

Modulation of histone H3 lysine 56 acetylation as an antifungal therapeutic strategy

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Abstract

Candida albicans is a major fungal pathogen that causes serious systemic and mucosal infections in immunocompromised individuals. In yeast, histone H3 Lys56 acetylation (H3K56ac) is an abundant modification regulated by enzymes that have fungal-specific properties, making them appealing targets for antifungal therapy. Here we demonstrate that H3K56ac in *C. albicans* is regulated by the *RTT109* and *HST3* genes, which respectively encode the H3K56 acetyltransferase (Rtt109p) and deacetylase (Hst3p). We show that reduced levels of H3K56ac sensitize *C. albicans* to genotoxic and antifungal agents. Inhibition of Hst3p activity by conditional gene repression or nicotinamide treatment results in a loss of cell viability associated with abnormal filamentous growth, histone degradation and gross aberrations in DNA staining. We show that genetic or pharmacological alterations in H3K56ac levels reduce virulence in a mouse model of *C. albicans* infection. Our results demonstrate that modulation of H3K56ac is a unique strategy for treatment of *C. albicans* and, possibly, other fungal infections.

H3K56ac is an abundant post-translational modification found in newly synthesized H3 molecules deposited throughout the genome during DNA replication^{1,2}. Originally discovered in yeast^{2–6}, H3K56ac also occurs in human cells^{7–10}. In *Saccharomyces*

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COMPETING FINANCIAL INTERESTS

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AUTHOR CONTRIBUTIONS

H.W. and S.T. designed and performed most experiments; G.L. constructed the yeast strains used in this study; J.T. and S.T. performed the fungal load and cytokine analyses; A.M. and M.R. designed and supervised the mouse studies; P.D., P.T. and A.V. designed and performed mass spectrometry experiments; E.-H.L. provided technical support; all authors participated in manuscript preparation; M.R. and A.V. supervised the study.

cerevisiae, H3K56ac peaks when new histones are synthesized during S phase² and is mediated by the histone acetyltransferase Rtt109p^{11–13}. H3K56ac is removed genome-wide by the histone deacetylases Hst3p and Hst4p during the G2 and M phases^{1,14}. Tightly regulated H3K56ac plays a key part in the DNA damage response, as mutants that cannot acetylate or deacetylate Lys56 are extremely sensitive to genotoxic agents^{1,2,12,14,15}.

C. albicans is a major human fungal pathogen in terms of both its clinical importance and its use as an experimental model for fungal pathogenesis^{16,17}. It is one of the leading causes of infections affecting immunodeficient individuals, including HIV-infected individuals and patients undergoing cancer therapy. *C. albicans* is the fourth most common cause of nosocomial bloodstream infections in the US and is associated with high mortality rates^{18–20}. Treatment of candidiasis involves the use of azole or echinocandin drugs that target ergosterol and cell wall biosynthesis, respectively, but their efficacy can be compromised by the emergence of drug-resistant strains^{21,22}. As the fungal enzymes that regulate H3K56ac have diverged considerably from their human counterparts^{23,24}, we sought to determine whether these enzymes could be potential targets for treatment of *C. albicans* infections.

RESULTS

H3K56ac modulates fungicidal agent sensitivity in *C. albicans*

We first verified the presence of H3K56ac in *C. albicans*. Immunoblots of total lysates from SC5314 and SN152 strains probed with an antibody against H3K56ac^{1,2} revealed a band of the expected molecular weight (~15 kDa) (Fig. 1a). We also isolated histones from *C. albicans* cells and analyzed them by mass spectrometry. The product ion spectrum of a doubly protonated precursor peptide (m/z 638.9²⁺) shows a y -type fragment ion series that confirms acetylation of Lys56 on the basis of the 42-Da mass shift between the y_7 and y_8 fragment ions (Fig. 1b). We also estimated the stoichiometry of H3K56ac by derivatization of nonmodified lysine ϵ -amino groups with deuterated acetic anhydride before trypsin digestion^{1,2}. In asynchronously growing cultures, the Lys56-acetylated peptide was present in 20% to 27% of H3 molecules (Fig. 1c,d). Thus, H3K56ac is an abundant modification in *C. albicans*. This is in stark contrast to the low stoichiometry of H3K56ac (1%) in human cells^{7,9}.

The *RTT109* gene encodes an acetyltransferase responsible for H3K56ac^{11–13}; its ortholog in *C. albicans* is orf19.7491 (*RTT109*). Deletion of both alleles of *RTT109* in strain SN152 (Supplementary Fig. 1) led to nearly complete disappearance of H3K56ac on the basis of immunoblots and mass spectrometry (Fig. 1a,d and Supplementary Table 1). Deacetylation of H3K56 requires the sirtuins Hst3p and Hst4p in *S. cerevisiae*. Deletion of both genes results in DNA damage sensitivity and thermosensitivity, but the double mutant remains viable^{1,14,15}. We only found one gene in *C. albicans* (orf19.1934; *HST3*) encoding a potential ortholog of *S. cerevisiae* *HST3* and *HST4*. We generated a heterozygous deletion mutant for *HST3* in SN152, but were unable to obtain an *hst3* /*hst3* (*hst3* / hereafter) homozygous mutant, suggesting that the *C. albicans* *HST3* gene is essential. Although immunoblots of whole-cell extracts from *hst3* /*HST3* (*HST3*+ / hereafter) cells failed to detect an increase in H3K56ac (Fig. 1a), the stoichiometry of H3K56ac monitored by

quantitative mass spectrometry was twofold higher than in wild-type cells (Fig. 1d and Supplementary Table 1).

We studied the survival of *C. albicans* *rtt109* and *hst3* mutants treated with genotoxic and antifungal agents. Deletion of both alleles of *RTT109* conferred sensitivity to hydroxyurea and methyl methane sulfonate (Fig. 1e). *HST3*^{+/} heterozygous mutants were also sensitive to these agents (Fig. 1e). We also tested the susceptibility of these mutants to two families of antifungal agents: azoles (fluconazole) and echinocandins (caspofungin, micafungin). We found that *rtt109* / *rtt109* (*rtt109* / hereafter) and *HST3*^{+/} cells were no more susceptible to fluconazole than wild-type cells, but the *rtt109* / strain was very sensitive to both echinocandins (Fig. 1e). Because echinocandins induce oxidative stress²⁵, we tested whether the echinocandin sensitivity of *rtt109* / mutants could be suppressed by antioxidants. The antioxidants *N*-acetyl-L-cysteine and vitamin C restored viability of *rtt109* / mutant cells exposed to micafungin (Fig. 1e). These results show that genetic perturbation of H3K56ac sensitizes *C. albicans* to genotoxic and antifungal drugs.

