Histone acetyltransferase Rtt109 is required for *Candida albicans* pathogenesis

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Candida albicans is a ubiquitous opportunistic pathogen that is the most prevalent cause of hospital-acquired fungal infections. In mammalian hosts, *C. albicans* is engulfed by phagocytes that attack the pathogen with DNA-damaging reactive oxygen species (ROS). Acetylation of histone H3 lysine 56 (H3K56) by the fungal-specific histone acetyltransferase Rtt109 is important for yeast model organisms to survive DNA damage and maintain genome integrity. To assess the importance of Rtt109 for *C. albicans* pathogenicity, we deleted the predicted homolog of Rtt109 in the clinical *C. albicans* isolate, SC5314. *C. albicans* *rtt109*^{−/−} mutant cells lack acetylated H3K56 (H3K56ac) and are hypersensitive to genotoxic agents. Additionally, *rtt109*^{−/−} mutant cells constitutively display increased H2A S129 phosphorylation and elevated DNA repair gene expression, consistent with endogenous DNA damage. Importantly, *C. albicans* *rtt109*^{−/−} cells are significantly less pathogenic in mice and more susceptible to killing by macrophages in vitro than are wild-type cells. Via pharmacological inhibition of the host NADPH oxidase enzyme, we show that the increased sensitivity of *rtt109*^{−/−} cells to macrophages depends on the host's ability to generate ROS, providing a mechanistic link between the drug sensitivity, gene expression, and pathogenesis phenotypes. We conclude that Rtt109 is particularly important for fungal pathogenicity, suggesting a unique target for therapeutic antifungal compounds.

acetylation | chromatin | fungal pathogenesis | DNA damage resistance | macrophage

The basic repeating unit of eukaryotic chromatin, termed the nucleosome, is composed of 146 base pairs of DNA wrapped around an octamer of core histone proteins containing two copies each of H2A, H2B, H3, and H4 (1). Posttranslational modifications (PTM) of histone molecules promote various chromatin functions, including replication, repair, transcription, and silencing (2–4). In the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, newly synthesized histone H3 molecules are abundantly acetylated on lysine 56 (5–7). In *S. cerevisiae*, H3K56 acetylation is required for replication fork stability (8, 9), reassembly of chromatin after DNA damage repair, and histone association with chromatin assembly proteins (10–13). A fungal-specific histone acetyl-transferase (HAT) enzyme, termed Rtt109, and its stimulatory histone chaperone cofactor, Asf1, are required for H3K56 acetylation (8, 14–16). *S. cerevisiae* mutants lacking Rtt109 or Asf1 display delayed cell-cycle progression (15), spontaneous DNA damage (5, 17, 18), unstable replication forks, and are extremely sensitive to DNA-damaging agents (8). In accordance, point mutation of H3 lysine 56 to arginine, which cannot be acetylated and mimics a positively charged, unacetylated lysine, results in similar phenotypes (5, 14). Therefore, acetylation of H3K56 is a particularly important PTM for fungal growth.

C. albicans is an opportunistic pathogen that poses a considerable public health problem, with an estimated 40% mortality rate for systemic candidiasis (19, 20). Antifungal drug resistance is a major clinical problem, and few drugs are available to battle *Candida* infections (21). H3K56 acetylation appears to be much less abundant in mammals than in yeasts (22–24), and close homologs of Rtt109 are not detected outside of the fungal kingdom (25, 26). Therefore, we hypothesized that Rtt109 might

provide a unique target for antifungal therapeutics, and we began to investigate the importance of H3K56 acetylation in fungal pathogenicity.

