The Paralogous Histone Deacetylases Rpd3 and Rpd31 Play Opposing Roles in Regulating the White-Opaque Switch in the Fungal Pathogen Candida albicans

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ABSTRACT Chromatin modifications affect gene regulation in response to environmental stimuli in numerous biological processes. For example, N-acetyl-glucosamine and CO2 induce a morphogenetic conversion between white (W) and opaque (O) cells in MTL (mating-type locus) homozygous and heterozygous (a/α) strains of the human fungal pathogen Candida albicans. Here, we identify 8 histone-modifying enzymes playing distinct roles in the regulation of W/O switching in MTL homozygous and heterozygous strains. Most strikingly, genetic removal of the paralogous genes RPD3 and RPD31, both of which encode almost identical orthologues of the yeast histone deacetylase (HDAC) Rpd3, reveals opposing roles in W/O switching of MTLa/α strains. We show that Rpd3 and Rpd31 functions depend on MTL genotypes. Strikingly, we demonstrate that Rpd3 and Rpd31, which are almost identical except for a divergent C-terminal extension present in Rpd3, exert their functions in distinct regulatory complexes referred to as CaRpd3L and CaRpd31S complexes. Moreover, we identify the Candida orf19.7185 product Ume1, as a shared core subunit of CaRpd3L and CaRpd31S. Mechanistically, we show that the opposing roles of Rpd3 and Rpd31 require their deacetylase activities. Importantly, CaRpd3L interacts with the heterodimeric transcriptional repressor a1/a2, thus controlling expression of WOR1 encoding the master regulator of W/O switching. Thus, our work provides novel insight about regulation mechanisms of W/O switching in MTLa/α strains. This is the first example of two highly conserved HDACs exerting opposing regulatory functions in the same process in a eukaryotic cell.

IMPORTANCE RPD3-like histone deacetylases (also called class I HDACs) are conserved from unicellular eukaryotes to mammals. Specifically, the genome of the human fungal pathogen Candida albicans, the most frequent cause of invasive fungal infections of high morbidity and mortality, harbors two almost identical paralogous HDACs, Rpd3 and Rpd31. We show here for the first time that Rpd3 and Rpd31 acquired functional divergence related to a distinct C-terminal domain. Rpd3 and Rpd31 associate with different complexes in the control regions of the master regulator gene WOR1, which is required for white-opaque (W/O) morphogenesis, respectively. The ability to switch is important for fungal pathogenesis, since it enables distinct host niche colonization. This work is to the best of our knowledge the first description of two paralogous HDACs playing opposing functional roles in the same developmental process. Our work adds a new angle concerning the molecular understanding of HDACs in the regulation of cell fate decisions.

n eukaryotic cells, posttranslational modifications (PTMs) of histones dynamically modulate chromatin architecture and function, which play pivotal roles in numerous biological processes in response to environmental stimuli (1, 2). In fungal pathogens, adaptive chromatin changes promote phenotypic plasticity implicated in many development processes. For example, chromatin remodeling in human fungal pathogens such as Candida spp. enables their rapid adaptation to host niches and allows for colonization or infection (3). Candida albicans, the most common human opportunistic fungal pathogen (4), can cause diseases ranging from superficial to life-threatening systemic infections (5). The ability to mount morphogenetic changes such as filamentation in response to different host stimuli is a key virulence trait of C. albicans (6, 7). Moreover, in addition to filamentation (6), C. albicans can undergo a reversible switch between two morphologies known as white (W) and opaque (O) phases (8). W cells are oval and form dome-shaped colonies, while O cells are elongated and form flatter and darker colonies, which can be selectively stained on plates containing the dye phloxine B (9). Although the W and O cell types share the same genome, they display distinct phenotypes, including filamentation, adhesion to human tissues and plastics, proteinase production, and mating competence, as well as antifungal drug susceptibility (10, 11). More importantly, O cells acquire hypervirulence phenotypes, at least in cutaneous infections, and appear less well recognized by the host immune surveillance (12, 13).

For decades, white-opaque (W/O) switching was presumed to occur exclusively in MTL homozygous mating-type (a/a, α/α)
strains of C. albicans (11). The heterodimeric a1/a2 transcriptional regulator acts in heterozygous MTLα/α strains to repress the expression of Wor1, the key regulator of W/O switching. Thus, a1/a2 blocks cells in the W state (14). However, recent work discovered O cell formation in a number of natural MTLα/α strains in response to environmental cues such as N-acetylgalactosamine (GlcNAc) or CO2, suggesting that phenotypic switching competence is a common trait shared by all MTL types of C. albicans (12).

