Low Dosage of Histone H4 Leads to Growth Defects and Morphological Changes in *Candida albicans*

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**Abstract**

Chromatin function depends on adequate histone stoichiometry. Alterations in histone dosage affect transcription and chromosome segregation, leading to growth defects and aneuploidies. In the fungal pathogen *Candida albicans*, aneuploidy formation is associated with antifungal resistance and pathogenesis. Histone modifying enzymes and chromatin remodeling proteins are also required for pathogenesis. However, little is known about the mechanisms that generate aneuploidies or about the epigenetic mechanisms that shape the response of *C. albicans* to the host environment. Here, we determined the impact of histone H4 deficit in the growth and colony morphology of *C. albicans*. We found that *C. albicans* requires at least two of the four alleles that code for histone H4 (HHF1 and HHF22) to grow normally. Strains with only one histone H4 allele show a severe growth defect and unstable colony morphology, and produce faster-growing, morphologically stable suppressors. Segmental or whole chromosomal trisomies that increased wild-type histone H4 copy number were the preferred mechanism of suppression. This is the first study of a core nucleosomal histone in *C. albicans*, and constitutes the prelude to future, more detailed research on the function of histone H4 in this important fungal pathogen.

**Introduction**

*Candida albicans* is a major human fungal pathogen and is the fourth most common cause of nosocomial bloodstream infections [1]. *C. albicans* is a common commensal of the skin and mucosa, and often causes superficial, non-life threatening infections at these sites [2]. However, in immune-compromised individuals *C. albicans* can cause systemic infections, which have a mortality rate of >30% even in patients undergoing antifungal therapy [3]. The steady increase in the population of immune-compromised individuals due to modern medical practices such as chemotherapy and organ transplantation, as well as because of the AIDS epidemic continues to provide niches for the development of *C. albicans* systemic and mucosal infections.

*C. albicans* pathogenesis has been increasingly linked to alterations in chromosome structure and dynamics. *C. albicans* strains with altered karyotypes are frequently isolated from clinical samples, from passage through mammalian hosts, and by growth in specific carbon sources or antifungals in *vitro* [4,5]. *C. albicans* has a high tolerance to aneuploidies, perhaps because they provide a source for phenotypic variation, critical for survival and pathogenesis [6]. Aneuploidies are associated with antifungal resistance, metabolic changes, and mating [7–13]. Altered karyotypes have also been associated with variations in colony morphology [4,6,14,15]. However, the mechanisms that promote ploidy changes and genomic rearrangements are not well understood.

Histone modifying enzymes and chromatin remodeling proteins also contribute to the regulation of pathogenesis traits. For example, mutants in histone deacetylases, methylases, acetyltransferases, and members of chromatin remodeling complexes show defects in yeast-hyphal transitions, white-opaque switching, adhesion to epithelial cells, and/or antifungal and stress resistance [16–23]. Therefore, changes in the structure and function of the chromatin leads to epigenetic defects and potentially to karyotypic variations that have a direct impact on *C. albicans* virulence.

Chromatin is a dynamic structure composed of DNA and DNA binding proteins that allows for an efficient storage and usage of the genetic information. The basic unit of chromatin architecture is the nucleosome, which is composed of the evolutionary conserved histones H2A, H2B, H3 and H4 assembled in a hetero-octamer of two H2A/H2B dimers and one H3/H4 tetramer. The DNA is wrapped around the nucleosome, constituting the first level of chromatin compaction. Due to this intimate relationship with the DNA, histones are involved in all processes associated with chromatin structure and function, including transcription, replication, DNA repair, recombination, and chromosome segregation. Histones participate in the regulation of these processes by providing a platform to transport information to other proteins (e.g. DNA and RNA polymerases) through posttranslational modifications in their residues [24,25] and through nucleosomal occupancy of regulatory regions in the DNA [26,27]. Thus, histones constitute the primary regulators of chromatin activity.
Alterations in histone availability have profound effects on the cell. Unbalanced histone dimer stoichiometry causes defects in the segregation of mitotic chromosomes, increases recombination and genetic instability, and leads to sporoulation defects in *Saccharomyces cerevisiae* [28–37]. Furthermore, incomplete meiosomal occupancy due to histone dosage defects directly impacts transcriptional regulation [39–44]. Therefore, alterations in histone stoichiometry have pleiotropic effects in cells.