H3K56 deacetylation by Hst3p is essential in *C. albicans*

Because we were unable to obtain a homozygous *hst3* / mutant, we generated strains in the CaSS1 background²⁶, in which we deleted one copy of the *HST3* gene and placed the other copy under the control of a promoter that can be repressed with doxycycline (*hst3* / *pTET-HST3*, Supplementary Methods and Supplementary Fig. 2c–e). Addition of doxycycline to *hst3* / *pTET-HST3* cells resulted in a strong decrease in *HST3* mRNA levels and a concomitant increase in H3K56ac (Fig. 2a,b). To confirm this result, we isolated histones either before or after doxycycline addition and determined the stoichiometry of modification by mass spectrometry. We found that the fraction of H3 molecules that were Lys56-acetylated increased from 48% to 87% when we treated the *hst3* / *pTET-HST3* strain with doxycycline for 7.5 h (Fig. 2c and Supplementary Table 1). The degree of acetylation of other lysine residues in the N-terminal tail of H3 did not increase upon *HST3* repression with doxycycline (Supplementary Table 1). To obtain equal amounts of histone H3 for immunoblotting, we needed to load increasing amounts of total protein as a function of time after doxycycline addition (Fig. 2b). Immunoblots of whole-cell lysates from equal numbers of cells confirmed that the amounts of both H3 and H4 declined over time after doxycycline addition to *hst3* / *pTET-HST3* cells (Fig. 2b). This time-dependent loss of H3 and H4 suggests that *HST3* repression leads to pronounced changes in chromatin structure.

Repression of *HST3* expression with doxycycline severely impaired the growth of *hst3* / *pTET-HST3* cells (Fig. 2d). To determine whether repression of *HST3* was fungistatic or fungicidal, we performed colony formation assays after transient doxycycline exposure. We grew tenfold serial dilutions of *hst3* / *pTET-HST3* and control cells in liquid cultures in the presence or absence of doxycycline for 24 h and then transferred the cells to solid medium lacking doxycycline. We found that the number of colony-forming units in *hst3* / *pTET-HST3* cultures was much lower after a 24 h exposure to doxycycline (Fig. 2e) compared to control cells, suggesting that transient *HST3* repression led to cell death. To confirm this, we used propidium iodide staining of nucleic acids as a measure of cell death; live cells are not permeable to propidium ions²⁷. Almost all *hst3* / *pTET-HST3* cells killed with heat (65 °C

for 30 min) or treated for 24 h with doxycycline stained with propidium iodide (Fig. 2f). We also observed that *HST3* repression with doxycycline triggered anomalous filamentous growth (Fig. 2f). It was possible that filamentous growth in itself could increase propidium iodide staining, irrespective of cell viability. To rule out this possibility, we grew *hst3* / *pTET-HST3* cells in the absence of doxycycline but under conditions that induce a switch to filamentous growth. We found that the resulting filaments were negative for propidium iodide staining (Fig. 2f). These results demonstrate that, unlike in *S. cerevisiae* and *Schizosaccharomyces pombe*, repression of *HST3* in *C. albicans* leads to a loss of cell viability.

We hypothesized that the high levels of H3K56ac induced by repression of *HST3* were causing cell death. This model predicts that deletion of the *RTT109* acetyltransferase gene should rescue the lethality of the *hst3* / mutant. Consistent with this, we were able to delete both alleles of the *HST3* gene in the *rtt109* / genetic background (Fig. 2g and Supplementary Fig. 2f–h). The *rtt109* / *hst3*^{+/} and *rtt109* / *hst3* / mutants were not more susceptible to genotoxic agents than the *rtt109* / strain, although they grew more slowly than either *rtt109* / or *hst3*^{+/} mutants (Fig. 2g and Supplementary Fig. 2h). These results demonstrate that the lethality of *hst3* / mutants depends upon the *RTT109* gene. Therefore, we conclude that H3 Lys56 hyperacetylation is toxic to *C. albicans* cells and that H3K56ac is a major physiological substrate of *C. albicans* Hst3p.

Under conditions that normally maintain the yeast form of *C. albicans*, *HST3* repression with doxycycline triggered a transition to filamentous growth (Fig. 3a). True hyphae typically lack constrictions at sites of septation and have narrow (~2- μ m) parallel cell walls along their entire length²⁸. In contrast, pseudohyphae have visible constrictions at sites of septation²⁸. To determine whether *HST3* repression produced true hyphae, we stained *hst3* / *pTET-HST3* cells grown in doxycycline-containing medium with DAPI and calcofluor white to visualize DNA, cell walls and septa. Cells initially appeared to divide normally, as we observed many budded cells after 3 h of doxycycline treatment (Fig. 3b). After 6 h, we observed pseudohyphae-like cells with septal junctions (Fig. 3b). Notably, at later time points (9 and 24 h), most cells appeared as abnormal ‘V-shaped’ filaments (Fig. 3b). In contrast to *HST3* repression, *hst3* / *pTET-HST3* cells in spider medium lacking doxycycline (conditions that preserve *HST3* expression) produced hyphae with parallel-sided, narrow germ tubes and no clear septal junctions (Fig. 3a). These features are clearly different from the morphological changes observed after repression of *HST3* (Fig. 3b). After 9 h and 24 h of doxycycline treatment, *hst3* / *pTET-HST3* cells showed diffuse or absent DAPI staining (Fig. 3b), suggesting that *HST3* repression resulted in DNA fragmentation and degradation. This is consistent with the presence of high levels of spontaneous DNA damage previously reported in *S. cerevisiae* *hst3* *hst4* mutants¹. These results indicate that inappropriately high levels of H3K56ac lead to DNA fragmentation, histone H3 and H4 degradation and an anomalous morphological transition that culminate in cell death. Remarkably, all of these events occurred when the stoichiometry of H3K56ac increased from about 48% to 87% after *HST3* gene repression (Fig. 2c and Supplementary Table 1). Some of these pronounced phenotypes were not reported in *S. cerevisiae* and *S. pombe* cells that lack H3K56 deacetylases^{1,14,15,29,30}. In addition, we observed that a fraction of *rtt109* / cells (about 10%) spontaneously formed pseudohyphae with abnormal nuclear DNA staining

(Supplementary Fig. 1g), indicating that tightly regulated levels of H3K56ac are crucial for proper chromatin structure and morphogenesis in *C. albicans*.

