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Large-Scale Chromosomal Changes and Associated Fitness Consequences in Pathogenic Fungi

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Abstract

Pathogenic fungi encounter many different host environments to which they must adapt rapidly to ensure growth and survival. They also must be able to cope with alterations in established niches during long-term persistence in the host. Many eukaryotic pathogens have evolved a highly plastic genome, and large-scale chromosomal changes including aneuploidy, and loss of heterozygosity (LOH) can arise under various in vitro and in vivo stresses. Both aneuploidy and LOH can arise quickly during a single cell cycle, and it is hypothesized that they provide a rapid, albeit imprecise, solution to adaptation to stress until better and more refined solutions can be acquired by the organism. While LOH, with the extreme case of haploidization in *Candida albicans*, can purge the genome from recessive lethal alleles and/or generate recombinant progeny with increased fitness, aneuploidy, in the absence or rarity of meiosis, can serve as a non-Mendelian mechanism for generating genomic variation.

Keywords

Fungal pathogens; *Candida*; *Cryptococcus*; Aneuploidy; Loss of heterozygosity; Fluconazole resistance; Genome plasticity; Adaptation; Fitness

Introduction

A multitude of microbial species inhabit the human body, and they have evolved mostly as commensals inhabiting different niches in their hosts. While impaired host immune function allows some commensal and noncommensal species, such as *Candida* and *Cryptococcus*, to become opportunistic pathogens, these species also must possess characteristics that facilitate the transition from a harmless commensal to an aggressive pathogen [1, 2]. During the course of infection, the pathogen encounters many different host environments to which it must adapt rapidly to ensure growth and survival. Furthermore, it must be able to cope with alterations in established niches during long-term persistence in the host [3–5].

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Compliance with Ethics Guidelines

Human and Animal Rights and Informed Consent All studies by the author involving animal subjects were performed after approval by the appropriate institutional review boards.

Understanding the mechanisms by which the transition from commensal to pathogen takes place, and the relative contributions of host and pathogen factors in this tightly interwoven interaction, represents a continuing challenge. Most studies have focused on transcriptional analysis of host–pathogen interactions [6–11], and have identified systems for detecting and responding to changes in temperature, carbon source, metal ion availability, pH and gas tension [10, 12, 13]. While these studies investigated the different gene products expressed by commensal and pathogenic isolates, they do not provide an explanation as to how the transition from one state to the other occurs.

Recent studies in nonpathogenic and pathogenic fungi have shown that genome instability caused by large-scale chromosomal changes including gross chromosomal rearrangements (GCRs), supernumerary chromosomes (SNCs), aneuploidy and loss of heterozygosity (LOH) are triggered by in vitro and in vivo stresses [14–16, 17•, 18, 19•, 20]. Most of these changes cause fitness reduction or have no effect on fitness [21–23], consistent with the general observation that many aneuploid strains exhibit reduced growth rates [22, 24]. Nonetheless, some of these large-scale chromosomal changes are beneficial, causing improved adaptation and fitness of the fungus [25–28]. In this review, I first introduce the different types of large-scale chromosomal changes with the focus on ploidy changes and LOH. Then I address the effect of these changes on fungal fitness in general and fluconazole resistance in particular, with an emphasis on *Candida* and *Cryptococcus* species.

Types of Large-Scale Chromosomal Changes

Two major types of large-scale chromosomal changes that can arise from genome instability are aneuploidy and LOH. LOH plays a major role in diploid species such as *Candida albicans* and *Cryptococcus* hybrids, generating genetic diversity at the population level [16, 19•, 29•, 30–33]. There are three types of LOH: short-range LOH (mitotic recombination of short chromosome domains due to gene conversion or double crossovers); long-range LOH (longer regions of a chromosome arm from the site of recombination to the telomere due to a single crossover event and subsequent segregation of the homologs or due to break-induced replication) [34, 35]; and whole chromosome LOH (thought to arise via chromosome nondisjunction events during mitosis due to improper/unequal segregation of the daughter chromosomes) [36]. LOH arises frequently in somatic cells (about 10^{-6} /cell division) [17•, 37], and is elevated after exposure to oxidative, temperature, and antifungal drug stress, as well as after passage through the host [16, 19•, 38].

