Rapid Mechanisms for Generating Genome Diversity: Whole Ploidy Shifts, Aneuploidy, and Loss of Heterozygosity

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Human fungal pathogens can exist in a variety of ploidy states, including euploid and aneuploid forms. Ploidy change has a major impact on phenotypic properties, including the regulation of interactions with the human host. In addition, the rapid emergence of drug-resistant isolates is often associated with the formation of specific supernumerary chromosomes. Pathogens such as *Candida albicans* and *Cryptococcus neoformans* appear particularly well adapted for propagation in multiple ploidy states with novel pathways driving ploidy variation. In both species, heterozygous cells also readily undergo loss of heterozygosity (LOH), leading to additional phenotypic changes such as altered drug resistance. Here, we examine the sexual and parasexual cycles that drive ploidy variation in human fungal pathogens and discuss ploidy and LOH events with respect to their far-reaching roles in fungal adaptation and pathogenesis.

Ploidy change is a common attribute of eukaryotes and is often associated with sexual reproduction. During mating, fungal cells in one ploidy state typically conjugate with cells of identical ploidy, resulting in an overall doubling in genome size. Subsequent ploidy reduction can occur via meiosis, in which one round of DNA replication is followed by two successive rounds of DNA division, effectively halving DNA content in the cell. Ploidy changes can affect basic cellular properties such as cell

size, surface-to-volume ratio, genome stability, and transcriptional output (Galitski et al. 1999; Storchova et al. 2006; Otto 2007; Wu et al. 2010). Furthermore, many species preferentially exist in one ploidy state over another. This is exemplified by the model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, in which *S. cerevisiae* favors propagation in the diploid state, whereas *S. pombe* prefers to propagate in the haploid state (Gerstein and Otto 2009). Accordingly, *S. cerevisiae* cells show a

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transient haploid state, whereas *S. pombe* cells show a transient diploid state. The fitness advantages associated with propagation in higher or lower ploidy states, however, remain debated in the field (Gerstein et al. 2006; Gerstein and Otto 2009; Zörgö et al. 2013).

Cell ploidy changes can be introduced by mechanisms other than a conventional sexual cycle. For example, in *Candida albicans*, *Cryptococcus neoformans*, and some mammalian cell types, endoreduplication effectively doubles genome content via DNA replication without subsequent segregation of chromosomes (Puig et al. 2008; Lee et al. 2009; Ullah et al. 2009; Fox and Duronio 2013; Zielke et al. 2013). Parasexual reproduction, in which ploidy reduction occurs via a nonmeiotic mechanism, also occurs in several fungal species (Papa 1973, 1978; Geiser et al. 1998; Bennett and Johnson 2003; Lee et al. 2010; Dyer and O’Gorman 2012; Seervai et al. 2013). In *C. albicans*, mating between diploid cells of opposite mating type yields tet-

raploid cells (Hull and Johnson 1999; Hull et al. 2000; Magee and Magee 2000) that return to the diploid state by a parasexual program of “concerted chromosome loss,” yielding progeny cells with a wide variety of karyotypes (Bennett and Johnson 2003; Forche et al. 2008).

Aneuploidy also is frequently observed in fungal species and is defined as cells containing an unbalanced number of chromosomes. This includes both imbalances in the number of whole chromosomes and chromosome segments (Fig. 1). Interestingly, aneuploidy has been observed in somatic mammalian tissues including the liver and brain (Iourov et al. 2010, 2013; Duncan et al. 2012; Duncan 2013). Although the presence of aneuploid chromosomes often compromises fitness when measured under optimal growth conditions (Torres et al. 2008; Pavelka et al. 2010), aneuploidy promotes phenotypic variation and specific aneuploidies can confer growth advantages under specific conditions (Fig. 1). This is well docu-

Figure 1. Comparison of fitness attributes in a euploid diploid and aneuploid derivatives. *Upper panel:* (A) A euploid diploid with four chromosomes. Aneuploidies can occur in the form of supernumerary chromosomes (B), segmental aneuploidies (gains or losses) (C), or loss of whole chromosomes (D). *Lower panel:* In general, most aneuploid strains show fitness defects under replete (nonstressful) culture conditions relative to the euploid strain. Although any given aneuploidy is unlikely to provide a fitness advantage under a particular condition, specific aneuploidies may provide a fitness advantage under specific environmental stress conditions.

mented for *S. cerevisiae*, as well as for *C. albicans* and *C. neoformans* (Berman 2010; Pavelka et al. 2010; Sionov et al. 2010; Semighini et al. 2011; Sheltzer et al. 2011; Ni et al. 2013). Importantly, in multiple fungal species, specific aneuploidies are directly responsible for the drug-resistant phenotypes of clinical isolates (Selmecki et al. 2006, 2008; Sionov et al. 2010, 2013; Ngamskulrungs et al. 2012a). Overall, aneuploidy appears to be a conserved mechanism by which eukaryotic cells can rapidly adapt to stressful environments (Selmecki et al. 2006, 2008; Rancati et al. 2008; Pavelka et al. 2010; Yona et al. 2012; Zhu et al. 2012; Tan et al. 2013).