Hst3p inhibition with nicotinamide is cytotoxic to *C. albicans*

Hst3p is a member of a family of NAD⁺-dependent histone deacetylases known as sirtuins (Supplementary Fig. 3). These enzymes are inhibited by nicotinamide, a product of the NAD⁺-dependent deacetylation reaction. We reasoned that nicotinamide treatment of *C. albicans* cells should phenocopy *HST3* repression. Addition of nicotinamide to wild-type cells caused an increase in H3K56ac (Fig. 4a) and led to robust growth inhibition (Fig. 4b,c).

Furthermore, growth inhibition was not observed with nicotinic acid, which does not inhibit Hst3p¹, and was *RTT109* dependent (Fig. 4b,c). This suggests that nicotinamide exerts its effect through inhibition of H3K56 deacetylation. Finally, incubation of wild-type cells for 24 h with nicotinamide triggered the formation of V-shaped filaments with abnormal DNA staining (Fig. 4d), similar to those observed in doxycycline-treated *hst3* /*pTET-HST3* cells (Fig. 3b).

These results prompted us to determine whether other clinically relevant isolates of *C. albicans* and fungal species are susceptible to growth inhibition by nicotinamide. Several azole- and echinocandin-resistant clinical isolates of *C. albicans* were as sensitive to nicotinamide as a wild-type strain (Figs. 4c and 5a and Supplementary Fig. 4). We also found that other pathogenic *Candida* species were susceptible to nicotinamide. We observed robust growth inhibition of *C. krusei* at 25 mM nicotinamide, whereas growth of *C. tropicalis*, *C. glabrata* and *C. parapsilosis* was inhibited at higher nicotinamide concentrations (Fig. 5b). Nicotinamide also strongly inhibited the growth of *Aspergillus fumigatus* and *Aspergillus nidulans*, which are pathogenic to humans and plants, respectively (Fig. 5c). These results demonstrate that nicotinamide has broad antifungal properties.

Modulation of H3K56ac reduces *C. albicans* virulence in mice

We investigated the effect of H3K56ac modulation on *C. albicans* virulence in A/J mice, which are very sensitive to *C. albicans* infection^{31,32}. This phenotype results, at least in part, from a deficiency in the C5 component of the complement system³³. A/J mice accumulate high fungal loads within 24 h, providing a convenient model to rapidly and accurately measure fungal proliferation *in vivo*³². We first investigated the effect of *HST3* repression on *C. albicans* virulence. A/J mice were injected with wild-type (SC5314 or KTY3) or *hst3* /*pTET-HST3* cells, and tetracycline was included in the drinking water of the mice to repress *HST3*. The kidney fungal loads of mice injected with *hst3* /*pTET-HST3* cells were markedly lower than those of mice injected with wild-type strains (Fig. 6a). In addition, clinical symptoms typical of a *C. albicans* infection were not visible in mice injected with *hst3* /*pTET-HST3* cells (data not shown). We next determined whether nicotinamide treatment would mimic the effect of *HST3* repression *in vivo*. We gave mice nicotinamide 30 min before and 8 h after infection with *C. albicans*. Nicotinamide greatly lowered kidney fungal loads and clearly alleviated clinical signs of disease progression in mice injected with wild-type cells (Fig. 6b). In contrast, nicotinamide failed to protect mice injected with *rtt109* / cells, both in terms of fungal load and in terms of symptoms of candidiasis (Fig. 6b). As expected, introduction of a wild-type *RTT109* gene into *rtt109* / cells reestablished

sensitivity to nicotinamide (Fig. 6b). Nicotinamide has been reported to have complex immunomodulatory effects in mammals³⁴. However, under our experimental conditions, nicotinamide had no obvious side effects, as judged by the absence of various inflammation markers (Supplementary Table 4) and by careful monitoring of the mice. Furthermore, the fact that the anti-fungal effect of nicotinamide requires *RTT109*-dependent H3K56ac firmly establishes that nicotinamide exerts its therapeutic effect through inhibition of Hst3p-mediated H3K56 deacetylation, rather than by enhancing the host immune response. These results indicate that genetic or pharmacological inhibition of Hst3p leads to a loss of virulence in mice.

We also investigated the effect of the *RTT109* deletion on virulence. We injected A/J mice intravenously with wild-type, *rtt109* / or *rtt109* / +*RTT109* revertant cells. At the time of killing (24 h after infection), only mice injected with wild-type and revertant cells showed clinical manifestations of advanced candidiasis (data not shown). This prompted us to investigate the effect of *RTT109* deletion on other parameters of *C. albicans* virulence. A/J mice suffer from cardiac dysfunction and accumulate high fungal loads in kidneys and the heart³². We found reduced fungal loads in the heart and kidneys of mice infected with *rtt109* / as compared with wild-type or revertant cells (Fig. 6c). We also observed that, compared with wild-type and revertant strains, the *rtt109* / mutant elicited a weaker inflammatory response in A/J mice, as evidenced by significantly lower amounts of interleukin-6, a key proinflammatory cytokine, and keratinocyte-derived chemokine, which is crucial for granulocyte recruitment (Fig. 6d). These results show that loss of Rtt109p reduces the virulence of *C. albicans* in A/J mice.

