The ‘obligate diploid’ *Candida albicans* forms mating-competent haploids

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*Candida albicans*, the most prevalent human fungal pathogen, is considered to be an obligate diploid that carries recessive lethal mutations throughout the genome. Here we demonstrate that *C. albicans* has a viable haploid state that can be derived from diploid cells under *in vitro* and *in vivo* conditions, and that seems to arise through a concerted chromosome loss mechanism. Haploids undergo morphogenetic changes like those of diploids, including the yeast–hyphal transition, chlamydospore formation and a white–opaque switch that facilitates mating. Haploid opaque cells of opposite mating type mate efficiently to regenerate the diploid form, restoring heterozygosity and fitness. Homozygous diploids arise spontaneously by auto–diploidization, and both haploids and auto–diploids show a similar reduction in fitness, *in vitro* and *in vivo*, relative to heterozygous diploids, indicating that homozygous cell types are transient in mixed populations. Finally, we constructed stable haploid strains with multiple auxotrophies that will facilitate molecular and genetic analyses of this important pathogen.

The opportunistic fungal pathogen *C. albicans* has been studied extensively since the 1800s and has been considered a strictly diploid organism with no haploid state. The diploid nature of the organism has complicated genetic and genomic analyses of *C. albicans* biology and virulence. Studies in the 1960s through the early 1980s debated the ploidy of *C. albicans*, and proposed that a haploid state existed based on cell size heterogeneity, parasexual genetics and estimates of DNA content by fluorometry. Haploid clinical isolates were reported, but were later found to be diploid in genome content or were shown to be non-*albicans* *Candida* species. The ‘obligate diploid’ nature of *C. albicans* was proposed to be due to recessive lethal mutations dispersed throughout the genome. However, studies of *C. albicans* chromosome monosomy and recombination demonstrated that homeozygosis of certain chromosomes can occur, arguing against this balanced lethal mutation hypothesis. A diploid–tetraploid parasexual cycle was also discovered in *C. albicans* and shown to involve a switch to the ‘opaque’ physiological state that renders cells mating-competent. Congaigation between opaque diploids to form tetraploids, and subsequent ploidy reduction resulting in diploid progeny, often carry multiple aneuploid chromosomes. Importantly, specific aneuploid chromosomes can provide a selective advantage under stress conditions such as exposure to antifungal drugs. Parasexual ploidy reduction in tetraploids occurs via a non-meiotic process termed ‘concerted chromosome loss’. This process can facilitate the rapid generation of diversity through the production of homozygous and aneuploid progeny, under conditions where outcrossing in the host is unlikely. If a haploid state for *C. albicans* were to exist, it could facilitate the elimination of lethal alleles from the population. Furthermore, mating between different haploids could promote adaptation to changing conditions within the mammalian host. With the advent of whole-genome approaches that can distinguish ploidy states, it is now possible to ask if *C. albicans* exists, even transiently, in the tetraploid or the haploid state.

**Detection of haploid *C. albicans* cells**

Haploid *C. albicans* was serendipitously discovered during experiments to follow loss of heterozygosity (*LOH*) at multiple independent loci. Using a multiply marked derivative of SC5314, the laboratory reference strain, we selected for an initial *LOH* event at the *GAL1* locus (encoding galactokinase) by growth on 2-deoxygalactose (2-DOG) and subsequently screened for additional *LOH* events at four other heterozygous loci. Importantly, growth in 2-DOG does not affect *LOH* rates and as such, the selection for cells with an *LOH* event does not artificially induce *LOH*. Amongst the approximately 2,500 galactokinase-negative (Gal⁻) isolates, 42 showed additional *LOH* events and intriguingly one strain, Haploid I, was homozygous for all of the markers tested, as well as for multiple single nucleotide polymorphisms (SNPs) on every chromosome. Analysis of DNA content in Haploid I by flow cytometry indicated that the genome had half the amount of DNA of that in a diploid control (Fig. 1a).