During the course of a systemic infection, *C. albicans* cells are engulfed by host phagocytes, where they are exposed to ROS (27). ROS contribute to efficient killing of *C. albicans* both in cultured cells and whole organisms (28–31). ROS directly damage DNA, as well as cellular proteins and lipids (32, 33). Upon incubation with macrophages, *C. albicans* DNA repair genes are transcriptionally induced (34), suggesting that DNA damage indeed occurs in the phagosome and that hypersensitivity to genotoxic stress would be disadvantageous to the pathogen. Because H3K56 acetylation is essential for yeast to survive genotoxic stress, we investigated the role of Rtt109 in *C. albicans* pathogenicity.

Results

***C. albicans* ORF19.7491 Encodes the Rtt109 Functional Homolog.** We identified *C. albicans* ORF19.7491 as the likely Rtt109 functional homolog through primary sequence homology. Also, the recent high-resolution structures of *S. cerevisiae* Rtt109 (25, 35) highlight several catalytic domains that are well conserved between the two species (Fig. S1A). *C. albicans* is an obligate diploid that lacks a classical sexual cycle, so two sequential rounds of gene deletion are required to generate homozygous mutants. For this, we employed the *Candida*-adapted Flp/FRT sequence-specific recombination system to replace the target locus (36). This system leaves behind a single 34-bp FRT site after excision (Fig. S1B). All genotypes were confirmed using PCR (Fig. S1C and Table S1). Immunoblot analysis showed that H3K56ac was lost in homozygous *rtt109*^{−/−} mutant cells, whereas in *RTT109*^{+/−} heterozygous cells H3K56ac was maintained (Fig. 1A). To verify that the phenotypes observed resulted from the intended deletion, we generated a “complemented” strain, *RTT109*^{FH/+}, by reintroducing a 2xFlag-6xHIS-tagged *RTT109* gene at the endogenous locus of the homozygous mutant (Fig. S1B and C). As anticipated, H3K56ac was restored in the complemented *RTT109*^{FH/+} strain (Fig. 1A). We conclude that *C. albicans* ORF19.7491 encodes a functional Rtt109 homolog.

***C. albicans* *rtt109*^{−/−} Cells Are Sensitive to Genotoxic Agents.** *C. albicans* can grow in multiple morphological states. Individual cells appear as budded or filamentous cells (37). The ability to switch among these morphologies is required for *C. albicans* pathogenicity (38, 39), suggesting that multiple morphologies contribute to host infection. Filamentous growth can be triggered by various

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Data deposition: The matrix files for gene expression microarray experiments reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, <http://www.ncbi.nlm.nih.gov/geo/> (accession no. GSE18936).

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strains (Fig. 3A). The difference in lethality caused by *rtt109*^{-/-} and wild-type strains was even more significant ($P < 0.001$) when mice were infected with 1.5×10^5 cells (Fig. 3B). To determine whether these phenotypes correlated with proliferation of the pathogen in the animal, we retrieved single kidneys from infected animals and assessed fungal burden at 3 and 20 days post-infection by serial dilution of tissue homogenates on YPD agar (Fig. 3C and D). We observed that mice infected with wild-type or complemented *C. albicans* strains had comparable renal fungal loads. In contrast, mice infected with the *rtt109*^{-/-} mutant carried negligible amounts of yeast. We conclude that *C. albicans* requires Rtt109 for efficient pathogenesis in mice.

***C. albicans* *rtt109*^{-/-} Cells Are More Susceptible to ROS-Mediated Killing by Macrophages.** Macrophages play an important role in controlling *C. albicans* infections (27). To determine whether macrophage-mediated growth inhibition could contribute to the observed decrease in *C. albicans* *rtt109*^{-/-} pathogenicity, we quantified yeast cell survival after exposure to a mouse-derived macrophage-like cell line, ZBM2. After a 15- to 16-h coinocubation at a ratio of 1 fungal cell per 15 ZBM2 cells, proliferation of *rtt109*^{-/-} cells was significantly more inhibited by macrophages than were wild-type *C. albicans* cells ($P = 0.025$; Fig. 3E). We repeated these experiments in the presence of an NADPH oxidase inhibitor (DPI; diphenyleneiodonium chloride) to inhibit

generation of ROS by the macrophages (50). Previous work has shown that inhibition of NADPH oxidase in phagocytes by DPI decreases the fungicidal activity against *C. albicans* (29, 30). We determined that in the presence of DPI, survival of macrophage killing by the *rtt109*^{-/-} cells was increased to the same level observed for wild-type cells (Fig. 3E). We conclude that without Rtt109, *C. albicans* is more susceptible to killing by mammalian host macrophages because of the ROS deposited into phagosomes of macrophages.