Of note, regulation of W/O switching involves a dual layer of both transcriptional and epigenetic networks (15). At least seven transcriptional regulators, Wor1, Wor2, Wor3, Wor4, Czf1, Efγ1, and Ahr1, form a network of positive and negative feedback loops to control W/O conversion (16–18). Furthermore, at the epigenetic level, several histone-modifying enzymes strongly impact W/O switching frequencies (15, 19, 20). For example, the SET3C histone deacetylase (HDAC) complex operates in concert with transcription factors to modulate W/O switching (15), as well as filamentation (21) and biofilm formation (22). However, the impact of HDACs on the modulation of W/O switching in MTLα/α cells has not been extensively investigated, although at least 5 transcription factors, Wor1, Rfg1, Brg1, Efγ1, and Wor4, have been implicated to date (12, 18).

Environmental stimuli may help a/α cells to override the inhibition of WOR1 by the a1/a2 repressor complex and thus facilitate W/O switching. Of note, emerging evidence suggests the epigenetic integration of extracellular and intrinsic signaling events (23, 24). Here, we use a systematic reverse-genetics approach to show that 8 out of 20 deletion mutants lacking histone-modifying enzymes play different roles in the regulation of W/O switching in MTL homozygous versus heterozygous strains. Most remarkably, genetic ablation of RPD3 and RPD31, two paralogous genes encoding almost identical HDACs, shows that these genes play opposing roles in W/O switching in MTLα/α strains. Moreover, we demonstrate for the first time that Rpd3 and Rpd31 form distinct regulatory complexes recruiting distinct proteins, most likely due to a short divergent C-terminal tail present in Rpd31. Rpd3-mediated regulation of W/O switching requires the master regulator gene WOR1 (25). Interestingly, the regulatory role of Rpd3 in MTL homozygous versus heterozygous strains requires HDAC activity, which modulates recruitment of the heterodimeric a1/a2 repressor to the WOR1 promoter. We propose a model whereby Rpd3 and Rpd31 control W/O switching in MTLα/α strains. Thus, in addition to novel insights into the epigenetic regulation of developmental switches, our data exemplify how paralogous genes in a pathogen can maintain the same enzymatic activity but undergo functional diversification following gene duplication events. In the case of C. albicans, we propose that this diversification allows for flexible adaption to changing host niches and promotes or sustains host colonization.

RESULTS

Deletion of histone-modifying genes affects W/O switching in MTLα/α strains. Previous studies have shown that host environmental stimuli can override the inhibition by the a1/a2 complex and induce W/O switching in MTLα/α strains (12). However, the underlying mechanisms remain unclear. Since chromatin modifications play important roles in response to environmental stimuli, we systematically tested the roles of histone modifiers in modulating W/O switching in MTLα/α strains. In total, we constructed 20 homozygous deletion mutants lacking histone-modifying enzymes involved in histone acetylation, deacetylation, methylation, and dephosphorylation in an MTLα/α background. W/O switching assays under optimal inducing conditions (12) showed that 13 out of 20 genes altered W/O switching frequencies compared to the wild-type (WT) control (Fig. 1).

Five mutants (hda1Δ/Δ, rpd31Δ/Δ, set2Δ/Δ, hos2Δ/Δ, and set3Δ/Δ) showed similar effects in MTL homozygous and heterozygous strains (15, 20). However, dot1Δ/Δ strains lacking an H3K79 methylase (26), the sas2Δ/Δ mutant devoid of a histone acetyltransferase (HAT) (27), and the set2Δ/Δ methyltransferase mutant (28) showed increased O cell formation. Conversely, elp3Δ/Δ mutants lacking a putative HAT (29) and the hos1Δ/Δ mutant lacking an HDAC (20) displayed significantly decreased W/O switching. Of note, deletion of SPT10, encoding a putative histone H3 acetyltransferase (30), completely blocked O cell formation in MTLα/α strains (Fig. 1). Notably, none of these six genes influenced switching in MTL homozygous cells (15). Removal of the NAT4 HAT increased W/O switching, while it showed decreased conversion in MTL homozygous strains (15). Most surprisingly, deletion of RPD3 strongly decreased W/O switching in MTLα/α strains, whereas the paralogous gene RPD31 displayed an opposing function (Fig. 1). Since the putative HADCs Rpd3 and Rpd31 share a high primary sequence identity of 74% (see Fig. S1 in the supplemental material), we wondered how almost identical HDACs can play inverse functions in W/O switching in C. albicans.

Opposing effects of RPD3 and RPD31 on W/O switching are MTL dependent. The W/O switching assays used media containing GlcNAc as the carbon source, while glucose was used in previous studies (15, 20). Hence, the impact of different carbon sources on W/O switching was tested in different culture media (Fig. 2A). Both rpd3Δ/Δ and rpd31Δ/Δ mutants showed consistent results in Lee’s glucose medium and Lee’s GlcNAc medium. While lack of Rpd3 strongly decreased switching, loss of Rpd31 dramatically increased the W/O conversion, showing that the opposing roles of Rpd3 and Rpd31 are medium independent (Fig. 2A). Importantly, genomic reintegration of RPD3 and RPD31 into the corresponding homozygous deletion mutants fully restored wild-type phenotypes (Fig. 2A). To exclude the possibility that the rpd3Δ/Δ and rpd31Δ/Δ mutant phenotypes are
strain specific, we deleted both genes in another clinical MTLα/a strain from an entirely different source (12). Importantly, we confirmed the results for both genes, showing that lack of RPD3 decreased W/O switching, whereas lack of RPD31 increased the conversion (see Fig. S2 in the supplemental material). We also quantified the reverse switching frequency, namely, the O/W conversion under different culture conditions. Only rpd3Δ/Δ mutants displayed insignificant increases in the O/W switching on Lee’s glucose medium plus 5% CO2 (Fig. 2B), suggesting that both Rpd3 and Rpd31 exert their regulatory roles in W rather than in O cells.

