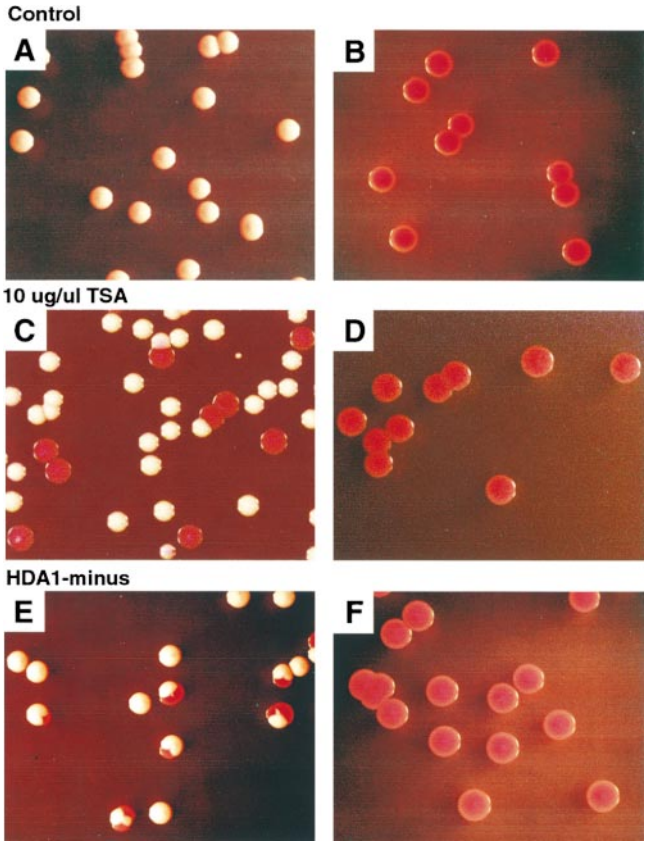

Figure 1.—White and opaque colony morphologies of untreated wild-type cells (A and B, respectively), TSA-treated wild-type cells (C and D, respectively), and *HDA1*-minus (HDho19) cells (E and F, respectively) are displayed. Colonies were grown on Lee's agar medium plates at 25° and photographed after 4 days.

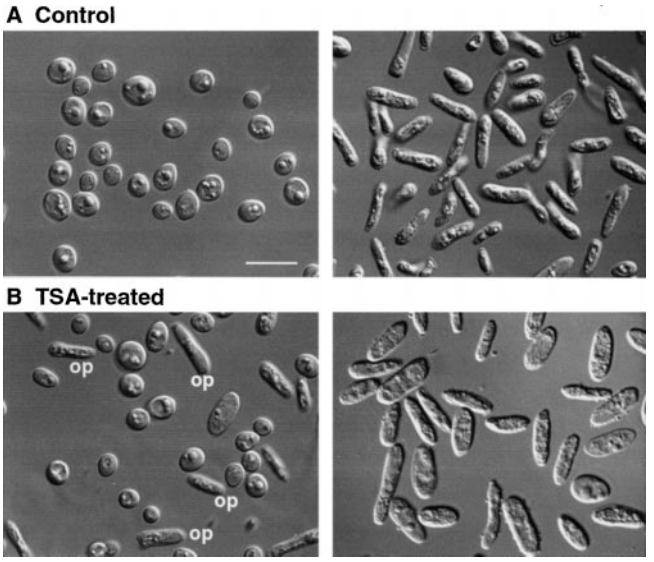

Figure 2.—TSA treatment of white phase cells induces a change from the round budding cell phenotype to the cigar-shaped opaque cell phenotype. White or opaque cells of the wild-type strain WO-1 were mixed with 3 μl of either DMSO alone (A, control) or 10 μg/ml of trichostatin A in DMSO (B, TSA-treated), spotted on agar medium, and incubated for 48 hr. Cells were then compared using phase-contrast microscopy. "op" represents opaque phase cells in the TSA-treated sample. Parts on right represent opaque phase switches of control or TSA-treated samples.

To demonstrate that TSA affected the frequency of switching and did not cause an irreversible change, such as the mutation in a transacting gene in *S. cerevisiae* that locks the cell in the switched phenotype (Klar *et al.* 1981), we tested the reversibility of 20 TSA-stimulated opaque phase colonies by inducing the opaque-to-white transition through a shift from 25° to 37° (Slutsky *et al.* 1987). All 20 preparations switched *en masse* back to white, demonstrating that reversible switching was fully operational in TSA-induced opaque phase cells.