rtt109^{-/-} Cells Display an Altered Profile of Metabolic Gene Expression and Constitutively Induce DNA Repair Genes.

To gain further insight into the altered phenotypes of the *rtt109*^{-/-} mutant cells, we performed microarray analysis of gene expression. We first compared logarithmically growing wild-type and *rtt109*^{-/-} mutant cells in rich (YPD) media, focusing on genes that displayed a greater than 2-fold change with $P < 0.01$ (see *Materials and Methods*). Among this group were a large number of genes involved in the response to DNA damage, almost all of which displayed elevated expression in the *rtt109*^{-/-} mutant cells (Table S2A). In contrast, many of the genes most highly down-regulated in the mutant cells were related to carbohydrate metabolism (Table S2B). To extend these observations, GO-term analysis was performed to quantify the enrichment of genes associated with biological processes. Many of the enriched GO-terms significantly overlapped, such that all of the enriched biological process terms can be generally categorized as being involved in carbohydrate metabolism, DNA damage and repair, or mitochondrial function (Fig. 4). Together, the transcription data suggests two major trends: complex misregulation of energy metabolism in mutant cells and constitutive DNA damage signaling.

To investigate the sensitivity of *rtt109*^{-/-} cells to ROS, we also analyzed gene expression changes in cells exposed to 0.4 mM hydrogen peroxide for 10 min—a treatment that has previously been shown to induce a DNA damage response (51, 52). Under these conditions, we observed that there were many more genes with elevated rather than reduced RNA levels in the *rtt109*^{-/-} mutant cells (Table S3). We note that the broad group of DNA repair genes that was elevated in the YPD-grown mutant cells was not observed in the comparison of strains grown in the presence of H₂O₂, likely because both strains in this experiment

Fig. 3. *rtt109*^{-/-} mutant cells display reduced pathogenicity in mice and increased sensitivity to macrophages. BALB/cByJ female mice were infected with 1.0×10^5 (A) or 1.5×10^5 (B) yeast cells via venous tail injection with the indicated *C. albicans* strains. Renal fungal load of mice infected with 1.0×10^5 of the indicated strains 3 dpi (C) or 20 dpi (D). Five-fold serial dilutions of homogenized left kidney were plated on rich media agar for 24 h at 30°C. (E) ZBM2 macrophage-derived cells were incubated for 15–16 h with the indicated *C. albicans* strains at an MOI of 1:15 in the presence or absence of 0.75 μ M diphenyleneiodonium chloride (DPI). The macrophages were osmotically lysed, and dilutions of the coculture were plated onto rich media. Colony forming units (cfu) were compared with cultures of yeast incubated in parallel in the absence of macrophages. Results are mean $\pm$ SEM (*Rtt109*^{+/+} n = 5; *rtt109*^{-/-} n = 5; $P = 0.025$).

Fig. 4. *rtt109*^{-/-} mutant cells have an altered transcription profile significantly enriched in carbohydrate metabolism and DNA repair-related GO terms. Enriched gene ontology terms were calculated from all genes with $P < 0.01$ and fold change of $\log_2 \leq -1.0$ or ≥ 1.0 . These GO terms have an adjusted B.H. P value from $6.58E-4$ (Upper) to $8.00E-3$ (Lower) and represent overlapping genes (see Dataset S1). The percent of genes increased or decreased in *rtt109*^{-/-} is represented within each GO term bar.

are responding to H₂O₂-mediated DNA damage. Notably, genes that specifically respond to oxidative stress (e.g., superoxide dismutases encoded by *SOD3*, *SOD4*, and *SOD5*) were more highly induced in mutant cells, leading to significant enrichment of multiple GO terms related to oxidative stress (Fig. 5). Therefore, *rtt109*^{-/-} mutant cells appear able to sense and mount a vigorous transcriptional response to oxidative stress, but appear unable to survive in phagocytes or mice.