Aneuploidy, which is defined as an unbalanced number of chromosomes, is widespread in the pathogenic fungi especially in *C. albicans*, *C. glabrata*, *Cryptococcus neoformans* and *C. gattii* [39–43]. Aneuploidy usually involves entire chromosomes and/or chromosomal segments [16, 20, 44•]. Cellular stresses caused by exposure to the host environment, heat shock, transformation and exposure to antifungal drugs all can induce aneuploidy [16, 20, 45–48]. Mechanistically, aneuploidy can arise via several routes including improper/unequal segregation of sister-chromatids during mitosis or meiosis and defects in double-strand break repair [23, 49]. Aneuploid cells can also arise from unstable tetraploid intermediates (e.g. tetraploid mating products in *C. albicans*) via a nonmeiotic process

termed “concerted chromosome loss” [33, 50]. Surprisingly, *C. albicans* is quite tolerant of aneuploidies [33, 48], and can be stable in its haploid state [19••].

Both aneuploidy and LOH can arise rapidly within a few cell cycles. They provide a rapid, albeit imprecise, solution for adaptation to stress until more refined solutions can be acquired by the organism. However, while aneuploidy is a reversible process, LOH is not, as chromosome loss results in homozygosity of all genes in the region. The one way to regain the diversity lost during homozygosity is for the cell to outcross to regain the lost heterozygous alleles. The frequency with which such outcrosses occur is not known.

Candida Albicans

Fitness Consequences of LOH

Candida albicans is a predominantly diploid, highly heterozygous fungus [51, 52] with a few large regions of homozygosity on several chromosome arms [51]. Early population genetic studies indicated that *C. albicans* is a primarily clonal organism that propagates by mitosis [53–55]. In predominantly clonal organisms, heterozygosity is “fixed”, and this results in high genetic diversity at individual loci but in lower numbers of different genotypes within populations [56]. The fact that *C. albicans* has maintained a high level of heterozygosity as well as high genotypic diversity within populations is therefore quite puzzling [53, 55, 57]. It suggests that perhaps heterozygosity itself is advantageous [58] or that limited outcrossing, which has yet to be identified in this species, maintains the high genetic diversity. Another possible mechanism for the generation of genotypic and phenotypic variation is the parasexual mating program [33, 50], and the parasexual cycle coupled with concerted chromosome loss could serve as a capacitor for increasing genotypic and phenotypic variation [59].

Since the acquisition of novel heterozygosity is only possible via outcrossing, recent studies have started to study the process of LOH, and have begun to analyze the frequency and types of LOH, the contribution of LOH to genetic diversity at the population level, and how LOH affects the fitness of the fungus [17••, 19••, 60]. There are many processes that can affect LOH rates and types in *C. albicans*. For example, the frequency of LOH is enhanced by passage through a murine model of infection [16], indicating that it occurs in response to in vivo signals. In vitro, environmental stress also increases LOH: exposure to oxidative stress, febrile temperatures, or fluconazole all increase LOH in derivatives of strain SC5314 [17••]. Oxidative stress increases the proportion of short-range LOH, while high temperatures or fluconazole increase the proportion of whole chromosome LOH. This presumably reflects the nature of the different stresses. Oxidative stress introduces DNA breaks that must be resolved via recombination, whereas heat shock and fluconazole presumably exhibit a stronger effect on chromosome segregation mechanisms. Whole chromosome LOH also is prevalent in parasexual progeny [33, 50], and could be a consequence of the stress conditions (high glucose or sorbose) used to induce concerted chromosome loss [33, 59].