In this work, we review how sexual, parasexual, and asexual mechanisms contribute to karyotypic variation through ploidy shifts, aneuploidy, and loss of heterozygosity (LOH) events in human fungal pathogens. We also address the wide-ranging consequences of these changes for fitness of the fungus, resistance to environmental stress, and interaction with the human host.

PLOIDY SHIFTS VIA SEXUAL AND PARASEXUAL CYCLES

Ploidy Changes Mediated by Sex

The best-characterized mechanism of ploidy change is sexual reproduction, which is thought to have evolved once very early in the eukaryotic lineage. Thus, factors regulating meiosis and recombination are conserved from fungi to man (Keeney 2001; Ramesh et al. 2005; Schurko and Logsdon 2008). Our understanding of sex has been greatly bolstered by studies in unicellular model yeasts such as *S. cerevisiae* and *S. pombe*. In both species, mating occurs between haploid cells of opposite mating type, generating diploid products that subsequently undergo meiosis. Completion of meiosis is accompanied by sporulation and the formation of recombinant, haploid progeny (Fig. 2) (Yamamoto 1996; van Werven and Amon 2011).

In *S. cerevisiae* and *S. pombe*, as in most sexual fungal species, mating and meiosis are regulated by genes encoded at the mating-type (*MAT*) locus. This locus contains transcription

factors that are master regulators of cell-type specification, ensuring that only haploid cells of opposite mating type undergo conjugation and that only diploid cells are competent to undergo meiosis (Yamamoto 1996; Galgoczy et al. 2004; Bennett et al. 2005; Sherwood and Bennett 2009; van Werven and Amon 2011). Complete sexual cycles also have been established for two of the most prominent human fungal pathogens, the basidiomycete *C. neoformans* (Kwon-Chung 1975, 1976a,b) and the filamentous ascomycete *Aspergillus fumigatus* (O’Gorman et al. 2008) (for detailed reviews of sex in fungi, see Lee et al. 2010; Ni et al. 2011; Ene and Bennett 2014). In both pathogens, heterothallic mating between opposite mating types takes place, and mating is strictly regulated by nutritional cues.

In *C. neoformans*, pheromone signaling drives fusion of α and α cells and results in the formation of a heterokaryon, in which binucleate cells grow as filaments. Formation of the dikaryotic stage is dependent on the Sxi α /Sxi2 α homeodomain regulatory complex encoded at the mating locus (Hull et al. 2002, 2005; Kruzel and Hull 2010). Nuclei in the dikaryon subsequently fuse during basidium development to form four meiotic products, which then divide by mitosis to generate chains of basidiospores.

Interestingly, homothallic mating has been observed in a number of human fungal pathogens including *C. neoformans*. In this species, unisexual mating of α cells can produce α/α diploids, and subsequently results in the formation of recombinant haploid α progeny (Fraser et al. 2005; Lin et al. 2005). Homothallism has been observed in other human fungal pathogens as well (discussed below), indicating that it is a frequent mechanism used by pathogens to produce diversity. It is perhaps particularly beneficial for *C. neoformans* for which the vast majority of clinical and environmental isolates are of the α -mating type (Kwon-Chung and Bennett 1978; Casadevall and Perfect 1998).

In *A. fumigatus*, heterothallic mating between *MAT1-1* and *MAT1-2* cells results in the formation of sexual fruiting bodies (cleistothecia) containing ascospores. Progeny analy-

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Figure 2. Genomic changes that can accompany sexual, parasexual, and asexual processes. (A) Sexual mating and subsequent meiosis typically results in the formation of recombinant euploid cells, although aneuploidy also is observed in a subset of progeny. (B) Parasexual reproduction involves fusion of complementary cells (by mating or anastomosis) followed by concerted chromosome loss, resulting in the formation of many aneuploid forms in addition to euploid progeny. Recombination is less frequent than in meiosis. (C) Asexual mitotic divisions also can change genomic content, by either chromosome missegregation, endoreduplication, or titan cell formation. Subsequent (asexual) ploidy reduction is expected to include aneuploid products.

sis revealed extensive genetic recombination, a hallmark of conventional meiosis (O’Gorman et al. 2008). Mating was discovered following coincubation of parent cells for 6 months on parafilm-sealed oatmeal plates at 30°C in the dark (O’Gorman et al. 2008), showing the highly stringent conditions often required for fungal mating. Recently, more fertile mating strains of *A. fumigatus* have been identified (Sugui et al. 2011).