In a previous study, the genetic removal of RPD3 in MTLα/a strains increased W/O switching, but the parent strain used in our study differs from the one used in the previous study (20). Thus, to confirm that the regulation by Rpd3 is indeed different between MTL homozygous strains and heterozygous strains, we removed the RPD3 and RPD31 genes in an MTLα/a strain obtained through amplification using genomic DNA directly prepared from colonies. Strains were grown on YPD plates at 30°C for 2 days and then plated on Lee’s GlcNAc medium and grown at 25°C with 5% CO2 for 5 days. *, P < 0.05, and **, P < 0.01, compared to the WT strain.

Rpd3 and Rpd31 act in distinct regulatory complexes to control W/O switching. In Saccharomyces cerevisiae, Rpd3 constitutes the catalytic subunit of two well-known HDAC complexes, ScRpd3L and ScRpd3S, and of the newly characterized complex ScRpd3μ (31). In C. albicans, Rpd3 and Rpd31 are two putative orthologues of ScRpd3 sharing sequence identities of 75% and 84%, respectively (Fig. S1). Based on the Candida Genome Database, except for Ume1, all other subunits of the ScRpd3L and ScRpd3S complexes are conserved in C. albicans (Fig. 3A; see Table S4 in the supplemental material). For example, ScPHO23 encodes an Rpd3L-specific subunit, whose deletion did not affect Rpd3L complex integrity but significantly decreased the HDAC

FIG 2 RPD3 and RPD31 play opposing roles in W/O switching of MTL heterozygotes. (A) Genetic deletion of RPD3 and RPD31 in MTLα/a cells showed consistent W/O switching frequencies under different culture conditions. The indicated strains were grown on YPD plates at 30°C for 2 days. Cells were plated on Lee’s glucose medium and Lee’s GlcNAc medium, respectively, and incubated at 25°C with 5% CO2 for 5 days. (B) O/W switching of the WT (J4-2.1) and rpd3Δ/Δ and rpd31Δ/Δ mutant MTLα/a strains under different growth conditions. Pure opaque cells were plated on Lee’s glucose medium and Lee’s GlcNAc medium and cultured under normal aeration or at 25°C with 5% CO2 for 5 days, respectively. (C and D) Comparison of W/O switching frequencies of WT, rpd3Δ/Δ, and rpd31Δ/Δ cells in MTLα/a (1130) and MTLα/a (J4-2.1) background genotypes. All strains were grown on YPD plates at 30°C for 2 days. Then cells were plated on SC-glucose medium at 25°C with 5% CO2. After 5 days, cells from white colonies were replated on fresh SC-glucose medium at 25°C with 5% CO2 for 5 days. *, P < 0.05, and **, P < 0.01, compared to the WT strain.

FIG 3 Lack of RPD3 and PHO23 debilitates W/O switching, whereas deletion of RPD31 and RCO1 promotes switching of MTLα/a cells. (A) Schematic cartoons of Rpd3L and Rpd3S complexes from S. cerevisiae (31). Green ovals indicate three core components shared by both complexes; gray and red ovals indicate the components specific to each complex, wherein ovals with white and bold letters indicate homologous components identified in C. albicans via immunoprecipitation in the present work. (B) Single and double deletion mutants were constructed in the WT strain (J4-2.1) and confirmed by PCR amplification using genomic DNA directly prepared from colonies. Strains were grown on YPD plates at 30°C for 2 days and then plated on Lee’s GlcNAc medium and grown at 25°C with 5% CO2 for 5 days. *, P < 0.05, and **, P < 0.01, compared to the WT strain.
activity (32). Furthermore, ScRCO1 encodes an Rpd3S-specific subunit required for the integrity or stability of the Rpd3S complex (33). Since we hypothesized that C. albicans Rpd3 and Rpd31 may operate in separate complexes, we deleted the orthologues of ScPHO23 and ScRCO1 in C. albicans a/α strains and tested their roles in W/O switching.