LOH can have profound effects on clinically relevant phenotypic traits including increased drug resistance. In *C. albicans*, resistance can be altered due to LOH of *TAC1*, which

encodes a transcription factor that upregulates the ABC-transporter genes *CDR1* and *CDR2* [61], or *MRR1*, which encodes a transcription factor that regulates expression of the Mdr1 multidrug resistance transporter [62]. For both *TAC1* and *MRR1*, hyperactive mutant alleles increase the expression of the corresponding transporters, and homozygosity of these alleles therefore increases drug resistance. LOH after the acquisition of a point mutation also has been detected at *ERG11* [63], which is the direct target of fluconazole, and at *UPC2*, which encodes a transcription factor that upregulates *ERG11* expression [64]. Thus, LOH of mutant alleles appears to be a common mechanism by which strains can acquire stable drug resistance.

An extreme case of whole genome LOH (and aneuploidy) was recently reported by Hickman et al. [19••]. Viable haploid forms of *C. albicans* were isolated from both in vitro and in vivo experiments. Analysis by flow cytometry and comparative genome hybridization (CGH) confirmed that true haploid cells are monosomic for each of the eight chromosomes of *C. albicans*. It had been thought that *C. albicans* was an obligate diploid because recessive lethal alleles on multiple chromosomes caused inviability of haploids; the surprising result was that *C. albicans* cells can indeed tolerate homozygosity on all eight chromosomes. However, a bias of homozygosity for certain chromosomes was apparent, as strains homozygous for the 'a' homolog of chromosomes 1, 4, 6, and 7 have not yet been recovered [17••, 19••, 33], suggesting that lethal recessive alleles may exist on these homologs [19••].

All haploids grow significantly more slowly than the diploid heterozygous progenitor strain (strain SC5314). The progenitor is also more fit than the diploid mating products, which could be due to incomplete restoration of genome heterozygosity in the related haploid mating products (inbreeding depression). Importantly, autodiploids (homozygous diploids that arose from the haploids) have similar growth rates to the haploids, suggesting that recessive alleles within the strain, rather than haploidy per se, limit fitness. Interestingly, and similar to hybrid vigor seen in AD hybrids in *C. neoformans* (see below), the mating products grow significantly more quickly in rich medium than either haploid parent. Similar to growth in vitro, haploids and their autodiploids showed similar, lower fitness in a model of hematogenously disseminated candidiasis [19••], with both being outcompeted by the heterozygous diploid progenitor. This strongly suggests that it is not the diploid state of the cell but rather the level of heterozygosity that determines fitness, and supports the hypothesis that heterozygosity in *C. albicans* is maintained because of its advantage in adaption to the mammalian host.

Fitness Consequences of Aneuploidy

Until recently, aneuploidy was difficult to detect and was mostly inferred via semiquantitative methods (e.g. CHEF-Southern [20, 65]). The development of new genotyping methods such as CGH coupled with flow cytometry [66, 67] has facilitated the detection of aneuploidy and the new conclusion that aneuploidy is much more frequent than originally thought. Monosomic strains tend to be less fit under nonselective conditions and commonly reduplicate the monosomic chromosomes [19••, 47]. Surprisingly, at least some aneuploidies are quite stable in *C. albicans*.

Aneuploidy of specific chromosomes can result in phenotypic changes [68], a phenomenon recently reported for natural *Saccharomyces cerevisiae* isolates as well [44••]. For example, GCRs of chromosomes 2 and 6 allow *C. albicans* to grow better on D-arabinose [69], and monosomy of chromosome 5 enables it to grow on sorbose as the sole carbon source [47, 70]. Trisomy of chromosome 3 appears to be sufficient to confer thermotolerance in evolved *S. cerevisiae* [27]. Perepnikhatka et al. originally reported an association between aneuploidy and fluconazole resistance, in which very high levels of the drug yielded strains with different aneuploidies [46]. More recent studies of multiple clinical isolates by CGH have revealed a direct connection between drug resistance and the presence of isochromosome 5L [i(5L)] [20]. The isochromosome consists of the two left arms of chromosome 5, and contains the *ERG11* and *TAC1* genes, encoding the target of fluconazole and a transcriptional regulator of drug efflux pumps, respectively. The isochromosome can be lost when isolates are cultured in drug-free medium, demonstrating that the isochromosome is responsible for resistance [20]. Furthermore, the increased copy number of *ERG11* and *TAC1* is sufficient to explain the levels of azole resistance conferred by i(5L) [20, 71]. To ask whether aneuploidy-acquired drug resistance is associated with a fitness cost, the genomic structure of strains experimentally evolved with and without fluconazole was investigated [25]. An i(5L) arose in all drug-treated populations within a few generations. All clones with i(5L) were fitter in fluconazole than the non-i(5L) clones, which showed fitness comparable to the progenitor strain. Many isolates carrying i(5L) had acquired other aneuploidies on chromosomes that also harbor genes implicated in drug resistance, and the accumulation of aneuploidies correlated with the highest minimum inhibitory concentration. Importantly, extra chromosomes did not impair fitness either with or without fluconazole. Another interesting finding was the appearance of a SNC that consisted of an i(5L) and the right arm of chromosome 3 (termed i(5L)-3R). Surprisingly, this SNC incurred a fitness cost since isolates carrying it grew more slowly with and without the drug.