DISCUSSION

Two major classes of chemicals are commonly used to treat candidiasis: azoles and echinocandins. However, the emergence of strains that are resistant to these agents requires the development of new approaches to clinical intervention. In this report, we have identified two enzymes as attractive new therapeutic targets to treat *C. albicans* infections and possibly those caused by other pathogenic fungi. These enzymes, Rtt109p and Hst3/Hst4p, respectively acetylate and deacetylate histone H3K56 in *C. albicans*, *S. cerevisiae* and *S. pombe*^{1,11–15,29,30}, and potential orthologs exist in several other pathogenic fungi. In *C. albicans*, disruption of the *RTT109* gene conferred acute sensitivity to two distinct echinocandin family members, and this sensitivity was suppressed by antioxidants. Given that *RTT109* mutations render *C. albicans* and other yeasts sensitive to genotoxic agents that impede DNA replication^{11–13,29,30}, it seems likely that the marked echinocandin sensitivity of *rtt109* mutants arises from oxidative damage to DNA³⁵. Deletion of *RTT109* also leads to pseudohyphae formation and reduces the growth rate of *C. albicans* in rich medium. Concordant with this, we found that *rtt109* / mutants are less virulent than wild-type *C. albicans* cells in the A/J mouse model. Given these results, Rtt109p inhibitors would be expected to enhance the cytotoxicity of echinocandins and reduce the virulence of *C. albicans*. CREB-binding protein (CBP) and p300 acetylate H3K56 and other substrates in human cells^{7,23}. Despite similarities in their polypeptide backbones suggesting an ancestral relationship, the catalytic cores, catalytic mechanisms and substrate specificity of yeast Rtt109p and human p300/CBP are vastly different²³. Therefore, it is likely that chemical

library screens could identify specific inhibitors of Rtt109p that do not affect human p300/CBP.

We found that repression of the *C. albicans* *HST3* gene is lethal even in the absence of antifungal agent. In A/J mice, Hst3p inhibition by gene repression or nicotinamide treatment considerably reduced *C. albicans* proliferation and clinical manifestations of candidiasis. Nicotinamide is a vitamin that serves as a precursor of NAD⁺. It is well tolerated and used systemically to treat pellagra and skin lesions^{36,37}. Despite its beneficial effects, complex immunomodulatory effects of nicotinamide have been reported in mammalian cells³⁴. This suggests that the ability of nicotinamide to curtail *C. albicans* infection might be due to an enhanced immune response of the host. However, three lines of evidence firmly establish that the main therapeutic effect of nicotinamide is exerted through inhibition of Hst3p, thus blocking H3K56 deacetylation. First, nicotinamide is not cytotoxic to *C. albicans* cells lacking Rtt109p and H3K56ac. Second, the antifungal property of nicotinamide in A/J mice depends on the presence of the *RTT109* gene. Third, as judged by the absence of various inflammation markers, short-term nicotinamide treatment has no obvious immunomodulatory effects under our experimental conditions.

There are sound biochemical arguments to indicate that selective inhibitors of Hst3p may not have major side effects in humans. Despite the fact that sirtuin-1 (SIRT1) and SIRT2 have been implicated in H3K56 deacetylation in human cells^{7,10}, there are sequence motifs conserved among fungal Hst3p family members that are absent from human sirtuins²⁴. For instance, differences occur in the peptide binding channel of sirtuins³⁸. Unlike human SIRT1 and SIRT2 (refs. 39–41), Hst3p seems to be highly substrate specific. In *S. cerevisiae*¹ or *C. albicans* (Supplementary Table 1), acetylation of several sites in the N-terminal tails of H3 or H4 does not increase in the absence of Hst3 or Hst4p. Hence, it is conceivable that small molecules that specifically block the deacetylase activity of fungal Hst3p orthologs, without affecting human sirtuins, might be identified. Indeed, small molecules that inhibit even closely related sirtuins with high selectivity have been identified by screening chemical libraries⁴².

We determined the sensitivity to nicotinamide of a broad spectrum of fungal species. All of the *C. albicans* clinical isolates tested, including azole- and echinocandin-resistant strains, were very sensitive to nicotinamide. This is clinically important because, among the numerous species that cause candidiasis, *C. albicans* is responsible for the majority of cases⁴³ and frequently develops resistance to azoles and echinocandins^{21,22}. The non-*albicans* *Candida* strains that we tested showed various degrees of sensitivity. The strong sensitivity of *C. krusei* to nicotinamide is of particular interest, because *C. krusei* is intrinsically resistant to many antifungal drugs⁴⁴. We also found that two different *Aspergillus* species, *A. fumigatus* and *A. nidulans*, were very sensitive to nicotinamide. This is noteworthy because *A. fumigatus* causes the second most common invasive mycoses in humans, after candidiasis⁴⁵. Our data thus support the notion that nicotinamide or future fungal-specific sirtuin inhibitors may prove useful to treat various fungal infections and provide a solid physiological and molecular basis for the development of new antifungal agents that inhibit the enzymes that regulate H3K56ac. Although further *in vivo* studies will be necessary to assess the true therapeutic potential of nicotinamide and related chemicals,

our results constitute a proof of principle that this strategy represents a useful therapeutic avenue.

ONLINE METHODS

Strain construction

Detailed methodologies for genetic manipulations, Southern blot and northern blot analyses are presented in the Supplementary Methods. The *C. albicans* strains constructed in this study are listed in Supplementary Table 2, and the primers used for their construction are shown Supplementary Table 3. The molecular characterization of the mutant strains is described in detail in the Supplementary Results.

Immunoblots

We prepared whole-cell extracts for SDS-PAGE by an alkaline method⁴⁶ or a glass bead method³⁰. Antibodies against H3K56ac (AV105), the H3 N-terminal domain (AV71/72) and recombinant *S. cerevisiae* histone H4 (AV 94) have been previously described^{2,30,47}.

Identification of H3 K56 acetylation by mass spectrometry

We isolated histones from *C. albicans* as previously described for *S. cerevisiae*⁴⁸. Acid-extracted histones were further purified by reverse phase HPLC⁴⁹. Nano liquid chromatography–tandem mass spectrometry analyses of H3 tryptic peptides with multiple reaction monitoring were performed on an AB/MDS Analytical Technologies 4000 Q-Trap mass spectrometer equipped with a Nanospray II interface. We injected samples in triplicate, using multiple reaction monitoring for relative quantification.

RT-PCR

We extracted total RNA from 2×10^7 cells by the hot phenol method⁵⁰. One microgram of total RNA was reverse-transcribed to cDNA with the SuperscriptIII reverse transcriptase enzyme (Invitrogen). Quantitative PCR was performed in triplicate with a SYBR green master mix containing Jumpstart Taq DNA polymerase (Sigma) and SYBR green nucleic acid stain (Invitrogen). We performed quantitative PCR on a Step One qPCR instrument (Applied Biosystems). Results were normalized to the *ACT1* gene signal. The Supplementary Methods contain primer sequences.