Once we became aware that haploid *C. albicans* cells could be detected, we used flow cytometry to screen isolates from many sources, including *in vitro* stresses, from which one haploid isolate (Haploid IV) was identified amongst small colonies growing in the presence of fluconazole (in the halo of an E-test strip), a commonly

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used antifungal drug. We also screened C. albicans cells isolated from in vivo mouse models of candidaemia\textsuperscript{27} and candidiasis\textsuperscript{28} and discovered several additional isolates with 1N DNA content. The overall population of post-in vivo isolates screened for ploidy included approximately 300 isolates from YPD plates (Gal\textsuperscript{+}), and approximately 740 isolates from the 2-DOG selection plates (Gal\textsuperscript{-}), and only the 2-DOG-resistant (2-DOG\textsuperscript{R}) colonies contained isolates that were haploid. We found that 3.2\% of 2-DOG\textsuperscript{R} colonies from the OPC model were haploid (10 out of 312), whereas 1.2\% of 2-DOG\textsuperscript{R} colonies from the systemic model were haploid (5 out of 431). Because LOH frequencies in vivo are $\sim 10^{-3}$ (ref. 29), this indicates that detectable haploids arose at a frequency of 1–3 per 100,000 cells. Loss of GAL1 is often, but not always, associated with the haploid state (for example, Haploid IV), especially in diploid progenitors that are heterozygous for this locus. In total, we characterized eleven states (for example, Haploid IV), especially in diploid progenitors that are heterozygous for this locus. In total, we characterized eleven isolates from multiple independent and varied sources. Because previous reports of haploid C. albicans\textsuperscript{3} included misidentified species (Supplementary Fig. 1), we determined if these newly identified isolates were bona fide C. albicans by PCR amplifying and sequencing the mating type-like (MTL) locus. All isolates contained sequences identical to either the MTL\textalpha or MTL\textbeta alleles of SC5314 (Supplementary Fig. 4 and data not shown). Next, we analysed the haploid genomes by hybridization to SNP/CGH (comparative genome hybridization) arrays\textsuperscript{30} to determine allele ratios and relative chromosome copy numbers. Six isolates were completely euploid (Fig. 1b and Supplementary Fig. 5); in the remaining five haploids, one or two chromosomes (chromosomes 6 and 7) were disomic. These disomic chromosomes were heterozygous except in one isolate (Haploid IV) where the disomic chromosome (chromosome 6) was homozygous.

Based on the C. albicans haplotype map\textsuperscript{10}, each haploid chromosome primarily contained alleles from only one parental homologue with few, if any, obvious crossovers detectable (Fig. 1b and Supplementary Fig. 5). This implies that haploids did not arise through conventional meiosis, which usually requires at least one crossover per chromosome\textsuperscript{31}. The few crossovers detected probably arose by mitotic recombination before selection for LOH events. For example, Haploid I was isolated following selection for loss of GAL1, which is within the crossover region on chromosome 1. Furthermore, in C. albicans haploids (Fig. 1b and Supplementary Fig. 5), the majority of disomic chromosomes were heterozygous, which would arise after mis-segregation of homologues in meiosis I. Such meiosis I mis-segregation is not seen in Drosophila male meioses, which lack recombination\textsuperscript{32}; it is seen in Candida lusitaniae meiotic progeny, which undergo high levels of recombination\textsuperscript{33}. The low level of genetic recombination together with the presence of heterozygous disomic chromosomes remains most consistent with random homologue segregation in C. albicans. Accordingly, we propose that haploids arise through a concerted chromosome loss mechanism similar to that described for the diploid–tetraploid C. albicans parasexual cycle\textsuperscript{23}, although a non-conventional meiotic program cannot be completely discounted.

The existence of haploids refutes the argument that recessive lethal alleles are present on each C. albicans homologue\textsuperscript{11,14}. Nonetheless, the absence of some chromosomal homologues from the haploid progeny indicates that recessive lethal alleles may exist. Indeed, only the ‘b’ homologues for chromosomes 3, 4, 6, 7 and most of chromosome 1 were detected in the haploids analysed (Fig. 1b and Supplementary Fig. 5). Interestingly, a similar homologue bias was seen in diploid parasexual progeny derived from SC5314 (ref. 18). In contrast, homologues of chromosome 5, which carries the MTL, appeared in equal numbers (six MTL\textalpha and five MTL\textbeta) and were entirely of one or the other parental homologue. Similarly, both homologues of chromosomes R and 2 were observed. As such, homologues from more than half the chromosomes (chromosomes 3, 4, 6, 7 and most of chromosome 1) potentially carry at least one recessive lethal mutation and may limit the frequency with which viable haploids arise. Accordingly, we propose that haploidization may provide an effective mechanism for eliminating recessive lethal mutations from the predominantly diploid populations of C. albicans.