LOH and aneuploidy are often found within the same isolates [33, 66], and hence both could potentially alter the fitness of strains to varying degrees. Single nucleotide polymorphism/CGH arrays revealed multiple aneuploidies and LOH events that had been acquired during numerous rounds of yeast transformation [48] and/or strain handling [39]. To distinguish between the effects of LOH and aneuploidy on fitness, doubling times were determined in strain SC5314 and its derivatives under standard laboratory conditions. Significantly increased doubling times were observed in aneuploid strains compared to a euploid control, and even small aneuploidies (about 40 kb) resulted in increased doubling times. Large LOH tracts (>100 kb) also resulted in increased doubling times while shorter LOH tracts did not. This was likely due to the specific alleles involved in LOH and not necessarily the size of the LOH tract [66].

In conclusion, *C. albicans* is exposed to constant selective pressure in its natural environment, the human host. Its ability to quickly acquire large-scale genomic changes such as LOH and aneuploidy to adapt to adverse conditions may contribute to its success as both a commensal and an opportunistic pathogen. More in-depth population-level studies, particularly under in vivo growth conditions, are required to identify specific LOH and

aneuploidy events that enable this fungus to survive and persist in its diverse host niches and/or to cause infection.

Candida Glabrata

Genome Plasticity, Drug Resistance and Aneuploidy

Candida glabrata has emerged as the second most common pathogenic *Candida* species, partly due to its inherited tolerance to the most commonly used antifungal drug fluconazole [41, 72, 73]. Unlike *C. albicans*, *C. glabrata* is normally haploid and more closely related to the nonpathogenic yeast *S. cerevisiae* [74]. The few multilocus sequence typing analyses of the population structure have demonstrated an overall clonal population structure with limited evidence of recombination [75]. Nonetheless, the *C. glabrata* genome exhibits high levels of genome plasticity [18, 75–78]. Karyotype analysis of ten isolates of the type strain CBS138 from different laboratory stocks have revealed GCRs, including translocations and possible aneuploidies [77]. Besides the apparent karyotypic variation, not much else is known about genotypic variation. The discovery of aneuploidy-associated acquired drug resistance (see below) supports the need to expand studies on genome plasticity and its underlying mechanisms in *C. glabrata* [18, 76].

In an early study, a patient carrying *C. glabrata* was treated with 400 mg fluconazole once daily over a period of 9 days, which resulted in an increased tolerance of this particular strain to the antifungal drug [79]. A matched set of drug-sensitive isolate (prior to drug treatment) and drug-tolerant isolate was then analyzed to elucidate possible mechanisms of increased drug tolerance. Both isolates were analyzed for uptake of the drug, sterol synthesis, and for NADPH-cytochrome *c* reductase activity. The increased ergosterol synthesis of the resistant isolate together with its decreased susceptibility to 14 α -demethylase inhibitors suggested higher constitutive P-450 levels or overexpression of the enzyme [80]. Interestingly, extensive subculturing (120 times) of the drug-resistant isolate led to the loss of resistance and P-450 levels comparable to those of the drug-sensitive isolate. In a follow-up study, Marichal et al. analyzed the expression of the cytochrome P-450 14 α -lanosterol demethylase in both isolates and detected an increase in copy number for *CYP51* (now *ERG11*) of about fourfold as well as mRNA levels eight times higher in the drug-resistant isolate [81]. The analysis of copy number by hybridization to chromosomal blots revealed that the increased expression of *ERG11* was caused by an amplification of the entire chromosome that harbors *ERG11* (chromosome E) [51]. In addition, a gradual loss of resistance during culturing in drug-free medium coincided with the loss of the extra copy of the duplicated chromosome [81].