Drug susceptibility assays

We performed spot and liquid microtiter plate assays as described previously⁵¹. We monitored cell growth after 48 h of incubation at 30 °C, unless otherwise specified. We purchased genotoxic and antifungal drugs from Sigma unless otherwise stated. Caspofungin was a gift from Merck Frosst Canada and micafungin was purchased from Astellas Pharma Canada.

Microscopy

We performed DIC and epifluorescence microscopy with a Zeiss Axio-Imager Z1 microscope. Image analysis was carried out with the Zeiss AxioVision 4.8 software. DAPI

staining of DNA, CFW staining of cell walls and septa and propidium iodide staining of dead cells were performed as previously described⁵².

***In vivo* disseminated candidiasis model**

Eight to twelve week old A/J mice were purchased from The Jackson Laboratories. Housing and all experimental procedures were approved by the Biotechnology Research Institute Animal Care Committee, which operates under the guidelines of the Canadian Council of Animal Care. To turn off the expression of the *HST3* gene (Fig. 6a), 5% sucrose and 2 mg ml⁻¹ tetracycline were added to the drinking water of the mice 3 d before infection with *C. albicans*. Inoculums of the *C. albicans* strains were grown overnight in YPD medium. Mice were injected via the tail vein with 3×10^5 blastospores, except for the conditional repression of *HST3* with tetracycline (Fig. 6a), where a lower dose was used (5×10^4 blastospores) to allow time for tetracycline to act. Mice were closely monitored for clinical symptoms of candidiasis such as lethargy, hunched back and ruffled fur. Mice showing extreme lethargy were deemed moribund and were killed by exsanguination. This was typically performed at 24 h and 48 h after injection for 3×10^5 and 5×10^4 *C. albicans* blastospores, respectively. For determination of fungal load, kidneys and hearts were homogenized in PBS, and appropriate dilutions were plated on Sabouraud broth-agar plates. For the *in vivo* nicotinamide treatments (Fig. 6b), mice (six per treatment) were injected intraperitoneally with PBS containing nicotinamide (500 mg per kg body weight) 30 min before the *C. albicans* infection, as it takes 20–30 min for the nicotinamide concentration to peak in the blood stream⁵³. A second dose of nicotinamide was injected 8 h after *C. albicans* infection to maintain high nicotinamide concentrations in circulation.

Cytokine analysis

We collected mouse blood in 1.1 ml Z-gel microtubes (Sarstedt), and samples were processed as recommended by the manufacturer. To determine the levels of cytokines in circulation, we analyzed sera (12.5 µl) with the BD CBA Flex sets (BD Biosciences) according to the manufacturer's instructions. Fluorescence levels were recorded with the BD LSRII and data analysis was carried out with the FCAP Array software (BD BioSciences).

Statistical analysis

Statistical comparisons used two-tailed Student's *t* test (kidney and heart fungal load estimations and cytokines analyses). Error bars are expressed as the s.e.m.