In this study we performed serial deletions of *C. albicans* histone H4 genes and determined the effect of a deficit in histone H4 on growth. We found that reduced histone H4 dosage caused a severe growth defect and the formation of colony morphology variants. *C. albicans* primarily counterbalanced the low dosage of histone H4 by increasing histone H4 gene copy number through the formation of aneuploidies. Suppression of the growth defect associated with low histone H4 dosage also restored colony morphology to the wild-type morphology. This is the first study on core histones in *C. albicans*, which provides background genetic information for future experiments that address the role of chromatin structure and function in *C. albicans* biology and pathogenesis.

**Materials and Methods**

**Strains and plasmids**

All strains used in this study are listed in Table 1. Strain DAY1069 was generated as follows. BWP17 was transformed with a hht2-hhf22::URA3-dpl200 disruption cassette amplified in a PCR using primers HHT2-HHF22 5′ detect, HHF1 3′ detect, HHT2-HHF22 5′ detect-new that flank the integration site (Table 2), and by Southern blot. This study

| Strain       | Parent/Background | Genotype                                                                 | Reference |
|--------------|-------------------|---------------------------------------------------------------------------|-----------|
| DAY1         | BWP17             | ura3::Jimm434/ura3::Jimm434 his1::hisG his1::hisG arg4::hisG/arg4::hisG   | [97]      |
| DAY286       | DAY1              | ura3::Jimm434/ura3::Jimm434 his1::hisG ARG4::URA3/arg4::hisG/arg4::hisG   | [98]      |
| DAY963       | SC5314            | Prototrophic clinical isolate                                             | [99]      |
| DAY1066      | DAY1              | ura3::Jimm434/ura3::Jimm434 his1::hisG arg4::hisG/arg4::hisG HHF1::URA3-dpl200 | This study |
| DAY1067      | DAY1              | ura3::Jimm434/ura3::Jimm434 his1::hisG ARG4::URA3/arg4::hisG/arg4::hisG   | This study |
|              |                   | HHT2-HHF22/hht2-hhf22::URA3-dpl200                                       | This study |
| DAY1068      | DAY1066           | ura3::Jimm434/ura3::Jimm434 his1::hisG arg4::hisG/arg4::hisG HHF1::URA3-dpl200 | This study |
|              |                   | HHT2-HHF22/hht2-hhf22::ARG4                                             | This study |
| DAY1069      | DAY1067           | ura3::Jimm434/ura3::Jimm434 his1::hisG arg4::hisG/arg4::hisG HHF1::URA3-dpl200 | This study |
|              |                   | HHT2-HHF22/hht2-hhf22::ARG4/htt2-hhf22::ARG4/htt2-hhf22::ARG4/htt2-hhf22::ARG4 | This study |
| DAY1070      | DAY1068           | ura3::Jimm434/ura3::Jimm434 his1::hisG arg4::hisG/arg4::hisG HHF1::URA3-dpl200 | This study |
|              |                   | HHT2-HHF22/hht2-hhf22::ARG4                                             | This study |
| DAY1071      | DAY1069           | ura3::Jimm434/ura3::Jimm434 his1::hisG arg4::hisG/arg4::hisG HHF1::URA3-dpl200 | This study |
|              |                   | hht2-hhf22::ARG4/htt2-hhf22::ARG4/htt2-hhf22::ARG4/htt2-hhf22::ARG4      | This study |
| DAY1072      | DAY1071           | ura3::Jimm434/ura3::Jimm434 his1::hisG arg4::hisG/arg4::hisG HHF1::URA3-dpl200 | This study |
|              |                   | hht2-hhf22::ARG4/htt2-hhf22::ARG4/htt2-hhf22::ARG4/htt2-hhf22::ARG4      | This study |
| DAY1074      | DAY1070           | ura3::Jimm434/ura3::Jimm434 his1::hisG arg4::hisG/arg4::hisG HHF1::URA3-dpl200 | This study |
|              |                   | hht2-hhf22::ARG4/htt2-hhf22::ARG4/htt2-hhf22::ARG4/htt2-hhf22::ARG4      | This study |
| DAY1075      | DAY1074           | ura3::Jimm434/ura3::Jimm434 his1::hisG arg4::hisG/arg4::hisG HHF1::URA3-dpl200 | This study |
|              |                   | HHT2-HHF22/hht2-hhf22::ARG4                                             | This study |
| DAY1076      | DAY1070           | ura3::Jimm434/ura3::Jimm434 his1::hisG arg4::hisG/arg4::hisG HHF1::URA3-dpl200 | This study |
|              |                   | HHT2-HHF22/hht2-hhf22::ARG4                                             | This study |
| DAY1078      | DAY1070           | ura3::Jimm434/ura3::Jimm434 his1::hisG arg4::hisG/arg4::hisG HHF1::URA3-dpl200 | This study |
|              |                   | HHT2-HHF22/hht2-hhf22::ARG4                                             | This study |
| DAY1079      | DAY1070           | ura3::Jimm434/ura3::Jimm434 his1::hisG arg4::hisG/arg4::hisG HHF1::URA3-dpl200 | This study |
|              |                   | HHT2-HHF22/hht2-hhf22::ARG4                                             | This study |
| DAY141 (L40) | S. cerevisiae     | MATs his3::200 trp1-901 leu2-3, 112 ade2 LYS2::(lexAop)_8 HIS3::(lexAop)_8 URA3::(lexAop)_8 LacZ GAL4 | [100]     |
Plasmid DDB383 was generated by in vivo recombination as follows. An hhf1::URA3 disruption cassette was amplified in a PCR from DDB245 [45] using primers HHF1 5’-taaacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttccccgctacgcgagtt and HHF1 3’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttccccgctacgcgagtt (Table 2). The two flanking HHF1 regions of 570 bp and 523 bp with homology upstream (including the first 113 nucleotides of HHF1) and downstream of HHF1, respectively, were amplified in two high fidelity PCRs (Pfu Turbo DNA polymerase, Stratagene) from BWP17 genomic DNA using the primer pairs HHF1 5’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttcccagtcacgacgtt and HHF1 3’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttcccagtcacgacgtt, and HHF1 5’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttcccagtcacgacgtt and HHF1 3’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttcccagtcacgacgtt, respectively. The three PCR products were co-transformed with a NotI/EcoRI double digestion of DDB78 into the Trp- Saccharomyces cerevisiae L40 strain to generate DDB383.