Amplification and/or the formation of SNCs in *C. glabrata* have also been associated with an increase in virulence potential [18, 76]. Karyotype analysis of 15 isolates coupled with CHEF-Southern analysis using up to 100 gene probes revealed dramatic GCRs and two isolates with SNCs. Interestingly, the SNCs were composed of parts of larger chromosomes that harbor genes important for drug resistance such as *PDR5* (*MDR1* in *C. albicans*) and *ERG11* as well as other known virulence genes including secreted aspartic proteases and phospholipases [82, 83]. Furthermore, these isolates showed an increased resistance to fluconazole and the SNCs were lost during extended culture in drug-free medium [18].

Therefore, the generation of SNCs in *C. glabrata* may provide a fitness advantage under drug stress and may also be advantageous to its survival in the host.

Cryptococcus Neoformans

Loss of Heterozygosity in Diploids

Cryptococcus neoformans is typically a haploid, sexual species that has been classified into three serotypes based on an antibody-promoted slide agglutination assay of the polysaccharide capsule: serotypes A, D and AD [84]. Although the AD serotype was originally thought to be quite rare, a recent study has shown that 19% of cryptococcal infections in Europe are caused by AD hybrid strains [85]. AD hybrids are thought to have arisen through crosses between serotype A and D strains in nature but can easily be generated in the laboratory [32, 86]. Ploidy analysis of AD hybrids has shown that they are mostly diploid or aneuploid (between $1N$ and $2N$) [32]. CGH analysis of the genomes of A, D and AD strains has shown extensive variation among the three types, including regions of sequence divergence, deletions, and amplifications [87].

Li et al. recently demonstrated that AD hybrids are highly dynamic and undergo extensive chromosome loss, often followed by reduplication of the remaining homolog [30]. Interestingly, extensive LOH in AD hybrid strains results in preferential retention of the serotype A homolog of chromosome 1 [87]. Serotype A strains are generally more virulent and retention of the A homolog may therefore contribute to a selective advantage in the mammalian host [86].

To study the effect of ploidy, mating type and serotype on virulence and fitness, Lin et al. constructed AD hybrids with all possible mating type combinations. AD hybrids exhibited similar in vitro phenotypes regardless of their mating type configuration, yet compared to their haploid parents uniformly demonstrated hybrid vigor, and were more resistant to UV and higher temperatures [31, 86]. They found that higher ploidy had a minor adverse fitness affect on melanization, and on virulence in a murine inhalation model of cryptococcosis, which was independent of the genetic background of the hybrids [86]. Interestingly, the assessment of the virulence potential of AD hybrids produced contrasting results in various studies. For example, Cogliati et al. and Lengeler et al. found that, in a murine inhalation model of cryptococcosis, AD hybrids were less virulent than either A or D haploid strains [32, 88], while Lin et al. found that AD hybrids were as virulent as their more virulent haploid parent [31]. These contrasting results are likely the result of the different genetic backgrounds of the analyzed strains [31]. Taken together, the highly dynamic nature of AD hybrids, their hybrid vigor for many virulence factors, and the relatively high abundance among clinical and natural isolates demonstrate the advantage of hybridization in this species, and might be especially beneficial for the generally less virulent serotype D strains [31].