Additional methods

Detailed methodology is described in the Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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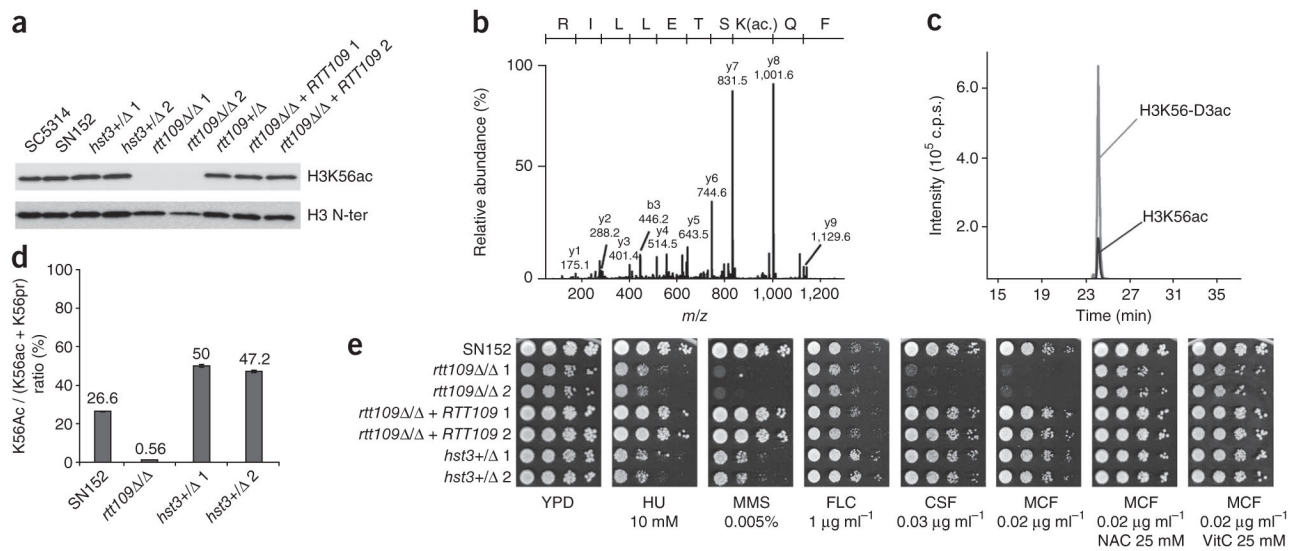
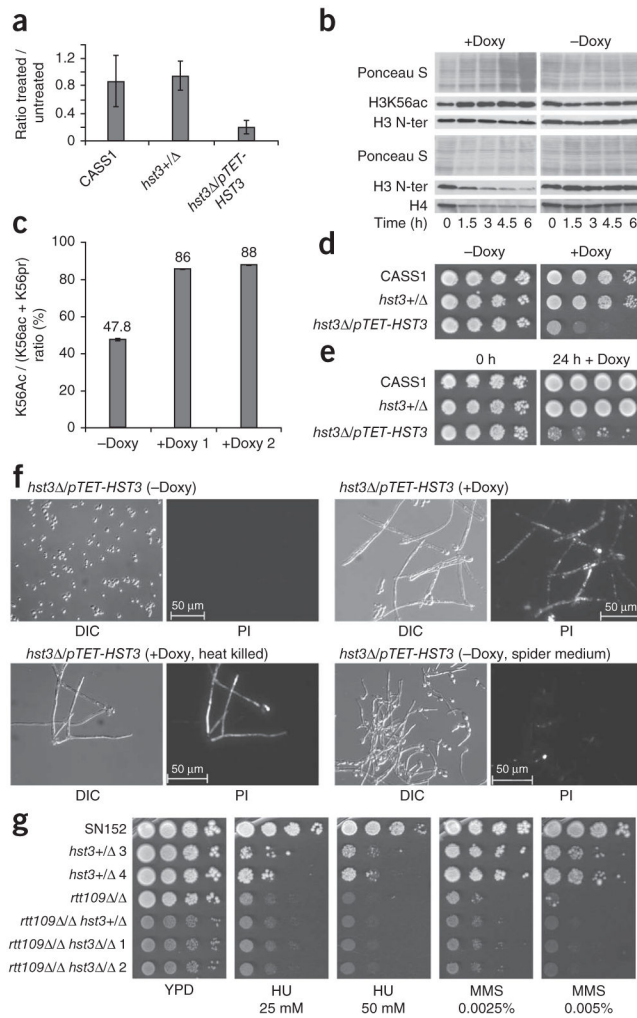
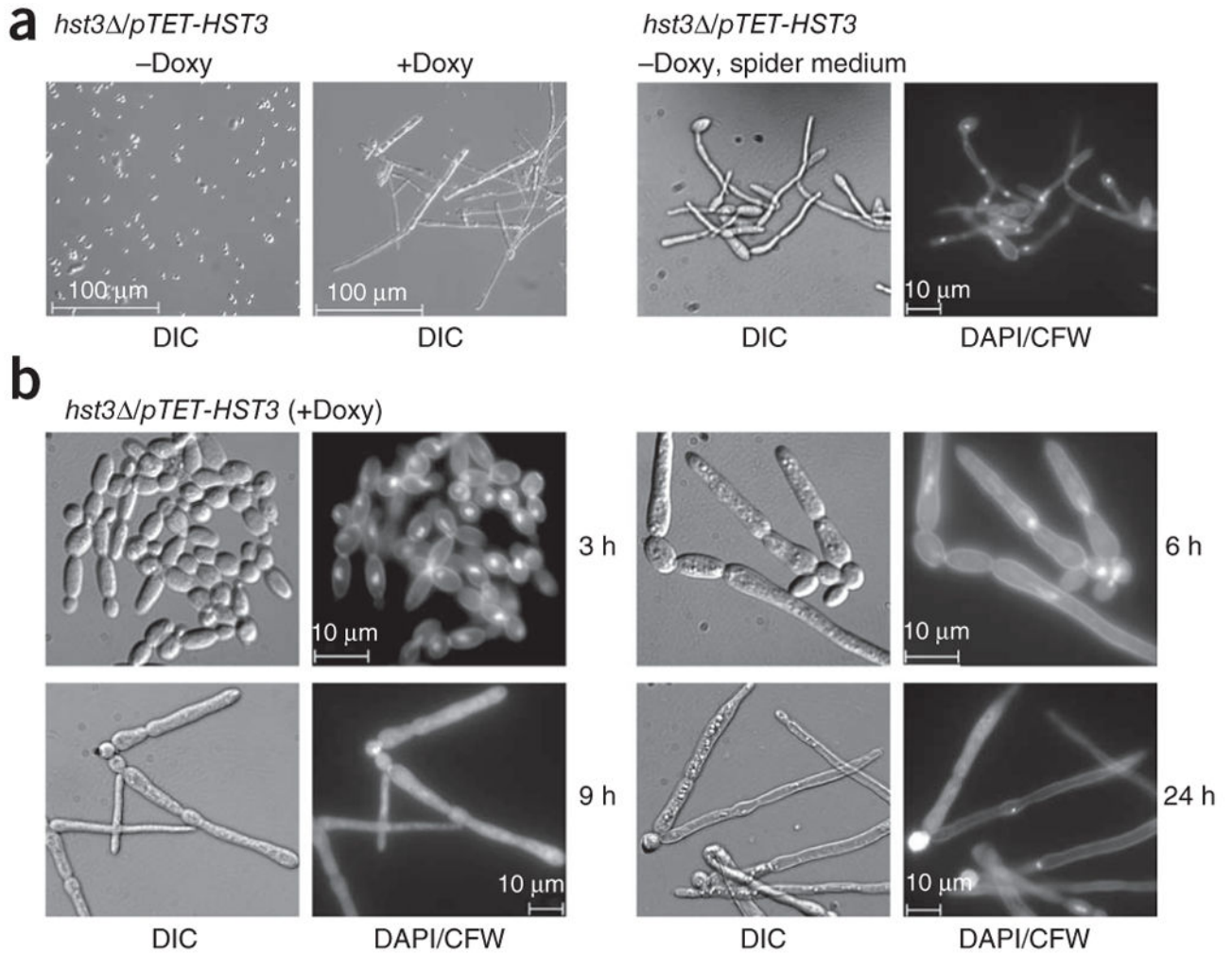