Media and growth conditions

C. albicans was routinely grown at 30°C in YPD supplemented with uridine (2% bacto-peptone, 1% yeast extract, 2% dextrose, and 80 µg ml⁻¹ of uridine). Mutants were selected on synthetic medium (0.17% yeast nitrogen base without ammonium sulfate (Q-BioGene), 0.5% ammonium sulfate, 2% dextrose, and supplemented with a dropout mix containing amino and nucleic acids except those necessary for the

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Table 2. Primers used in this study.

| Name | Sequence (5’ to 3’) | Reference |
|------|---------------------|-----------|
| HHF1 5’ DR 100 in | 5’-taaacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttccccgctacgcgagtt | This study |
| HHF1 3’ DR | 5’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttccccgctacgcgagtt | This study |
| HHF22-HHF2 5 DR | 5’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttccccgctacgcgagtt | This study |
| HHF1 5 detect | 5’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttccccgctacgcgagtt | This study |
| HHF1 3 detect | 5’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttccccgctacgcgagtt | This study |
| HHF1 5’ detect-new | 5’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttccccgctacgcgagtt | This study |
| HHF1 5’ fragm DDB78 | 5’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttccccgctacgcgagtt | This study |
| HHF1 5’ fragm 3’ | 5’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttccccgctacgcgagtt | This study |
| HHF1 3’ fragm 3’ | 5’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttccccgctacgcgagtt | This study |
| HHF1 5’ fragm 5 | 5’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttccccgctacgcgagtt | This study |
| HHF1 5’ fragm 100 in for DDB78 | 5’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttccccgctacgcgagtt | This study |
| HHF1 3’ for DDB78 | 5’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttccccgctacgcgagtt | This study |
| HHF22 5 SB | 5’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttccccgctacgcgagtt | This study |
| HHF22 3 detect | 5’-ttataacgtcacagaattttcagataaactcatgatggattacaaaccgagctatagtttccccgctacgcgagtt | This study |

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Figure 1. Genomic organization of the histone H3 and H4 loci in Candida albicans. The black boxes indicate the extent of the deleted regions in the mutants, which are replaced by the auxotrophic markers ARG4, URA3, URA3-dpl200 or the dpl200 loop-out. HHF1 is deleted from nucleotide +114 to the STOP codon (65% of the gene) and the HHF22-HHT2 cluster is deleted from the STOP codon of HHF22 to nucleotide +329 of HHT2 (80% of the HHT2 gene). orf19.1060 is a possible spurious ORF. In red, the regions recognized by the probes D and G used for determining HHF1 or HHF22 copy dosage by Southern Blot are shown. H: HindIII; N: Ncol.