Auto-diploidization of haploids

In the course of these studies, prolonged propagation of haploid isolates yielded cultures with a mixed population of haploid and diploid cells (Fig. 1c). Subsequent colony purification yielded distinct haploid and/or diploid populations (Fig. 1d). Surprisingly, some haploid isolates also diploidized during the process of strain shipping, which involved storage, transit and revival from partial dehydration on a solid surface. For example, a potential haploid isolate identified in Taiwan was sent to Minnesota on sterile filter paper. Once revived and grown in liquid culture, the genome was diploid (Fig. 1e) and homozygous for all SNPs (Fig. 1b, ‘auto-dip.’), indicating auto-diploidization from a haploid phase. Whereas homozygous diploids could arise through mitotic defects or by self-mating\textsuperscript{34}, mating between cells of the same mating type was not detectable, as discussed below. Thus, we suggest that auto-diploids arise through mitotic defects that may be analogous to the auto-diploidization events in vertebrate haploid stem-cell cultures\textsuperscript{35–37}.

Haploid morphogenesis and mating

Consistent with reduced ploidy content in other yeast species\textsuperscript{38}, haploid C. albicans cells were smaller, on average, for both cell and nuclear size compared to diploid cells (Fig. 2a and Supplementary Fig. 6). The
**Figure 2** | Morphology and mating competency of haploid *C. albicans*. 

**a.** Representative differential interference contrast microscopy (DIC) images of haploid, diploid and tetraploid cells overlaid with fluorescence images of their nuclei. **b.** Calcofluor white staining revealed primarily cells with the axial budding pattern, 15% with a bipolar budding pattern (n = 72) and 3% that were difficult to resolve. White arrows, previous bud scars; black arrow, newest bud. Scale bars, 5 μm. **c.** Haploids form true hyphae, pseudohyphae and chlamydospores in serum, RPMI and corn meal agar media, respectively. **d.** White-opaque switching detected as pink colony sectoring (top) and by microscopy of cells from white and pink/opaque sectors. Diploid, MTLα/MTLα; Haploid I, MTLα; Haploid IV, MTLα. α, Mating between haploid cells. ‘Parents’: Haploid I (MTLA NAT1 ade2Δ4), Haploid II (MTLA ADE2), Haploids III and IV (MTLA ADE2) showing growth on media indicated. ‘Crossed with Haploid I’: opaque (Op) or white (Wh) cells from Haploid I were mixed with opaque and/or white cells from Haploids II, III or IV and plated to medium selective for mating products. Ade, adenine; NAT, nourseothricin; YPAD, yeast peptone dextrose media with adenine.

**Figure 3** | Haploid growth in vitro and in vivo. 

**a.** Growth (doubling times in YPAD) of control diploid, haploids (pink), their auto-diploid derivatives (green), and heterozygous, mating products 1 × III and 1 × IV (purple). Error bars reflect one standard deviation from the mean. *P < 0.01, **P < 0.001, Student’s t-test. b.** Survival of mice (tail vein systemic candidiasis model) following inoculation with Haploid II (pink) or its diploid progenitor, YJB12419 (grey). c. Recovery of colony-forming units (CFUs) from mouse kidneys (three mice per yeast strain) 48 h post-infection.
would not re-establish complete genome heterozygosity. Thus, even though the formation of haploids potentially eliminates lethal mutations, continued inbreeding between related haploids would perpetuate homozygosity of most chromosomes and consequently would reduce overall fitness.

Consistent with reduced growth rates in vitro, Haploid II was avirulent in a mouse model of systemic candidiasis (Fig. 3b) and was cleared from the mouse after 10 days. However, after 48 h of infection with a haploid strain, cells could be recovered (Supplementary Fig. 9a) and the majority of these isolates remained haploid. Subsequent in vivo experiments compared the colony-forming units (CFUs) of haploids, auto-diploids and SC5314 isolated from mouse kidneys 48 h post-inoculation in individual and direct competition experiments (Fig. 3c and Supplementary Fig. 9b). The number of CFUs recovered from mice inoculated with haploids and auto-diploids was several orders of magnitude lower than the number of CFUs recovered from mice inoculated with SC5314 (Fig. 3c). Furthermore, competition experiments indicated that haploids and auto-diploids showed similar fitness in vivo, as both of these forms were outcompeted by heterozygous diploids (Supplementary Fig. 9b). Similar to growth rates in vitro, the low fitness of haploids and their corresponding auto-diploids in vivo indicates that it is not the diploid state per se that is beneficial to growth, but rather extensive allelic heterozygosity, as shown with SC5314. From these data, we propose that haploid formation may not be rare, but that haploids probably represent a very small fraction of the overall population owing to their low competitive fitness relative to heterozygous diploids.