Additionally, in the presence of H₂O₂, the mutant cells up-regulate genes related to cell wall synthesis (Fig. 5 and Dataset S2). In contrast, enriched GO terms related to cell adhesion, interaction, and biofilm formation mostly included genes down-regulated in the mutant cells, including the agglutinins *ALS1* and *ALS2* (Table S3 and Dataset S2). It remains a possibility that some of these complex increases and decreases could contribute to the loss of pathogenicity in *rtt109*^{-/-} mutant cells (Fig. 3). However, given the pharmacological data showing that *rtt109*^{-/-} mutant cells are sensitive to killing by macrophages because of host-generated reactive oxygen species (Fig. 3E), we favor the idea that increased sensitivity to oxidative stress is the major cause of this phenotype.

Several genes that displayed transcriptional differences by microarray were validated through RT-PCR experiments. These genes included *RTT109*, *RAD51*, *IFE2*, *HHO1*, *HTZ1*, *SOD5*, *DDR48*, *ALS1*, and *PKC1* (Fig. S2). Similar alterations in RNA levels were detected by the microarrays and by RT-PCR for both the unperturbed and H₂O₂-stressed samples. We conclude that we have accurately detected gene expression changes in our experiments.

Discussion

We have identified the *C. albicans* Rtt109 acetyltransferase that modifies H3K56 (Fig. 1A). Importantly, we show that Rtt109 is required for efficient pathogenesis in mice, and our in vitro

Fig. 5. Exposure to hydrogen peroxide results in an altered transcription profile significantly enriched in cell wall synthesis and oxidative stress-related GO terms in *rtt109*^{-/-} mutant cells. Enriched gene ontology terms were calculated from all genes with $P < 0.01$ and changed by $\log_2 \leq -1$ and ≥ 1 upon H₂O₂ exposure compared with wild type. These GO terms have an adjusted B. H. P value 3.56×10^{-5} (Top) to 7.76×10^{-3} (Lower) and represent overlapping genes (see Dataset S2). The percent of genes increased or decreased in *rtt109*^{-/-} is represented within each GO term bar. GO terms indicated with asterisks were merged in cases where they were represented by the exact same genes and were enriched with the same B.H. P value. (1) Chitin biosynthetic process and cell wall chitin metabolic process; (2) glucosamine biosynthetic process, N-acetylglucosamine biosynthetic process, and amino sugar biosynthetic process; (3) fatty acid oxidation and lipid oxidation; (4) amino sugar metabolic process, glucosamine metabolic process, N-acetylglucosamine metabolic process, and cell wall polysaccharide metabolic process.

data suggest that increased susceptibility to macrophages makes *rtt109*^{-/-} mutant cells less pathogenic (Fig. 3). Our findings are consistent with previous data suggesting that efficient DNA repair is required for pathogenesis. For example, *C. albicans* *rad52*^{-/-} mutants are unable to perform homologous recombination as a mode of DNA repair, and are less pathogenic in mice (53). Our data specifically emphasize the importance of reactive oxygen species generated in mammalian host phagosomes for killing *C. albicans*. Previously, mice that lack the NADPH oxidase enzyme responsible for generating ROS in phagocytes were shown to experience greater rates of mortality during systemic *C. albicans* infections (31). Here, we show that the increased sensitivity of *rtt109*^{-/-} cells to macrophages requires NADPH oxidase function (Fig. 3E). We conclude that Rtt109 is an important link between resistance to phagocyte-generated genotoxic agents and *C. albicans* pathogenesis.