Deletion of the deacetylase gene *HDA1* phenocopies TSA treatment: Although we interpreted the effect of TSA on switching through its known effect on deacetylases (Yoshida *et al.* 1995; Grewal *et al.* 1998), there is the question of target specificity. We therefore identified in *C. albicans* a homolog of the *S. cerevisiae* gene *HDA1*, which encodes a deacetylase in *S. cerevisiae* that was demonstrated to be highly sensitive *in vitro* to TSA (Grozinger *et al.* 1999) and engineered strains harboring the null mutation for *HDA1* by targeted gene disruption using a urablast protocol (Fonzi and Irwin 1993). To create homozygous *hda1*$^{-}$/*hda1*$^{-}$ mutants, two different deletion cassettes were constructed, each spanning the essential deacetylation motifs (T. Srikantha, L. Tsai, K. Daniels, A. Klar and D. R. Soll, unpublished results). To generate the heterozygote, a CAT-*URA3*-CAT-based cassette (Srikantha *et al.* 2000) was employed. Recovered transformants were tested for heterozygosity by Southern analysis. A confirmed heterozygous clone was subjected to 5-fluoroorotic acid treatment to induce the pop-out of the *URA3* gene. To generate the homozygote, a hisG-*URA3*-hisG cassette (Fonzi and Irwin 1993) was employed that targeted the functional *HDA1* allele. Transformants HDho15 and HDho19 were confirmed for homozygosity by Southern analysis (T. Srikantha, L. Tsai, K. Daniels, A. Klar and D. R. Soll, unpublished results). Mutant cells exhibited the same selective increase in the frequency of switching in the white-to-opaque direction as TSA-treated cells. In populations of white phase cells derived from single colonies of the parental strain *HDA1/HDA1*, the frequency of opaque phase colonies was 0.1% and the frequency of white phase colonies with opaque phase sectors was 0.4% (Table 2). In populations of white phase cells of the mutant HDho15 the frequency of opaque phase colonies was 3% and white phase colonies with opaque phase sectors 50% (Figure 1, E and F), representing increases over wild type of 22- and 322-fold, respectively (Table 2). As in the case of TSA treatment, there was no effect on the opaque-to-white transition. The deletion strain HDho19 showed a similar

TABLE 2

The effect of the deletion of the deacetylase gene *HDA1* on the white-to-opaque transition

Strain	Total no. of colonies	White phase colonies (%)	Opaque phase colonies (%)	White phase colonies with opaque sectors (%)
HDA1$^+$/*HDA1*$^+$	5895	5865 (99.5)	8 (0.1)	22 (0.4)
hda1$^-$/*hda1*$^-$	2868	1351 (47)	83 (3)	1434 (50)

Cells from homogeneous 3-day-old white phase colonies were plated on agar containing phloxine B and the phenotypes of single colonies were assessed after 5 days of growth at 25°. Similar experiments with opaque phase cells revealed no effect on the white-to-opaque transition, so those data were not included.

effect (data not shown). As in the case of TSA-treated preparations, opaque phase cells that formed at high frequency in white phase cell populations exhibited the unique signature opaque phase phenotype, including the elongate shape and wall pimples (data not shown).

Although TSA treatment and deletion of *HDA1* resulted in the same selective increase in switching in the white-to-opaque phase, white phase cell cultures treated with TSA exhibited higher proportions of primary opaque phase colonies than the mutants, and the mutants exhibited higher proportions of sectored colonies than TSA-treated cells (Figure 1 and Tables 1 and 2). Although the reason for this difference cannot be derived from our data, two alternative explanations should be considered. First, *C. albicans* contains at least five distinct members of the histone deacetylase family, *HDA1*, *RPD3*, *HOS1*, *HOS2*, and *HOS3* (T. Srikantha, L. Tsai, K. Daniels, A. Klar and D. R. Soll, unpublished results). Although *HDA1* is the most sensitive of the deacetylases to TSA, other deacetylases may be affected by TSA. In this case, the addition of TSA to the *hda1*$^-$/*hda1*$^-$ mutant should result in the TSA-treated phenotype similar to that obtained with the wild-type cells. We performed the experiment but did not obtain this result. As a control, white phase HDho15 cells treated with DMSO for 48 hr and then plated formed 5% opaque, 25% white, and 70% white/opaque sectored colonies. White phase HDho15 cells treated with TSA dissolved in DMSO for 48 hr and then plated formed 3% opaque, 7% white, and 90% white/opaque sectored colonies. Wild-type cells treated with DMSO formed 100% white phase colonies without sectors, and wild-type cells treated with TSA in DMSO formed 30% opaque phase colonies and 4% white phase colonies with sectors. Treatment of HDho15 cells, therefore, resulted in the phenotype of untreated HDho15 cells and not the phenotype of treated wild-type cells.