Interestingly, genetic removal of PHO23 decreased the W/O switching frequency compared to that of the WT strain, thus phenocopying rpd3Δ/Δ traits. In contrast, both rco1Δ/Δ and rpd31Δ/Δ mutants displayed increased switching frequencies. Notably, lack of PHO23 increased opaque formation in MTLα cells (17). Hence, the similar phenotypes displayed by Rpd3 and Pho23 in W/O conversion suggest that both proteins function in a complex. Thus, we constructed a series of double mutants to identify possible genetic interactions. The rpd3Δ/Δ pho23Δ/Δ double mutant showed the same phenotype as the corresponding single mutants. Furthermore, assaying the rpd31Δ/Δ rco1Δ/Δ strain yielded similar results (Fig. 3B). These data provide compelling evidence that Rpd3 and Pho23 act in the same complex or pathway and cooperate in the W/O switching control, while Rpd31 and Rco1 work together in a distinct complex to inhibit O cell formation.

We therefore constructed epitope-tagged Rpd3 and Rpd31 strains (Table S5), including the Rpd3 and Rpd31 tagged Rpd3-9myc and Rpd31-9myc proteins (see Table S5 in the supplemental material) in corresponding heterozygous knockout strains. We then used immunoprecipitation coupled with mass spectrometry, along with the silver-staining profiles of immunoprecipitable proteins from the strains. We wanted to test whether Rpd3 is part of a CaRpd3L complex, whereas Rpd31 forms a CaRpd3S1 complex in C. albicans. Therefore, we constructed strains expressing fully functional epitope-tagged Rpd3-9myc and Rpd31-9myc proteins (see Table S5 in the supplemental material) in corresponding heterozygous knockout strains. We then used immunoprecipitation coupled with mass spectrometry to identify possible interaction partners (Fig. 4A).

The silver-staining profiles of immunoprecipitable proteins from the RPD3-9myc- and RPD31-9myc-tagged strains were very similar (Fig. 4A). To identify coprecipitating proteins, the gel bands were cut out and subjected to mass spectrometry, along with proper control samples from the same position in the untagged sample. Indeed, four orthologues of the ScRpd3L complex, Sin3, Dep1, Pho23, and Rxt2, were identified as interaction partners of Rpd3-9myc and Rpd31-9myc. Thus, both Rpd3 and Rpd31 may be part of a CaRpd3L complex.

Interestingly, a hitherto uncharacterized Candida protein encoded by orf19.7185 was also identified (Fig. 4A). To test whether orf19.7185 functions in W/O switching, we subjected an MTLα homozygous deletion mutant to W/O switching control, while Rpd3 and Rco1 work together in a distinct complex to inhibit O cell formation. The rpd3Δ/Δ rpd31Δ/Δ strain and the rpd3Δ/Δ rco1Δ/Δ strain also displayed increased switching frequencies, suggesting that initial induction of switching by Rpd3 is no longer required in the absence of repression by Rco1 and Rpd31 (Fig. 3B).

Since Rpd3 and Rpd31 might operate in different complexes, we tested whether Rpd3 is part of a CaRpd3L complex, whereas Rpd31 forms a CaRpd3S1 complex in C. albicans. Therefore, we constructed strains expressing fully functional epitope-tagged Rpd3-9myc and Rpd31-9myc proteins (see Table S5 in the supplemental material) in corresponding heterozygous knockout strains. We then used immunoprecipitation coupled with mass spectrometry to identify possible interaction partners (Fig. 4A). The silver-staining profiles of immunoprecipitable proteins from the RPD3-9myc- and RPD31-9myc-tagged strains were very similar (Fig. 4A). To identify coprecipitating proteins, the gel bands were cut out and subjected to mass spectrometry, along with proper control samples from the same position in the untagged sample. Indeed, four orthologues of the ScRpd3L complex, Sin3, Dep1, Pho23, and Rxt2, were identified as interaction partners of Rpd3-9myc and Rpd31-9myc. Thus, both Rpd3 and Rpd31 may be part of a CaRpd3L complex.

Interestingly, a hitherto uncharacterized Candida protein encoded by orf19.7185 was also identified (Fig. 4A). To test whether orf19.7185 functions in W/O switching, we subjected an MTLα homozygous deletion mutant to W/O switching control. Surprisingly, deletion of orf19.7185 strongly impaired W/O switching compared to the WT control and mimicked the rpd3Δ/Δ switching phenotype (Fig. 4B). These data indicated that this protein might act in concert with Rco1 to yield a RCO1-9myc strain, which showed similar morphology and conserved domain architecture with ScUme1. We therefore designated the orf19.7185 gene product as Ume1.

Of note, we were unable to identify the putative orthologues Ea3 and Rco1 present in the Rpd3S complex. Thus, to test for possible physical interactions between Rco1 and Rpd3 or Rpd31, we constructed epitope-tagged strains (Table S5), including the rpd3Δ/Δ:RPD3-3HA rco1Δ/Δ::RCO1-9myc and rpd31Δ/Δ::RPD31-3HA rco1Δ/Δ::RCO1-9myc strains. Epitope tagging preserved the function and did not alter W/O conversion frequencies (Table S5). Coimmunoprecipitation (co-IP) assays demonstrated that Rco1 physically interacted with Rpd31 but not with Rpd3.
Rpd31-T2-3HA and Rpd31-3HA were expressed at comparable levels to Rpd31-T2. We replaced the Rpd31-T2 variant in the heterozygous strain. Co-IP experiments were performed as described in the legend to Fig. 4D. * * , P < 0.01 compared to the WT strain.