Genome-wide expression profiling of *C. albicans* in the presence of several types of host cells has been performed (reviewed in refs. 54 and 55). These studies indicate that alterations in metabolism and stress response genes often occur upon interaction with host cells. For example, after phagocytosis, glycolytic genes are down-regulated and fatty acid oxidation and glyoxylate cycle genes are up-regulated (34, 56). Phagocytic cells are particularly important for this response, because erythrocytes and mononuclear cells have little effect on *C. albicans* gene expression, but an enriched population of polymorphonuclear cells (mostly neutrophils and eosinophils) down-regulates glycolytic genes, including *HXK2* (hexokinase II) and *PGI1* (phosphoglucosomerase), as well as glyoxylate cycle genes, including *MLS1* (malate synthase). Neutrophils also induce an oxidative stress response, including activation of *SOD5*, a cell surface-associated superoxide dismutase (56). Similar trends are also seen in the *rtt109*^{-/-} mutant cells. Together, the coordinated down-regulation of glycolysis and up-regulation of the glyoxylate cycle in mutant cells grown in rich media containing a large amount of glucose suggests that *rtt109*^{-/-} mutant cells are less well able to use sugars for metabolism, and rely more on glyoxylate cycle enzymes to convert fats to carbohydrates. As noted previously, there are also complex alterations of gene expression related to mitochondrial and peroxisomal metabolism and cell wall synthesis. How much the metabolic alterations in *rtt109*^{-/-} mutant cells contribute to the pathogenesis phenotype has yet to be resolved. Some of these alterations may be via indirect changes to chromatin, because we also observed that two histone isoform genes, *HHO1* and *HTZ1*, display reduced RNA levels in the mutant cells in YPD (Fig. S2 and Table S2). However, because *rtt109*^{-/-} cells became as resistant to macrophages as were wild-type cells upon inhibition of host NADPH oxidase (Fig. 3E), we favor the hypothesis that the sensitivity of *rtt109*^{-/-} cells to exogenous DNA damaging agents (Fig. 2), reflected by their constitutive response to endogenous damage (Figs. 1C and 4 and Table S2), largely explains why Rtt109 is important for pathogenesis.

Recent analyses of telomere-proximal gene silencing in the budding yeast *S. cerevisiae* has suggested that elevated levels of H3K56 acetylation antagonizes epigenetic silencing of telomere-proximal genes (57, 58), although deletion of *RTT109* has little effect on silencing (58). To determine whether loss of Rtt109 in *C. albicans* might cause position-dependent alteration of gene expression at telomeres, we paid particular attention to telomere-proximal genes in these analyses (Table S4). Genes with elevated and reduced transcript levels in *rtt109*^{-/-} cells were observed, and there did not appear to be any smooth trends that originate from the chromosome ends in these data sets; this is especially evident at the chrR right telomere. Moreover, many of the altered genes near telomeres can be explained by trends suggested from the GO term analyses. For example, there are several stress-, damage- and hyphal-induced genes that display elevated expression in the mutant cells (e.g., *XYL2*, *DFM1*, orf19.7531, *SOD3*, and, to a

lesser extent, *RAD3* and *NAG1*). Although it remains an open possibility that Rtt109 may play a role in position-dependent gene expression at some of the altered loci we have detected, our data suggest that the most salient changes in gene expression are related to DNA damage induction and alterations in cellular metabolism rather than chromosomal position.

Because Rtt109 is well conserved among fungal species (Fig. S1), we propose that Rtt109 is a strong candidate target for therapeutic intervention against fungal pathogens, not limited to *C. albicans*. Although mammalian p300/CBP HAT enzymes are distant Rtt109 homologs, many catalytically important residues in p300/CBP are distinct from those in Rtt109 (25). In fact, compounds that inhibit p300/CBP have no effect on Rtt109 (25), indicating that the potential for discovering specific inhibitors of fungal Rtt109 enzymes is promising.