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Southern blot and comparative genomic hybridizations

For the HHT1 and HHF22 dosage experiments, genomic DNA was digested with HindIII or NcoI, respectively (Figure 1), separated in a 1.2% agarose gel by electrophoresis, and transferred by capillary action to a nylon membrane. Probes D (for HHT1) and G (for HHF22) were PCR amplified from \textit{C. albicans} BWP17 genomic DNA using primer pairs HHT1 5’ fragm 5’ and HHF1 5’ fragm 5’, and HHF22 5’ SB and HHF22 3’ detect, respectively (Figure 1 and Table 2). The probes were radiolabelled with \({^{32}P}\)-dCTP using the Prime-a-Gene labeling system (Promega). Blots were developed with a phosphoimager (STORM system). Denaturing analysis of the images was performed using ImageJ 1.30v (Wayne Rasband, NIH, USA). For comparative genomic hybridizations (CGH), genomic DNA was prepared from \textit{C. albicans} strains grown overnight to saturation in 5 ml YPAD medium using phenol/chloroform as described [47]. 3 \(\mu\)g of DNA was digested with HaeIII (Invitrogen), labeled with Cy3 (experimental strains) or Cy5 (reference strain, SC5314), and hybridized to microarrays as described previously [48]. The microarrays were printed in-house and contain 14,688 total spots, representing 6175 ORFs, designed using Assembly six microarrays were printed in-house and contain 14,688 total spots, except for the presence in \textit{C. albicans} of a single unpaired, divergent third histone H3 gene.

Since Hht1 has a divergent amino acid sequence compared to Htt2/21 and is expressed independent of a histone H4, we propose that Hht1 is a histone H3 variant. Histone variants are usually replication-independent, diverge from the canonical histone sequences, and have a different function [60,61]. For example, \textit{Drosophila melanogaster} and vertebrates express histone H3.3, a canonical histone H3 variant that differs in four amino acids, three of which are clustered in the histone fold domain [62–65]. Although all three \textit{C. albicans} histone H3s are of the H3.3 class and the amino acid differences in Hht1 are not within the histone fold domain, Hht1 might still constitute a histone H3 variant. In particular, the S2\(\nu\)V change in Hht1 could affect epigenetic regulatory events, as it eliminates a potential phosphorylation site in the N-terminus of Hht1 [66,67]. Thus, Hht1 may encode a histone H3 homomorph variant.

The \textit{C. albicans} genome contains two genes, HHT1 and HHF22, that encode identical histone H4 proteins. The \textit{S. cerevisiae} genome also encodes two identical histone H4 proteins. Comparison of \textit{C. albicans} Hht1/22 to \textit{S. cerevisiae} Hht1/2, revealed seven amino acid differences (Figure 2B). Therefore, the genomic arrangement of histone H4 genes in \textit{C. albicans} is similar to \textit{S. cerevisiae}. Since \textit{C. albicans} contains three non-allelic histone H3 genes as well as a heteromorphic histone H3 variant Cse4, which replaces histone H3 in centromeric chromatin [68,69], we focused our studies on histone H4, which is encoded by two genes and is present in all nucleosomes.

Reduced histone H4 dosage impairs \textit{C. albicans} growth

Previous work in \textit{S. cerevisiae}, \textit{S. pombe}, and \textit{D. melanogaster} showed that alterations in the dosage of the core nucleosomal histones can lead to pleiotropic phenotypes, including growth and cell cycle defects, chromosomal and telomere instability and gene expression deregulation [28,29,34,36,38,39,41,70–72]. In order to understand the effects of altering histone H4 dosage in \textit{C. albicans}, sequential deletions of three of the four histone H4 alleles were performed.