The divergent C-terminal domain of Rpd31 is important for function. In diploid C. albicans cells, Rpd3 and Rpd31 encode 480- and 577-residue proteins, respectively, both of which share highly similar primary sequence with ScRpd3 (433 amino acids [aa]). Rpd3 and Rpd31 share 74% identical residues, but a unique and major difference lies in the C-terminal stretch of about 170 residues, starting from amino acid 407 (Fig. S1). To determine whether the extended C terminus of Rpd31 containing a predicted glutamic acid-rich region (aa 461 to 572 [Fig. 5A]) is required for function in W/O switching, we reintegrated two C-terminally truncated Rpd31 variants, both still carrying the intact HDAC domain, into the rpd31ΔΔ mutant strain (Fig. 5A). The truncated RPD31-T1 encodes a 483-residue protein, which is almost identical in size and domain organization to Rpd3. Furthermore, to test whether the truncated Rpd31-T2 interacts with Rco1, we constructed a strain coexpressing partial Rpd31-T2-3HA and Rco1-9myc and performed coimmunoprecipitation assays. However, Rpd31-T2 does not interact with Rco1, as shown in a co-IP assay. Rpd31-T2 was epitope tagged with 3HA, and Rco1 was tagged with 9myc in the RPD31/ rpd31Δ heterozygous strain. Co-IP experiments were performed as described in the legend to Fig. 4D. ** , P < 0.01 compared to the WT strain.

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FIG 5 A C-terminal extension domain of Rpd31 is required for W/O switching. (A) Schematic representation of the predicted functional domains of Rpd3, Rpd31, and truncated Rpd31-T1 and Rpd31-T2 variants as predicted by the motif scan tool (http://myhits.isb-sib.ch/cgi-bin/motif_scan). (B) Effects of truncated Rpd31-T1 and Rpd3-T2 variants on the regulation of W/O switching. A long truncated RPD31 (RPD31-T1) and a shorter variant (RPD31-T2) were constructed and genomically integrated into the corresponding chromosomal location in rpd31ΔΔ mutants. Correct genomic integration was verified in independent transformants by colony PCR. W/O switching frequencies were quantified (A) as described and were inspected by light microscopy (B). The scale bar corresponds to 5 μm.

FIG 6 Ectopic WOR1 expression bypasses repression of the O phase in the absence of Rpd3. All strains were grown on YPD plates at 30°C for 2 days, and then cells were plated on Lee’s GlcNAc medium plus 5% CO2. (C) Rpd3ΔΔ/pNIM1-WOR1 strain showed 10-fold lower W/O switching frequencies than WT/pNIM1-WOR1 strain. WT/pNIM1-WOR1 strain showed 100% switching frequency.

 levels (see Fig. S3 in the supplemental material). This indicates that the region diverging between the two partial Rpd31 variants (residues 418 to 482) is critically required for the function of Rpd31 in modulating W/O switching. Furthermore, to test whether the truncated Rpd31-T2 interacts with Rco1, we constructed a strain coexpressing partial Rpd31-T2-3HA and Rco1-9myc and performed coimmunoprecipitation assays. However, Rpd31-T2 fails to coimmunoprecipitate with Rco1, suggesting that the C terminus may also be required for Rpd31 to associate with Rco1.

Ectopic WOR1 expression bypasses repression of opaque cell formation in MTLα/α rpd3ΔΔ cells. Next, we aimed to unravel how Rpd3 drives W/O switching in MTLα/α strains. In both MTL homozgyous and heterozygous strains, Wor1 plays a key role in the O cell formation, as well as maintaining O phase stability (12, 25). To study whether ectopic overexpression of Wor1 can bypass the requirement of Rpd3, we expressed WOR1 under control of a tetracycline-inducible promoter in both WT and rpd3ΔΔ cells (Fig. 6A). Strains containing the empty vector (WT/pNIM1 and rpd3ΔΔ/pPIM1) served as controls. Without induction, 31.4 ± 6.0% of W cells of the WT/pNIM1-WOR1 strain switched to the O phase, whereas the rpd3ΔΔ/pPIM1-WOR1 strain showed 10-
fold-lower switching frequencies of about 3.8% ± 1.5%. However, upon induction with 50 μg/ml doxycycline, both the WT/ pNIM1-WOR1 and rpd3ΔΔ/pNIM1-WOR1 strains regained 100% W/O conversion. There were no differences in the switching of control strains between inducible and noninducible conditions. Furthermore, cell morphologies upon ectopic WOR1 expression were verified by light microscopy (Fig. 6B), confirming both W and O phenotypes. In summary, our data indicate that ectopic expression of WOR1 derepresses O cell conversion in the rpd3ΔΔ strain, strongly suggesting that Rpd3 functions upstream or at least at the level of Wor1.