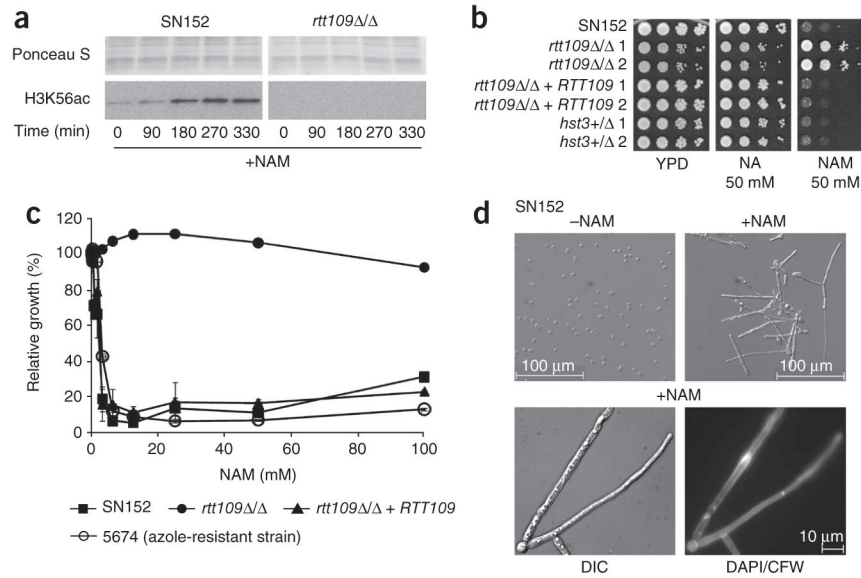
Figure 1. H3K56 acetylation modulates genotoxic and fungicidal agent sensitivity in *C. albicans*. **(a)** Immunoblot of H3K56ac and total H3 in whole-cell lysates from exponentially growing populations of each strain. **(b)** Fragmentation spectrum of precursor ion m/z 638.92⁺ showing the presence of H3K56ac. **(c)** Extracted ion chromatograms for m/z 638.92⁺ (H3K56ac) and its deuterated analog m/z 640.42⁺ (H3K56-D3ac). **(d)** Stoichiometry of H3K56ac determined by mass spectrometry for histones purified from exponentially growing cells. **e**-NH₂ of Lys56 is modified with either an acetyl (ac) or a deuterated acetyl (pr) group. **(e)** Colony formation assays (tenfold serial dilutions of each strain). YPD, control; HU, hydroxyurea; MMS, methyl methane sulfonate; FLC, fluconazole; CSF, caspofungin; MCF, micafungin; NAC, *N*-acetyl-L-cysteine; VitC, vitamin C.

**Figure 2.**

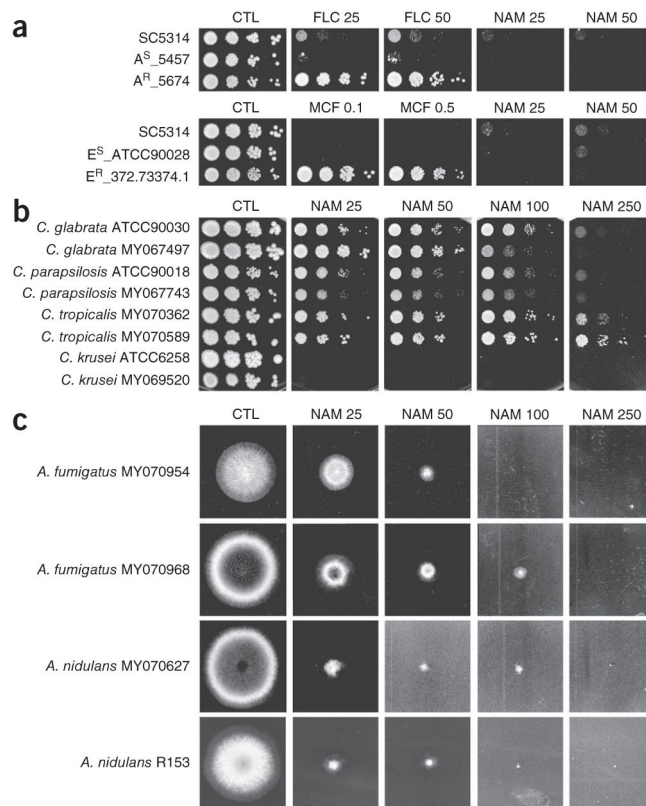
HST3 controls H3K56 deacetylation and is required for cell viability in *C. albicans*. **(a)** Repression of *HST3* mRNA by doxycycline treatment, as measured by quantitative RT-PCR. Results are the mean of three experiments \pm s.e.m. **(b)** Immunoblots of lysates from *hst3* / *pTET-HST3* cells treated with doxycycline (+Doxy, 40 $\mu\text{g ml}^{-1}$) or left untreated (-Doxy). Equal amounts of histone H3 (top) or of total proteins (bottom) were loaded for each time point. **(c)** Stoichiometry of H3K56ac determined by mass spectrometry of histones purified from *hst3* / *pTET-HST3* cells grown in the absence or presence of doxycycline (duplicates). **(d)** Growth of *hst3* / *pTET-HST3* cells on plates containing doxycycline (50 $\mu\text{g ml}^{-1}$) or control plates. CASS1, wild-type strain. **(e)** Growth of *hst3* / *pTET-HST3* cells transiently exposed to doxycycline (50 $\mu\text{g ml}^{-1}$) for 24 h and plated on YPD lacking doxycycline. **(f)** Lethality of *hst3* / *pTET-HST3* cells caused by doxycycline treatment (20 $\mu\text{g ml}^{-1}$ for 24 h), as assayed by fluorescence microscopy after propidium iodide staining. Control cells were either heat-killed or triggered to undergo filamentation (spider medium). DIC, differential interference contrast. PI, propidium iodide. **(g)** Susceptibility to genotoxic agents of WT (SN152), single (*rtt109* / , *hst3+ /*) and double (*rtt109* / *hst3+ /* , *rtt109* / *hst3* /) mutants (tenfold serial dilutions), as determined by colony formation assays.