First, we generated a deletion of the HHT2-HHT1 region, which lacks additional ORFs (Figure 1). HHT22 and HHT2 were simultaneously deleted in order to reduce any potentially harmful effects of changing the histone H3/histone H4 ratio [36,41,71,73]. The entire HHT22 ORF, 80% of the HHT2 ORF, and the intergenic region were replaced by an auxotrophic marker cassette (Figure 1). We were able to generate both HHT22-HHT1/hht2-hht2A heterozygous and hht22-hht2A/hht2A homozygous strains, and these strains had no overt growth or morphological phenotypes.
Figure 2. Amino acid sequence alignment of histone H3 and H4 genes. Comparison of the amino acid sequences of histone H3 (A) and histone H4 (B) alleles from C. albicans and S. cerevisiae using ClustalW. doi:10.1371/journal.pone.0010629.g002

(Figure 3A). This suggests that Hhf22 and Hht2 are not essential for growth.

S. cerevisiae diploids containing a single H3-H4 locus have a longer generation time and a more prolonged G1 phase than wild-type cells [70]. Further, deletion of HHT2-HHF2 in S. cerevisiae, but not HHT1-HHF1, causes greater minichromosome loss compared to wild-type [70], indicating that, although both H3-H4 loci are generally functionally redundant, there are differences between both loci. To determine if C. albicans has a different requirement for HHF1 or HHF22 for growth, we constructed a strain in which both copies of HHF1 were deleted. We were readily able to generate HHF1/hhf1D and hhf1D/D strains, and these strains did not show growth or morphological defects (Figure 3B and data not shown), indicating that Hhf1 is also not essential for growth in C. albicans. Therefore, unlike in S. cerevisiae, in C. albicans the presence of either one of the histone H4 gene is sufficient to ensure normal growth and colony morphology.

When we attempted to mutate one copy of HHF1 in the hhf22-hht2D background, we only recovered a single homologous recombinant (1 homologous recombinant/395 transformants screened). Similarly, when we attempted to mutate the remaining copy of HHF22-HHT2 from a HHF22-HHT2/hhf22-hht2D HHF1/hhf1A double heterozygote, we only recovered homologous recombinants 7% of the time (32 homologous recombinants/437 transformants screened). However, all of these recombinants retained a wild-type HHF22-HHT2 copy and arose by a marker exchange (29/32) or a by an increased HHF22-HHT2 copy number (3/32). To increase the rate of homologous recombination, we generated an hhf1::URA3 disruption cassette containing regions of homology ~9x larger than the original cassettes (Table 2). Using this extended disruption cassette we increased homologous recombination in the hhf22-hht2D strain to 50% (11/22). All hhf22-hht2A/A HHF1/hhf1A transforms had a pronounced growth defect (Figure 3A) and gave rise to both smooth and wrinkly colonies of heterogeneous size (Figure 4A and B). The difficulty in eliminating a third histone H4 gene and the severe growth defects observed in the mutant with only one HHF1 copy suggested that C. albicans might require at least two copies of histone H4 for normal growth. Alternatively, HHF22 might be the primary histone H4 gene in C. albicans.

If HHF22 is indeed the primary histone H4 gene in C. albicans, we predicted that we could construct an HHF22/hhf22A hhf1A/A mutant without impacting growth. To construct this mutant, we eliminated the last HHF1 copy from a HHF22-HHT2/hhf22-hht2A HHF1/hhf1A double heterozygous strain. As before, the use of longer regions of homology increased the rate of homologous recombination (44/45). However, in most cases (40/44) the wild-type HHF1 copy was retained. While the HHF22-HHT2/hhf22-hht2A HHF1/hhf1A strain had no overt growth defects compared to wild-type colonies, the four HHF22-HHT2/hhf22-hht2A hhf1A/A mutants obtained had severe growth defects (Figure 3B). HHF22-HHT2/hhf22-hht2A hhf1A/A strains also gave rise to
heterogeneous colony sizes with both smooth and wrinkly morphologies (Figure 4 and 5). The similar growth defects in the HHF22-HHT2/HHF1/hhf22-hht2 Δ/D strain suggest that HHF22 is not the primary histone H4 gene. Rather, C. albicans growth is dependent on the presence of at least two copies of histone H4.