**Loss of RPD3 increases acetylation of the WOR1 promoter and a1/a2 recruitment.** Our data have shown that deletion of RPD3 in MTL homozygous and heterozygous a/a strains exerted opposite effects on W/O switching. One possible explanation could be that Rpd3 interacts with different proteins in a/a and a/a cell types. Therefore, we performed immunoprecipitation analysis of RPD3-9myc tagged in an a/a and a/a strain background. Silver-staining profiles of immunoprecipitable proteins from both strains were identical (see Fig. S4 in the supplemental material), indicating that Rpd3 interacts and acts with the same protein complex in both a/a and a/a cells. In MTLa/a strains, expression of WOR1 is efficiently prevented by binding of the heterodimeric a1/a2 repressor to a single cis-acting motif located at around bp −5647 upstream of the translational start site (14). This repression is fully relieved in MTLa homozygous strains. Since loss of RPD3 decreases switching of MTLa/a W to O cells, we assumed that WOR1 expression is repressed to an even greater extent in rpd3ΔΔ cells compared to WT cells requiring a1/a2 complex recruitment. To test our hypothesis, we constructed strains expressing the functional MTLa2-myc variant in the WT and rpd3ΔΔ, rpd31ΔΔ, and rpd3ΔΔ rpd31ΔΔ mutant backgrounds (Table S5). We then performed chromatin immunoprecipitation analysis coupled to quantitative PCR (ChIP-qPCR) to verify a possible recruitment of the a1/a2 complex to the WOR1 gene in the W phase. As expected, a1/a2 binding to the upstream region of the WOR1 coding sequence was increased by more than 3-fold in rpd3ΔΔ and rpd31ΔΔ rpd31ΔΔ cells compared to the WT control, while rpd31ΔΔ cells and the WT control showed comparable levels of a1/a2 decoration (Fig. 7A). Importantly, a1/a2 binding was similar in the coding region of WOR1, which served as a negative control. These data strongly indicate that loss of RPD3 rather than RPD31 promotes binding of a1/a2 to the promoter region of WOR1, explaining the enhanced repression of WOR1 in rpd3ΔΔ cells.

Notably, Wor1 is stochastically expressed at very low levels, with levels varying even among individual cells in the W phase. Wor1 decorates its own DNA regulatory region, forming a positive feedback loop to activate its transcription (36). Therefore, we compared the average steady-state expression of WOR1 in W phase cells of the WT and rpd3ΔΔ and rpd31ΔΔ mutant backgrounds within one generation to ensure that all the cells remain in the W state. We used a permanent GlcNAc stimulus to increase chances of WOR1 transcription in individual cells. Preliminary experiments suggested a generation time of about 4 h for cells cultured in Lee’s GlcNAc medium at 25°C (data not shown). Figure 7B shows that expression of WOR1 in WT within one doubling time first increases and then decreases again. However, while showing expression patterns similar to those in the WT, average WOR1 levels were significantly reduced in rpd3ΔΔ cells at all time points compared to the WT control. Of note, rpd31ΔΔ cells displayed a distinct WOR1 expression pattern, showing sustained elevated expression up until 4 h. These data provide strong evidence that the loss of Rpd3 increases the probability of Wor1 to exceed the threshold levels required to activate its positive feedback loop, whereas the converse is true for the rpd3ΔΔ cells. These data are entirely consistent with the observed altered W/O switching frequencies of rpd3ΔΔ and rpd31ΔΔ strains and present a mechanistic explanation for the opposing regulatory phenotypes.

Rpd3 is a putative HDAC deacetylating lysine residues on histones H2A, H2B, H3, and H4. Hence, the histone acetylation status of promoter regions may control the access and affinity of transcription factors to their cognate cis-acting DNA sites (37). Therefore, we tested whether the increased binding of a1/a2 in rpd3ΔΔ cells can be attributed to higher acetylation levels around
the binding site. We used ChIP-qPCR analysis to assess histone acetylation levels at upstream and coding regions of the WOR1 gene in the WT and rpd3Δ/Δ and rpd31Δ/Δ mutant strains. Remarkably, deletion of RPD3 significantly increased the acetylation of histone H4, precisely at the unique putative a1/a2 binding site at positions −5667 to −5467, whereas deletion of RPD31 had no impact at all (Fig. 7C). Furthermore, acetylation at the transcriptional start site at bp −2000 and across the entire 5′-untranslated region (5′-UTR) was also increased in rpd3Δ/Δ cells. Interestingly, no significant changes in the acetylation status were detectable in the WOR1 coding region compared to WT and rpd31Δ/Δ cells. Taken together, these data indicate that Rpd3 might be recruited to the promoter region of WOR1 through a specific CaRpd3L complex, thereby deacetylating the WOR1 control region and modulating decoration by the a1/a2 repressor and possibly other transcription factors.