Haploid strains as genetic tools

The haploid state greatly facilitates experimental approaches such as classical genetic screens for recessive alleles and a single round of gene-knockout phenotyping. To illustrate this potential, a set of auxotrophic C. albicans strains was derived from a relatively stable haploid, GZY792 (Fig. 4a and Supplementary Fig. 10), and genes important for morphogenesis in diploids (HGC1, RVS167, SLA1, SEC3 and ACE2) were deleted in a single step (Fig. 4c). The resulting mutants showed morphogenesis defects closely resembling those of the corresponding diploid null mutants (Fig. 4d)13-16. Furthermore, because the deleted genes map to all eight chromosomes (Fig. 4c), the ability to delete each gene in a single step confirms that all chromosomes were monosomic in the parental haploid.

Concluding remarks

In summary, C. albicans can no longer be considered to be an obligate diploid. Rather, it has the ability to form haploids that subsequently mate to form diploids or undergo auto-diploïdization. The lower fitness of haploids and auto-diploids suggests they will not persist in the population and thus should be detected only rarely. Nonetheless, efficient mating between viable haploids can produce heterozygous diploïds that have increased fitness, presumably owing to the complementation of detrimental recessive alleles. Furthermore, the reduction to a haploid state can serve as a vehicle to eliminate recessive lethal alleles from a heterozygous diploid population. We propose that C. albicans rapidly generates genetic diversity by producing a broad range of different ploidy states, including haploid, diploid, tetraploid and aneuploid25. Each of these ploidy states are mating competent and genetic outcrossing can introduce, albeit infrequently, further genetic diversity into the population. Whereas the ploidy reduction mechanism(s) used by C. albicans remain elusive, the discovery of a haploid form and the potential for a haploid-diploid parasexual cycle significantly expands our ability to manipulate, and thereby better understand, this opportunistic pathogen. Furthermore, this study reveals how whole-genome analyses can lead to a re-evaluation of common assumptions about genomic structure in microbial organisms.

Figure 4 | Auxotrophic haploid strains enable one-step gene deletions. a, Series of strains constructed from a stable haploid isolate, GZY792 (MTLa, his4), which was isolated after propagation for 30 passages, screening for ploidy by flow cytometry and selection of isolates that were consistently haploid. GZY803 (ura3Δ) was constructed by disruption of URA3 with HIS4. Other auxotrophies were generated by the URA-flipper approach26. b, Flow cytometry of these auxotrophic strains. c, Genes disrupted in one-step map to all eight chromosomes. Circles, centromere position. d, Cell morphology phenotypes of haploid mutants grown in minimal media (yeast) or media supplemented with 20% FBS at 37 °C (hyphae) are similar to phenotypes seen for the corresponding diploid null mutants.

METHODS SUMMARY

For full details please refer to the Supplementary Methods. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals as defined by the National Institutes of Health (PHS Assurance #A3284-01) and National Advisory Committee For Laboratory Animal Research of Singapore (NAACLAR). Animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Brown University and ASTAR (#110686). All animals were housed in a centralized and AAALAC-accredited research animal facility that is fully staffed with trained husbandry, technical and veterinary personnel. Haploid isolates recovered from mouse models of bloodstream and oropharyngeal infections were identified by plating dilutions of kidney or tongue tissue homogenates onto YPD and onto 2-DOG medium to identify Gal+ cells. Colonies from YPD and 2-DOG plates were analysed by flow cytometry to determine cell ploidy. For preparation of samples for flow cytometry, cells were collected during mid-log phase, fixed with ethanol, stained with SybrGreen1 and analysed using a FACScaliber. Mating+, in vitro growth+ and in vivo+ assays were performed as previously described.

Full Methods and any associated references are available in the online version of the paper.

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**METHODS**

**Haploid screening from the mouse models of infection.** Bloodstream infections were performed by injecting $10^6$ cells of parent strain AF7 (gal1Δ::URA3/GAL1), into the tail vein of 13 outbred ICR male mice (22–25 g, Harlan)\(^2\). When moribund (at 5–7 days), mice were anaesthetized using isofluorane, euthanized, and both kidneys were removed. Kidneys were combined, homogenized with 1 ml of water. 1:1,000 dilutions of kidney homogenates were plated for total cell count onto YPD, and 1:10 dilutions of the same homogenate were plated onto 2-DOG medium to obtain Gal\(^+\) colony counts at 3 days.