We noticed that both hhf22-hht2 Δ/Δ HHF1/hhf1 Δ and hhf22-hht2 Δ/Δ HHF22-HHT2/hhf1 Δ strains gave rise to larger colonies at a low frequency (Figure 3 and 4). We considered the possibility that the larger colonies contained stable suppressor mutations. To address this, we re-isolated small and large colonies on fresh medium. When re-isolated, hhf22-hht2 Δ/Δ HHF1/hhf1 Δ and hhf22-hht2 Δ/Δ HHF22-HHT2/hhf1 Δ strains gave rise to primarily small colonies with distinct morphologies (Figure 5), and a few large and always smooth colonies (Figure 4C). However, hhf22-hht2 Δ/Δ HHF1/hhf1 Δ large colonies gave rise to uniformly large and smooth colonies (Figure 4C). Re-isolation of small colonies from the HHF22-HHT2/hhf22-hht2 Δ hhf1 Δ/Δ strain also gave rise to small colonies with distinct morphologies, and to large colonies (Figure 4D and 5). In contrast to the hhf22-hht2 Δ/Δ HHF1/hhf1 Δ strain (Figure 4E), re-isolation of large colonies from the HHF22-HHT2/hhf22-hht2 Δ hhf1 Δ/Δ strain gave rise to primarily large colonies, but also occasionally to small colonies (Figure 4F). When re-isolated, these small colonies behaved like the parental HHF22-

Figure 3. Growth of (A) wild-type (DAY286), HHF22-HHT2/HHF1/hhf22-hht2 Δ (DAY1067), hhf22-hht2 Δ/hhf22-hht2 Δ (DAY1069), and hhf22-hht2 Δ/HHF1/hhf22-hht2 Δ (DAY1072) or (B) wild-type (DAY286), HHF1/hhf1 Δ (DAY1066), HHF1/hhf1 Δ HHF22-HHT2/HHF1/hhf22-hht2 Δ (DAY1068), and hhf1 Δ/HHF1 HHF22-HHT2/hhf22-hht2 Δ (DAY1074) histone H4 mutants in rich medium. All strains were grown overnight at 30 °C in liquid YPD, streaked on YPD, and incubated at 30 °C for 48 hrs.

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HHT2/HHF22 small colonies (data not shown), indicating that the suppressor phenotype may be reversible. These results suggest that the severe growth defect caused by the low dosage of both HHT1 and HHF22 confers a strong selective pressure for the generation of secondary suppressor mutations, which restore growth. Further, the differences observed in the emergence of small colonies during re-isolation of large colonies in both type of mutants is likely a consequence of the stability of the suppressors that arose in the mutants.

Aneuploidy as a mechanism for H4 dosage compensation in C. albicans

Two copies of histone H4 are necessary and sufficient for wild-type growth. Thus, we reasoned that the increased colony size and phenotypic stability observed in the large suppressor colonies reflected an increase in histone H4 copy number. Since aneuploidies are common in C. albicans strains [5,7,8,10,11,13,48,74–78], we hypothesized that the large suppressor colonies had duplicated the remaining histone H4 gene, thereby restoring the number of histone H4 alleles to two, which supports normal and phenotypically stable growth (Figure 3). The duplication of large DNA fragments, including whole chromosomes, as a mechanism to suppress a slow growth defect has also been described in S. cerevisiae [79].

In order to determine if the large colonies of the hhf22-ht2/HHF1/hhf1A and HHF22-HHT2/hhf22-ht2A hhf1A/A mutants had increased the number of wild-type H4 allele copies, we performed quantitative Southern blot analysis. Two probes, Probe D and Probe G, were designed to anneal equally to both the wild-type and the mutated versions of HHF1 or HHF22, respectively (Figure 1). Densitometry analysis was performed on Southern blots of small and large colonies isolated from the mutants to determine the ratio of mutated to wild-type histone H4 allele (Figure 6). We included the HHF22-HHT2/hhf22-ht2A HHF1/hhf1A heterozygous strain DAY1070 as a control, which had a 1:1.1 hhf22-ht2A/HHF22 ratio by quantitative Southern blot (Figure 6B). Indeed, the small colonies of the histone mutants retained a 1:0.9–1.1 ratio of mutated to wild-type H4 alleles while the large colonies presented a 1:1.5–2.6 ratio of mutated to wild-type H4 alleles (Figure 6A and 6B). Thus, the large colonies of the hhf22-ht2/HHF1/hhf1A and HHF22-HHT2/hhf22-ht2A hhf1A/A mutants analyzed showed an increase in DNA associated with the wild-type histone H4 compared to the small colonies. Thus, the suppressor colonies arise by increasing the genomic dosage of histone H4.