Alternatively, the phenotypic difference between TSA-treated and HDho15 cells may stem from the different molecular consequences of TSA treatment and gene deletion. More than one type of histone deacetylase coexist in supra-molecular complexes that interact with promoters. Therefore, TSA treatment may leave the structure of a supramolecular complex intact and may leave other components in the complex functional, while deletion of *HDA1* may disrupt complexes, thus suppressing other functions. What should be considered remarkable is the similarity rather than the dissimilarity of the TSA and deletion effects.

The selective effect of both TSA and deletion of *HDA1* on switching in the white-to-opaque but not opaque-to-white direction suggests that the mechanisms in the two directions differ. Several other observations support this conclusion. An increase (Slutsky *et al.* 1987) or a decrease in temperature leads to a selective increase in switching in the opaque-to-white direction but has no effect on switching in the white-to-opaque direction (Slutsky *et al.* 1987; Rikkerink *et al.* 1988); white blood cells and oxidants cause a selective increase in switching in the white-to-opaque direction but not in the opaque-to-white direction (Kolotila and Diamond 1990); and misexpression of the white phase-specific gene *WH11* in the opaque phase leads to a selective increase of switching in the opaque-to-white direction (Kvaal *et al.* 1997). Similarly, the epigenetically controlled *mat* region of *S. pombe* is changed only in one direction when the cells are treated with TSA or when a mutation in a histone deacetylase is encoded by the *clr3* gene (Grewal *et al.* 1998).

We entertained several possibilities for the molecular mechanisms regulating reversible high frequency phenotypic switching in the white-opaque transition in *C. albicans* (Soll 1992, 2001). These include reversible DNA rearrangements, which are the basis of antigenic switching in pathogenic bacteria, trypanosomes, and yeast mating (Berg and Howe 1989); changes in chromatin state (Grewal and Klar 1996); a prion-based mechanism (Wickner *et al.* 1999); and regulatory cascades (Madhani and Fink 1998). Recently, Perez-Martin *et al.* (1999) demonstrated that deletion of a *C. albicans* homolog of *SIR2* resulted in an increase in switching in strain CAI8, which exhibits the more complex 3153A-type switching system that includes several phenotypes that differ as a result of the distribution of budding cells, pseudohyphae, and hyphae in their colony domes (Soll 1989). Deletion of *SIR2* in strain

WO-1 did not affect the frequency of the white-opaque transition in either direction (C. Pujol and D. R. Soll, unpublished results), suggesting that the basic mechanism of switching differs between these alternative switching systems. However, the recent observation by Imai *et al.* (2000) that Sir2 possesses NAD-dependent histone deacetylase activity raises the possibility that both Hda1 and Sir2 function to suppress switching in a similar fashion. The selective effects of TSA or the *HDA1* deletion on the white-to-opaque transition lead to two different models, both of which involve suppression of gene expression by chromatin modification through the deacetylation of histones at a key switch locus. In the first model, Hda1 suppresses switching by deacetylating histones at the site of the basic switch event for the transition from the white-to-opaque phenotype. Inhibition of Hda1 or deletion of *HDA1* results in upregulation at the site of the actual switch event. In the second model, Hda1 suppresses expression of an activator of the basic switch event. Inhibition of Hda1 or deletion of *HDA1* results in upregulation of the activator of the switch event, which in turn results in upregulation of the switch event. Results of this study should guide future research to identify the critical switch locus. We propose that the acetylation/deacetylation of histones at key switch loci could play the same roles in epigenetic switches in other eukaryotic organisms.

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