**DISCUSSION**

Eukaryotes possess numerous histone-modifying enzymes to modify nucleosomes and chromatin states to regulate various biological processes in response to environmental stimuli. Here, we show how histone modifications can aid the integration of environmental signals to control W/O morphogenesis in the most prevalent human fungal pathogen, C. albicans. We use a systematic reverse-genetics approach by analyzing the phenotypes of 20 homozygous deletion mutants. We identify 6 histone-modifying genes (DOT1, SAS2, SET2, ELP3, HOS1, and SPT10) with specific and hitherto unrecognized roles in W/O switching in MTL heterozygous strains. These data demonstrate a novel role for these epigenetic modifiers in the integration of environmental stimuli (GlcNAc and CO₂) into the cell fate decisions. Notably, we also provide the first evidence for a novel mechanism governing the interplay of epigenetic control and transcriptional repression involving orthologous but functionally distinct HDAC proteins. Specifically, we demonstrate that W/O morphogenesis in MTL heterozygous strains of C. albicans is critically and inversely controlled by the paralogous HDACs Rpd3 and Rpd31. Remarkably, our data are the first report of two HDACs with the same enzymatic function showing opposing regulatory functions in a single biological process such as W/O morphogenesis.

C. albicans harbors two paralogous genes, orf19.2834 (RPD3) located on chromosome R and orf19.6801 (RPD31) on chromosome III. The genes encode two RPD3-like class I HDACs, which are conserved from unicellular yeast cells to humans (37, 38). We show that Rpd3 and Rpd31 of C. albicans exert divergent functions in cell fate decisions by virtue of their association with distinct regulatory multiprotein complexes. We identify two Rpd3-related HDAC complexes in C. albicans: CaRpd3L and CaRpd3S. While CaRpd3L comprises either Rpd3 or Rpd31 and at least five other subunits (Sin3, Dep1, Pho23, Rxt2, and Ume1), as identified in the CaRpd3L complex, includes exclusively Rpd31 and at least one other subunit, Rco1. Remarkably, the CaRpd3L complex drives W/O switching, whereas the CaRpd3S complex acts as a negative regulator of W/O conversion in C. albicans.

Gene duplication has long been thought to constitute an evolutionary driving force promoting biodiversity (39), as it leads to either pseudogenes or paralogous genes displaying divergent expression patterns or functions (40). Previous studies mainly addressed the functional diversification of transcription factors, as well as their cognate cis-acting motifs (41). Here, we show that the opposing functions of paralogous HDAC genes like RPD3 and RPD31 arise from domain-restricted alterations in their coding sequences. RPD31 inherited all original functions from the ancestral RPD3-like mother gene, thus enabling its association with both CaRpd3L and CaRpd3S complexes. RPD3 may then have undergone a mutational truncation in the C-terminal coding with a selective impact on W/O switching.

Interestingly enough, like C. albicans, other diploid Candida spp., including C. dubliniensis, C. tropicalis, and C. parapsilosis in the so-called Candida clade (42), also possess two yeast-like Rpd3 HDACs. In contrast, all three haploid species (Candida guilliermondii, Candida lusitaniae, and Debaryomyces hansenii) in this clade harbor only one orthologue in their genome, sharing a higher sequence conservation with CaRpd31 rather than CaRpd3. Of note, diploid Candida species are more pathogenic than haploid species, and several gene families implicated in pathogenesis are highly enriched for gene duplications (42). Hence, it is tempting to speculate that coevolution with the human host may have triggered gene duplication events, affecting RPD31 in the diploid Candida spp., perhaps to facilitate escape from the immune surveillance or to promote niche colonization. Indeed, W and O cells show restricted niche occupancy, as well as different antifungal drug susceptibilities (10, 12). Hence, host immune surveillance or commensal colonization may have constituted a selective pressure driving the functional diversification of RPD31-derived genes in pathogenic Candida spp. At this point, it remains unclear how many other biological processes are affected by the RPD3-like twins, but host-induced hypha formation and drug resistance may be worthwhile candidates for in-depth investigation in future studies.

We also show that the modulation of W/O switching by Rpd3 requires regulatory genes carried by the MTL loci and Wor1, the master regulator of W/O switching. Based on the charge neutralization model (37), we propose that loss of Rpd3 results in hyperacetylated histones in the upstream WOR1 regulatory region. This increases or facilitates access of the heterodimeric a1/a2 repressor and perhaps other as yet unknown regulators to the WOR1 promoter region. Several transcription factors bind directly to the region upstream of WOR1 and trigger transcriptional regulation, including the Efg1 repressor binding at around bp −2400 (16) or the Wor4 transcription factor decorating several sites in the WOR1 upstream region (18). Our data show that the histone acetylation in this region is also elevated in the rpd3Δ/Δ strain, implying a possible increase in the binding of Efg1 directly or indirectly. Therefore, removal of RPD3 in MTLα/α cells may trigger increased recruitment of a1/a2 and Efg1 or other regulators such as Wor4, thereby enhancing WOR1 repression and efficient impairment of W/O switching.