We reasoned that an increase in histone H4 copy number could involve either a segmental aneuploidy or a trisomy. Using comparative genome hybridization (CGH) arrays, we found that, as expected, a small colony of hhf22-ht2/HHF1/hhf1A had a diploid content of chromosome 1 (Figure 7). However, CGH analysis of a large suppressor colony derived from the hhf22-ht2/HHF1/hhf1A small colony revealed a trisomy of chromosome 1. Thus, a reduction in histone H4 dosage causes a severe growth

Figure 4. Growth defect and phenotypic instability of mutants containing a single allele of histone H4. A small colony of a histone H4 mutant (DAY1072) was re-streaked on YPD medium and incubated 4 days at 30°C (A), close-up picture (B). Re-isolated small (C) and large (C, E) colonies of hhf22-ht2/HHF1/hhf1A (DAY1072), and small (D) and large (D, F) colonies of HHF22-HHT2/hhf22-ht2A hhf1A/hhf1A (DAY1074 and DAY1079) incubated on YPD for 48 hrs at 30°C. doi:10.1371/journal.pone.0010629.g004
defect that can be overcome through whole chromosome aneuploidy to increase histone H4 copy number. While we cannot rule out segmental aneuploidy as a mechanism to restore histone H4 dosage, whole chromosome aneuploidy is clearly one mechanism that can restore histone H4 dosage.

We noted two additional aneuploidies in our CGH analysis. First, a segmental monosomy of one end of chromosome 5 in both the small and large colony (Figure 7). This is an attribute of the RM1000 background from which these strains are descended, which is known to have a stable deletion in one arm of chromosome 5 [12,48]. Second, a whole chromosome monosomy in chromosome 3 was observed in the hhf22-hht2/hhf1/HHF1/hhf1 small colony, but not the large suppressor colony derivative. Defects on histone dosage has been implicated in the generation of aneuploidies and chromosome instability, and this may reflect evidence of that phenomenon in C. albicans. However, it is also possible that reduced chromosome 3 dosage provides some advantage to cells containing one histone H4 locus.

It is noteworthy that not all large colonies gave the expected 1:~2 ratio of mutant to wild-type histone H4 by quantitative Southern blot (Figure 6C). We found a 0:1 ratio (arbitrarily set to 1 as there was no mutant allele to normalize to), which lost the mutant histone H4 allele, a 1:1 ratio, which maintained the ratio of the starting strain, and a ~2:1 ratio, which duplicated the mutant histone H4 allele. The 0:1 ratio found in large colonies indicates that either the strain became tetrasomic, duplicating both loci, or that there exist alternative suppressor mechanisms. The ~2:1 ratio found in large colonies indicates that the mutated version of the histone H4 was duplicated, perhaps causing a trisomy of part or all of chromosome R. This type of aneuploidy may restore growth, however we cannot rule out the possibility that chromosome R is found in a 4:2 mutant to wild-type ratio in these cells. In fact this latter possibility seems to be supported by the increased hhf22A and HHF22 signals observed in this sample (Figure 6C). We noted that the large colonies carrying a 0:1, 1:1, and 2:1 ratio of mutant to wild-type histone H4 had the typical smooth morphology, but the colonies carrying a 2:1 ratio showed a wrinkly top and were more heterogeneous in size (Figure 6D). These differences in colony morphology are in agreement with the formation of alternative karyotypes (see below). The 0:1, 1:1, and 2:1 ratio for large colonies arose from the HHF22-HHT2/hhf22-hht2/HHF1/hhf1A mutant but not from the hhf22-hht2A/HHF1/hhf1A mutant, which suggests that Chromosome R is less stable than Chromosome 1.

Since maintenance of genomic integrity is critical for survival, cells have different mechanisms to compensate for histone dosage defects, including genomic rearrangements and, more commonly, transcriptional alterations. Genomic rearrangements have been observed in S. cerevisiae, which can increase histone H2A-H2B copy number by forming a small circular chromosome [80]. Dosage compensation through transcriptional up-regulation of histone
gene expression has been observed both in *S. cerevisiae*, where the expression of one of the H2A-H2B loci (HTA1-HTB1) is regulated by the availability of histones H2A-H2B in the cell [73], as well as in *S. pombe* [81]. Due to the formation of suppressors, the proportion of small to large colonies was verified for each overnight culture by colony count on YPD to ensure that the DNA extraction was representative of a small or a large colony population. Overnight cultures from small colonies with a minimum of 80% of small colonies were used to prepare DNA for the Southern blot. Representative colonies from these plates corresponding to different ratios of mutated to wild-type histone H4 ratio are shown (D). Probes D and G were used to detect the alleles: hhf1::URA3 and HHF1, and hhf22::ARG4 and HHF22, respectively. HHF22-HHT2/hhf22-hht2.1 HHF1/hhf1.1 (DAY1070) was used in (B) as a control for a 1:1 mutated to wild-type HHF22 ratio.