**Figure 3.**

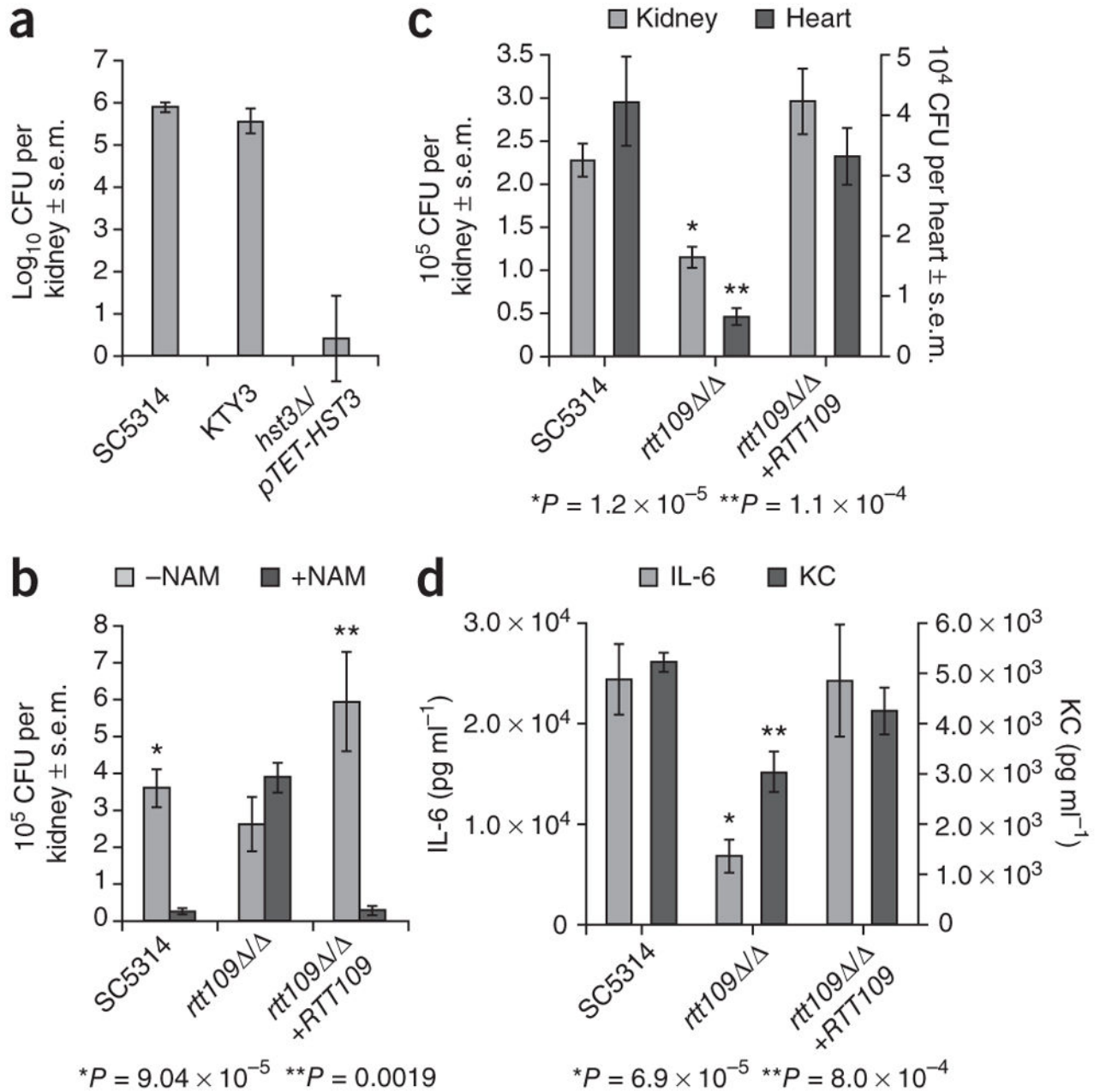
HST3 repression triggers abnormal changes in cell morphology and DNA staining. **(a)** Left, differential interference contrast (DIC) images of *hst3* Δ *pTET-HST3* cells grown in YPD medium at 30 °C in the absence or presence of doxycycline (20 $\mu\text{g ml}^{-1}$ for 24 h). Right, cells grown in spider medium at 37 °C (hyphae-inducing conditions), stained with DAPI and calcofluor white (CFW) to mark DNA and cell walls, respectively, and visualized by epifluorescence microscopy. **(b)** DIC and epifluorescence images showing the morphology, DNA, septa and cell walls of *hst3* Δ *pTET-HST3* cells monitored at various times after doxycycline addition.

**Figure 4.**

HST3 repression and nicotinamide are cytotoxic to *C. albicans* strains that express *RTT109*. (a) Immunoblots of H3K56ac in wild-type (SN152) and *rtt109* Δ/Δ cells treated with 100 mM nicotinamide (+NAM). (b) Colony formation assays performed in the absence (YPD) or presence of either nicotinic acid (NA) or nicotinamide (NAM). (c) The growth of each strain (monitored by OD₆₂₀ in a microtiter plate assay) at a given NAM concentration, as plotted as a percentage of its growth in the absence of NAM. (d) The effect of NAM on wild-type SN152 cells (50 mM NAM at 30 °C for 24 h), as monitored by DIC and fluorescence microscopy to detect either DNA (DAPI) or cell walls and septa (CFW).

**Figure 5.**

Nicotinamide inhibits growth of several clinically important pathogenic fungi. **(a)** Growth of azole-susceptible (A^S) and azole-resistant (A^R) strains (top) as well as echinocandin-susceptible (E^S) and echinocandin-resistant (E^R) strains (bottom), as tested in colony formation assays. Plates contained fluconazole (FLC; $\mu\text{g ml}^{-1}$), micafungin (MCF; $\mu\text{g ml}^{-1}$) or nicotinamide (NAM; mM). Ctl, control plates without NAM or antifungal agent. **(b)** Growth of two independent strains per *Candida* species on YPD medium containing NAM (mM). **(c)** Growth of two independent *A. fumigatus* or *A. nidulans* strains on complete medium containing NAM (mM).

**Figure 6.**

Modulation of H3K56ac levels reduces virulence in a mouse model of *C. albicans* infection.

(a) Kidney fungal loads (CFU, colony-forming units) of A/J mice injected with 5×10^4 cells of each strain. Mice were given tetracycline in their drinking water to repress the *pTET-HST3* allele. SC5314 and KTY3 are two distinct wild-type strains. (b) Kidney fungal loads of A/J mice injected with 3×10^5 cells of each strain. Mice were injected with nicotinamide 30 min before and 8 h after *C. albicans* infection. *P* values for two-tailed Student's *t*-test are indicated for comparison of fungal loads in NAM-treated and untreated mice. (c) Kidney and heart fungal loads of A/J mice injected with 3×10^5 cells of each strain. *P* values for two-tailed Student's *t* test are indicated for comparison of fungal loads in mice injected with

rtt109 / versus SC5314 WT cells. **(d)** Cytokine levels in the blood of the mice described in **c**. *P* values for two-tailed Student's *t* test are indicated for comparison of cytokine levels in mice injected with *rtt109* / versus SC5314 WT cells. IL-6, interleukin-6. KC, keratinocyte-derived chemokine.