Taken together, our data unequivocally demonstrate distinct and opposing regulatory functions of Rpd3 and Rps31 in W/O switching in MTLα/α heterozygous C. albicans. We would like to propose a hypothetical model for the modulation of W/O switching in MTLα/α cells (Fig. 8). Rpd3 binding is significantly associated with increased acetylation of histone H4 in rpd3Δ/Δ yeast cells (43). Therefore, based on our data (Fig. 7), we propose that the Rpd3-containing CaRpd3L complex is mainly recruited to the upstream region of WOR1, possibly along with as yet unknown coregulators. The increased deacetylation of histones may inhibit binding of negative regulators, eventually driving the stochastic switch from W to O morphotypes. Our data are consistent with
the fact that yeast ScRpd3L, in association with other coregulators such as Ume6 and Hog1, is exclusively recruited to the promoter regions of target genes to specifically regulate transcription (44, 45). In contrast, ScRpd3S only decorates coding regions and employs Ser5-phosphorylated polymerase II and Set2-methylated histone H3 to suppress intragenic transcription (46). Interestingly, a recent report shows that the dual PHD fingers of ScRco1 recognize the unmodified N terminus of H3, which restricts the recruitment of ScRpd3S to promoter histones normally carrying H3K4me3 marks (47). Of note, CaRco1 also contains two PHD fingers, and all residues critical for ScRco1 function are conserved in CaRco1 (see Fig. S5 in the supplemental material), suggesting that the orthologous CaRco1 and ScRco1 may share similar functions. Together with the results showing that expression of WOR1 in rpd3Δ/Δ W cells is strongly increased compared to that in WT control cells (Fig. 7B), we speculate by analogy that CaRpd3S decoration is restricted to the coding region of WOR1 to inhibit its intragenic transcription. Unfortunately, and despite intense efforts, we were unable to prove a direct interaction of either Rpd3 or Rpd3p with DNA (data not shown), making chromatin immunoprecipitation sequencing (ChIP-seq) efforts futile and obsolete. This may be caused by high off rates and/or the presence of low-affinity transient complexes or may simply be due to indirect interactions of Rpd3p with DNA mediated by other complex components. Interestingly enough, we also identify the putative histone acetyltransferase Spt10 as another key regulator of W/O transitions or may simply be due to indirect interactions of Rpd3p with DNA (data not shown), making chromatin immunoprecipitation sequencing (ChIP-seq) efforts futile and obsolete. This may be caused by high off rates and/or the presence of low-affinity transient complexes or may simply be due to indirect interactions of Rpd3p with DNA mediated by other complex components. Interestingly enough, we also identify the putative histone acetyltransferase Spt10 as another key regulator of W/O transitions. In any case, our data uncover for the first time a regulatory role for toneacetyltransferase Spt10 as another key regulator of W/O transitions. Interestingly enough, we also identify the putative histone acetyltransferase Spt10 as another key regulator of W/O transitions.

FIG 8 A hypothetical model for the regulation of W/O conversion by Rpd3p and Rpd3s. The Rpd3p-containing CaRpd3L complex decorates only the upstream region of the WOR1, perhaps in cooperation with as yet unknown additional or other coregulators to deacetylate histones. The altered acetylation status regulates affinity or binding of the a1/a2 repressor and possibly Efg1, thus promoting WOR1 transcription. Conversely, the CaRpd3S complex inhibits WOR1 expression, most likely by decorating the coding region of WOR1 to inhibit intragenic transcription. The oval with symbol “?” indicates unidentified complex components.
ples were cut at the same height on the silver-stained gel, digested with trypsin, separated on a liquid chromatography (LC) system, and then injected into the mass spectrometer in the mass spectrometry facility at the campus Vienna Biocenter. The raw spectra were matched against the Candida albicans database (http://www.candidagenome.org/) to identify proteins.

**ChIP-qPCR.** Overnight cultures were diluted to an OD_{600} of 0.15 in fresh YPD medium and then were grown at 30°C to an OD_{600} of 1. Cells were cross-linked with 1% formaldehyde and quenched by 125 mM glycine, and ChIP was carried out essentially as described previously (21). For histone ChIPs, 100 mM sodium butyrate was added to the ChIP lysis buffer. One milligram of whole-cell extract was used for immunoprecipitation. An antibody against the C terminus of histone H3 (ab1791 [Abcam]) was used to detect histone density, and anti-acetyl H4 (06-598 [Millipore]) was used to detect the total acetylated histone H4. MTA2α-myc ChIP was performed with an antibody against myc (4A6). All ChIP experiments were performed at least with three biological replicates.

For the qPCR quantification of WOR1 expression, overnight cell cultures were washed and diluted to an OD_{600} of 0.5 in fresh Lee’s GlcNAc buffer. One milligram of whole-cell extract was used for immunoprecipitation. An antibody against the C terminus of histone H3 (ab1791 [Abcam]) was used to detect histone density, and anti-acetyl H4 (06-598 [Millipore]) was used to detect the total acetylated histone H4. MTA2α-myc ChIP was performed with an antibody against myc (4A6). All ChIP experiments were performed at least with three biological replicates.

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