For the oropharyngeal model of infection\(^2\), Balb/C mice were immunosuppressed with cortisone on days 2, 1 and 3 of infection. Calcium alginate swabs were saturated with a suspension of $10^6$ cells per ml of YJB9318 (gal1Δ::URA3/GAL1) under their tongues for 75 min. Mice were euthanized at 1, 2, 3 and 5 days post-infection. 1:1,000 dilutions of tongue tissue homogenates were plated for total cell count onto YPD, and 1:10 dilutions of the same homogenate were plated onto 2-DOG medium to obtain Gal\(^+\) colony counts at 3 days. Importantly, to confirm that 2-DOG resistant cells only arose during in vivo passage and were not selected for by plating on 2-DOG medium, a gal1/gal1 mutant and a GAL1/gal1 heterozygote strain were plated onto 2-DOG medium and observed for growth. 2-DOG resistant colonies grew up from the gal1/gal1 mutant but not from the GAL1/gal1 heterozygote by day 3 after plating\(^2\). Therefore, we used a cut-off of day 3 for picking 2-DOG resistant colonies after plating.

Approximately 300 colonies were transferred from YPD plates and ~740 colonies from 2-DOG plates (312 from OPC and 431 BSI isolates) into 96-well plates containing 50% glycerol and stored at −80 °C. All C. albicans cells isolated following in vivo passaging were analysed by flow cytometry to determine cell ploidy.

**Flow cytometry preparation and analysis.** Mid-log phase cells were collected, washed and resuspended in 50:50 TE (50 mM Tris, pH 8: 50 mM EDTA) and fixed with 95% ethanol. Cells were washed with 50:50 TE and treated with 1 mg ml\(^{-1}\) RNase A and then 5 mg ml\(^{-1}\) proteinase K. Cells were washed with 50:50 TE and resuspended in SybrGreenI (1:85 dilution in 50:50 TE) incubated overnight at 4 °C. Stained cells were collected and resuspended in 50:50 TE and analysed using a FACScaliber. Whole-genome ploidy was estimated by fitting DNA content data with a multi-Gaussian cell cycle model that assumes the G2 peak has twice the fluorescence of the G1 peak and that minimizes S-phase cell contribution to the error function. Ploidy values were calculated by comparing the ratio of peak locations in experimental samples to those of diploid and tetraploid controls.

**Mating assays.** Opaque or white cells were mixed together in equal cell numbers and incubated on Spider media\(^3\) for 18 h before replica-plating onto SDC − Ade + NAT (to select for mating products), as well as YPAD, YPAD + Nat, SDC − Ade and SDC − Ade + NAT to detect parental auxotrophies and then photographed 24 h later.

**In vitro growth assays.** Strains were grown in YPD media supplemented with adenine, uridine and histidine in a 96-well microtitre plate and absorbance at 600 nm was measured every 15 min with a plate reader (Tecan Sunrise) for 24 h. Doubling times were calculated as previously described\(^3\).

**In vivo assays.** For virulence assays, eight mice per C. albicans strain were inoculated with $6.0 \times 10^5$ CFUs by tail vein injection\(^4\). For survival assays, three mice per strain were inoculated by tail vein injection, both kidneys were collected at 48 h and CFUs were determined by plating on YPAD. Ploidy of randomly selected isolates was determined by flow cytometry.
Corrigendum: The ‘obligate diploid’ *Candida albicans* forms mating–competent haploids

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In this Article, there were two errors in allele assignments. In the legend and colour key to Fig. 1b, the allele identity (‘a’ and ‘b’) is inadvertently reversed. Figure 1 of this Corrigendum shows the corrected panel b and legend for the original Fig. 1. A similar error is present in the colour key to Supplementary Fig. 5 (its legend is correct). The Supplementary Information to this Corrigendum shows the corrected Supplementary Fig. 5.

**Figure 1** | This figure shows the corrected panel b of the original Fig. 1. SNP/CGH array analysis of indicated strains (left) showing copy number (log₂ ratio; black) and SNP allele information (grey, heterozygous; cyan, allele ‘a’; magenta, allele ‘b’; white, no SNP data).

**Supplementary Information** is available in the online version of the Corrigendum.