Materials and Methods

Murine Candidiasis. BALB/cByJ female mice (Jackson Laboratory), 6–8 weeks old, were injected in the tail vein with log-phase *C. albicans* cells suspended in 400 μ L PBS. To assess fungal load, the left kidney was sterilely dissected and homogenized in 0.02% Triton-X 100. Five-fold serial dilutions of the homogenized tissue were plated on YPD agar for 24 h at 30 °C and photographed.

Macrophage Growth Inhibition Assay. A macrophage cell line, termed ZBM2, was derived from C57BL/6J bone marrow and immortalized by retroviral transduction of SV40 large T antigen. The cell line was maintained in DMEM supplemented with 10% heat-inactivated FBS, 10% L-929 cell-conditioned media, 100 units mL⁻¹ penicillin, and 100 μ g mL⁻¹ streptomycin.

Macrophage cells were plated at a density of 2×10^6 per 35 mm² dish and allowed to adhere for 5 h. Log-phase *C. albicans* grown in YEP were washed in DMEM media, plated to a 1:15 macrophage ratio in a final volume of 2 mL, and incubated at 37 °C in 5% CO₂ overnight. Samples were collected into 14-mL 0.02% Triton-X 100 (vol/vol) in water to osmotically lyse macrophages. Dilutions of each sample were plated onto YPD and placed at 37 °C to assess colony-forming units (CFU). Percent growth inhibition was calculated relative to yeast cultures incubated in parallel without macrophages. Experiments with diphenyleneiodium chloride (DPI) were performed similarly with the inclusion of 0.75 μ M DPI from a 31.8-mM stock solution in DMSO. Conditions without DPI included the same final concentration of 0.002% DMSO. All experiments were repeated at least four separate times.

Statistics. The survival data for the positive control and the experimental groups (Fig. 3 A and B) were compared using the Mantel-Cox method. Statistical analysis of macrophage growth inhibition assay (Fig. 3E) was determined by paired two-tailed Student *t* test. The software used was GraphPad Prism.

Microarray Experiments. Four independent pairs of biological replicate samples were analyzed to compare RNA levels in wild-type and *rtt109*^{-/-} cells. Two experiments were performed: first, in unperturbed cells grown in YPD, and second, in cells exposed to H₂O₂. To account for dye effects, two of the four samples for each experiment were analyzed using Cy3 labeling of the wild-type sample and Cy5 labeling of the mutant sample; the dyes were swapped for the other two biological replicates. Details regarding RNA extraction and array hybridization are in *SI Materials and Methods*.

Bioinformatic Analysis of RNA Expression. Limma package (limma_2.18.0) (59) from Bioconductor was used for preprocessing and model fitting. A linear model was fit to the background-corrected, loess-normalized, and log-transformed expression data. The dye effect and mutant effect were tested as explanatory variables to determine whether the expression level of each gene differed between mutant and wild-type samples, after the dye effect was removed statistically. Three features for each gene were printed on the array, resulting in three estimated log-fold changes for each gene. Genes with at least one feature having a *P* value <0.01 for mutant effect and a fold change ≥ 2 were considered potentially differentially expressed in mutant and were included for subsequent Gene Ontology (GO) enrichment test.

The gene and GO term association list was downloaded from <http://www.candidagenome.org/> (version 1.633 and taxon 5476) with duplicates removed. All of the ancestor GO terms were added to the association list using GO.db package (GO.db_2.2.11) from Bioconductor. GO enrichment analysis was performed using the hypergeometric test in R (V 2.9.0). GO terms with at least 10 associated genes in the genome, and a *P* value adjusted using the B-H method (60) <0.01, were considered significantly enriched.

Additional materials and methods are found in *SI Materials and Methods*.

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