Multiple studies indicate that ploidy change itself is a potent modulator of both cell behavior and pathogenesis. Comparison of haploid and diploid *C. neoformans* cells found that haploid cells were generally more virulent than diploid cells in a murine inhalation model of cryptococcosis (Lin et al. 2008). However, in an alternative infection model, diploids were as virulent, if not more virulent, than corresponding

haploid forms (Toffaletti et al. 2004). In the rare human pathogen *Aspergillus nidulans*, a close relative of *A. fumigatus*, diploid strains were more virulent in mice than the corresponding haploid strains (Purnell and Martin 1973).

Ploidy Change Mediated by Parasexual Reproduction

Parasexual reproduction is defined as ploidy change without meiosis and is often accompanied by mitotic recombination. Parasexuality was first described by Guido Pontecorvo in the 1950s while investigating *A. nidulans* (Pontecorvo et al. 1953a,b; Pontecorvo and Sermonti 1954). In this species, rare fusion of hyphae can occur (anastomosis), resulting in the formation of a heterokaryon. Subsequently, fusion of haploid nuclei yields relatively stable diploid

cells, which can produce haploid recombinants by mitotic crossing-over and loss of whole chromosomes (Fig. 2). Mitotic recombination during this parasexual cycle therefore results in the nonsexual exchange of genetic material. Since the pioneering work of Pontecorvo, similar parasexual cycles have been found in other fungal species such as *A. fumigatus* (Berg and Garber 1962), indicating that some species can undergo both sexual and parasexual reproduction.

The potential benefits of a parasexual cycle have been elegantly shown by Schoustra and coworkers who revealed that extended culturing of *A. nidulans* diploid strains resulted in higher fitness than equivalent culturing of haploid strains (Schoustra et al. 2007). In this case, diploids accumulated recessive deleterious mutations that only became beneficial when “unmasked” in recombinant haploid populations. This illustrates the phenomenon of sign epistasis, in which mutations that are individually neutral or deleterious to the organism can be beneficial when present in combination. Diploid cells can act as a reservoir of such recessive mutations, and subsequent reduction to haploidy allows unmasking of these mutations, enhancing overall fitness. Parasex, and its ability to mediate changes in ploidy, can therefore promote adaptation even in the absence of a conventional sexual program.

C. albicans, the most prevalent human pathogen, also possesses a parasexual mating program. This species was long thought to be an obligate diploid with no capacity for mating or sexual reproduction. However, *C. albicans* undergoes both homothallic and heterothallic mating, in which diploid cells of the same or opposite mating type undergo conjugation to form tetraploid mating products (Hull et al. 2000; Magee and Magee 2000; Alby et al. 2009). Mating in *C. albicans* is regulated by the white-opaque phenotypic switch. White cells can reversibly transition to the opaque state and only cells in the opaque state are competent for mating (Miller and Johnson 2002). In addition, the white-opaque switch is regulated by transcription factors encoded at the mating-type-like (*MTL*) locus, so that only α or α cells

efficiently switch to the opaque state under standard growth conditions (Miller and Johnson 2002; Xie et al. 2013). Comparisons of diploid and tetraploid *C. albicans* cells showed that tetraploid strains display a modest defect in virulence compared to diploids (Ibrahim et al. 2005).

Perhaps the most striking aspect of the *C. albicans* mating cycle is the apparent lack of a true meiosis. The *C. albicans* genome contains orthologs of many genes that are associated with meiosis in other species (Tzung et al. 2001), and yet meiosis has not been observed (Bennett and Johnson 2003). Indeed, the related species *Candida lusitanae* is able to undergo meiosis despite having a more limited repertoire of “meiosis-specific” genes (Butler et al. 2009; Reedy et al. 2009). Recent studies revealed that mating and meiosis are coupled regulatory processes in *C. lusitanae*, thereby promoting propagation in the haploid state (Sherwood et al. 2014). In place of meiosis, *C. albicans* tetraploid cells return to the diploid state via a parasexual program of concerted chromosome loss (Bennett and Johnson 2003). Thus, when cultured on certain media, tetraploids show genome instability, reducing their ploidy to that of a diploid, or near-diploid, cell. Genetic recombination between chromosome homologs is observed during chromosome loss, although the frequency of recombination is considerably lower than that during a conventional meiosis (Forche et al. 2008).