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with no obvious effects on growth or colony morphology [74]. Therefore, the alterations in colony size observed in both histone H4 mutants could be attributed to the gain and loss of a segment or an entire copy of Chromosome R or Chromosome 1.

Histone H4 deficit causes colony morphology changes

When growing on agar plates at 30°C, C. albicans normally forms smooth, round, cream-colored colonies composed of yeast cells. Wrinkly colonies are generally composed of a higher percentage of cells that are filamenting, a fact that explains why it is possible to exacerbate some colony morphology phenotypic differences at 37°C. Altered C. albicans colony morphologies have been detected in strains isolated from infected patients, from studies in mouse models, and can also be induced in the laboratory through UV irradiation and genetic manipulation [4,14,88–93]. The changes in colony morphology are a manifestation of underlying genomic changes that can involve a group of genes (like in the white-opaque switching) or major karyotypic rearrangements [9]. Thus, alterations in diverse factors can lead to changes in colony morphology.

When small, smooth colonies that carry only one H4 allele are sub-cultured onto a rich medium plate they give rise to colonies that have different morphologies (Figure 5A). The change in colony morphologies of small colonies was generally penetrant, although they also gave rise to colonies with other types of morphologies (Figure 5B). On the contrary, the large and smooth suppressor colonies did not produce colonies with altered morphology when they were sub-cultured, indicating that their colony morphology phenotype is stable (Figure 5C). One exception to this statement is the already mentioned formation of small colonies from re-streaking of the large colonies, which most likely arise by the loss of the duplicated chromosome R.

One explanation for the formation of semi-penetrant morphological variants in the histone mutants is karyotypic rearrangements. As previously mentioned, altered colony morphologies have been associated with altered karyotypes or with loss of heterozygosity [4,9,94]. Imbalances in histone dosage can lead to chromosome missegregation [28–31]. Further, the histone mutants grow slowly, a condition that might be more permissive to the accumulation and tolerance of aneuploidies [4]. This latter idea is supported by the presence of a monosomic chromosome 3 in a hhf22/hhf22/HHF1/hhf1/1 small colony (Figure 7). Thus, karyotypic rearrangements favored by the combination of the slow growth and the nucleosomal deficit of the histone H4 mutants might be one mechanism behind the formation of colony variants.

Candida albicans is the most important human fungal pathogen, causing serious infections in immunosuppressed individuals. C. albicans has a diploid genome with an unexpectedly high level of heterozygosity, given the primarily clonal reproductive style of this organism [95]. The genome of C. albicans has a remarkably high tolerance for genomic rearrangements. The ability to thrive with an altered karyotype may provide a profound advantage to this organism, because it represents a potential source of genetic variation [5]. Karyotypic rearrangements and aneuploidies in C. albicans are associated with pathogenesis: they affect cellular and colonial morphology, increase metabolic diversity, are required for mating, and, importantly, constitute a mechanism of antifungal resistance. Histone modifying enzymes and chromatin remodeling proteins are also required for pathogenesis in C. albicans [16–22,96]. The study of chromatin dynamics and structure in this fungus therefore is critical for understanding the nature of C. albicans pathogenicity and, furthermore, it may uncover potential targets for antifungal therapies. In this study, we have generated and characterized strains that can be used for future analysis of specific histone H4 mutant alleles, in order to begin to dissect the function and impact of epigenetic regulation in C. albicans lifestyle and pathogenesis.

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Author Contributions

Conceived and designed the experiments: LFZ DD. Performed the experiments: LFZ AMS. Analyzed the data: LFZ AMS DD. Contributed reagents/materials/analysis tools: AMS JB. Wrote the paper: LFZ DD.

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Figure 7. CGH analysis of a hhf22/hhf22/HHF1/hhf1/1 (DAY1072) small colony (A) and a spontaneous large colony suppressor (B). Genomic DNA was purified from both a DAY1072 small colony and large colony and compared to genomic DNA from SC5314 (DAY963). The DAY1072 small colony is monosomic for Chromosome 3 while the DAY1072 large colony is trisomic for Chromosome 1. Both isolates have a short segmental aneuploidy on Chromosome SR, which is present in the strain background [12,48].

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