Aneuploidy and Titan Cells

An extreme case of aneuploidy has recently been described in *C. neoformans*. In response to the host, this pathogen forms so-called titan cells that can reach a diameter of up to about 20-fold that of the original cell (100 μm vs. 6 μm) [89]. The enormous size of these cells

correlates with an increase in ploidy, which can range from tetraploid all the way to 64 or more genome copies. Interestingly, the progeny of titan cells are of normal size and are therefore able to disseminate into other parts of the host such as the brain [89]. The mechanism by which titan cells reduce their ploidy in off-budding daughter cells remains to be elucidated. However, the fact that titan cells are uninuclear and the daughter cells are haploid suggests that endoreduplication might play a significant role [90••]. Titan cell formation has been seen in several host-associated environments including the mammalian lung, macrophages, and even in an invertebrate infection model [91, 92]. The fact that titan cells are most frequently isolated during asymptomatic pulmonary infection suggests that they provide a means of latent persistence within the host until conditions become favorable for infection, thereby increasing the virulence potential of the fungus. Indeed, Crabtree et al. have recently found in an intranasal model of cryptococcosis that *C. neoformans* mutants defective in titan cell formation show attenuated virulence compared to the wild-type strain. The attenuation of virulence is accompanied by a lower fungal burden in the lungs and decreased levels of dissemination to the brain [93]. This study therefore established titan cell formation as a novel virulence factor in *C. neoformans*.

Aneuploidy and Heteroresistance

The emergence of drug resistance, especially to the most commonly used antifungal drug fluconazole, has become a major problem in the clinic for *Candida* species. Several routes of fluconazole resistance acquisition have been described, including an increased production of multidrug transporters [63], mutations in the ergosterol biosynthesis pathway genes [94], genome amplification of regions containing *ERG* genes and/or transcription factors [20, 25], and the activation of Hsp90 [95]. Interestingly, in *Cryptococcus* species, bona fide fluconazole-resistant strains have been rarely reported [96], and a survey of clinical isolates revealed that both *C. neoformans* and *C. gattii* are naturally heteroresistant (reversible adaptive response to the presence of fluconazole) [72, 97••].

To determine the frequency and the mechanism of heteroresistance among isolates, Sionov et al. analyzed about 100 clinical and environmental isolates of *C. neoformans*. Surprisingly, heteroresistance was intrinsic to all isolates, and the level of heteroresistance was correlated with an increased tolerance to other stresses [72]. Interestingly, the level of fluconazole where heteroresistance manifested itself correlates with the level of virulence [72]. CGH analysis of a series of H99 in vitro-derived resistant isolates with varying levels of resistance to fluconazole demonstrated a positive correlation between increased drug resistance and the number of aneuploidies [43]. A disomy of chromosome 1, which harbors the genes encoding Erg11 and the major azole transporter Afr1, was common to all resistant isolates; this aneuploidy was also acquired during drug treatment in vivo [41, 43]. Semighini et al. went on to show that disruption of the apoptosis-inducing factor *AIF1* increased the frequency of subpopulations with chromosome 1 disomies that were fluconazole-resistant in vitro and in vivo [98]. Interestingly, *aif1* strains exhibited stable drug resistance even in the absence of the drug while *AIF1* wild-type strains did not [98]. Chromosome 4 aneuploidies also were linked directly to heteroresistance. Genes on this chromosome encode a GTPase and two GTPase activating proteins that are involved in the regulation of endoplasmic reticulum morphogenesis and trafficking [97••]. In this series of strains, aneuploidy was associated

with a fitness cost: strain H99^{R64} (resistant to fluconazole at 64 µg/ml) exhibited slower growth in vitro and reduced virulence in vivo [43]. The sequential loss of the duplicated chromosomes when passaged under drug-free conditions correlated with the return to the original level of drug tolerance [43].

Conclusions

It has become quite apparent that many eukaryotic pathogens have evolved a highly dynamic genome. High levels of genome plasticity result in aneuploidy and LOH under a multitude of conditions that enable the organism to rapidly adapt to its constantly fluctuating host environments, thereby providing time for more refined adaptation strategies. While LOH, with the extreme case of haploidization in *C. albicans*, can purge the genome of recessive lethal alleles and/or generate recombinant progeny with increased fitness, aneuploidy, in the absence or rarity of meiosis, can serve as a non-Mendelian mechanism to generate large-scale, rapid and reversible genomic variation.

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