Multiple steps in the *C. albicans* parasexual cycle, including the white-to-opaque switch, sexual conjugation, and concerted chromosome loss, are stimulated by environmental stress or nutrient-poor conditions (Bennett and Johnson 2003; Ramírez-Zavala et al. 2008; Alby and Bennett 2009; Huang et al. 2009). This raises the possibility that parasex may act as a generator of genetic diversity precisely when adaptation to the environment is most beneficial (Berman and Hadany 2012). Recently, the related fungal pathogen *Candida tropicalis* was found to undergo a parasexual cycle similar to that in *C. albicans*. This includes white-opaque switching, efficient sexual conjugation between opaque cells, and concerted chromosome loss to achieve a diploid–tetraploid–diploid life cy-

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cle (Porman et al. 2011; Xie et al. 2012; Seervai et al. 2013).

In a surprising recent development, viable haploid forms of *C. albicans* were isolated from both in vitro and in vivo experiments (Hickman et al. 2013). Analysis by flow cytometry and comparative genome hybridization (CGH) confirmed that true haploid cells were formed that were monosomic for each of the eight chromosomes of *C. albicans*. Haploids were originally thought to be inviable because of the presence of recessive lethal alleles on multiple chromosomes. Although viable haploids exist, they show marked defects in fitness and virulence, presumably caused by loss of beneficial alleles from the heterozygous diploid. The formation of haploid cells appears to occur via parasexual chromosome loss rather than via meiosis, as haploid progeny showed few, if any, signatures of chromosome crossovers (Hickman et al. 2013). Haploids also spontaneously auto-diploidized and the resulting auto-diploids showed the same fitness defects as those of haploids, implying that decreased fitness was not a consequence of haploidy per se (Hickman et al. 2013). Despite their limited fitness, haploids showed phenotypic properties similar to those of diploid cells, including white-opaque switching, filamentation, chlamydospore formation, and mating. Thus, *C. albicans* haploids represent a potentially exciting tool for future genetic studies of the species.

Meiosis versus Parasex

What mechanistic differences distinguish fungal meiosis and parasex? Are these processes fundamentally different or are they variations on the same theme? And could there be advantages to undergoing parasex in place of meiosis? In this section, we compare the programs of meiosis and concerted chromosome loss and emphasize important differences between these two processes.

Several characteristics distinguish fungal meiosis from parasexual chromosome loss. First, meiosis is a complex developmental program, whereas parasex does not involve the same degree of complexity. For example, in

S. cerevisiae and *S. pombe*, signals from the mating locus, as well as environmental cues, converge onto the promoter of a master regulator of meiosis, Ime1 and Ste11, respectively (for reviews, see Sherwood and Bennett 2009; Neiman 2011; van Werven and Amon 2011). In contrast, parasexual chromosome loss in *C. albicans* and *A. nidulans* is mating-type locus independent, and there is no evidence for involvement of a master transcription factor (Bennett and Johnson 2003; Alby et al. 2009; Dyer and O’Gorman 2012).

Second, nutritional signals tightly regulate sexual programs, but not parasex, by controlling expression of the master transcriptional regulators. In both *S. cerevisiae* and *S. pombe*, nitrogen starvation promotes entry into meiosis, whereas the presence of glucose inhibits meiosis. In contrast, there is no evidence that defined nutritional signaling pathways activate parasexual chromosome loss, although environmental stress increases the frequency of chromosome loss in both *C. albicans* and *C. tropicalis* (Bennett and Johnson 2003; Alby and Bennett 2010; Berman and Hadany 2012; Seervai et al. 2013).

A third major difference between meiosis and parasex is that chromosome dynamics are highly coordinated during meiosis but are not obviously coordinated in parasex. Homologous chromosomes segregate during meiosis I, whereas sister chromatids split during meiosis II, such that meiosis II closely resembles a mitotic division. Coordinated movement of chromosomes has not been reported during parasex, and current models suggest that ploidy reduction occurs by chromosome nondisjunction, leading to an uneven segregation of chromosomes during cell division. Furthermore, in studies of parasex in *A. nidulans*, “haploidization” often is promoted by the addition of microtubule-destabilizing drugs to disrupt normal chromosome segregation (Schoustra et al. 2007). As a direct result of these processes, parasex often results in the formation of aneuploid progeny with varied phenotypes (Fig. 2, and discussed below).

Fourth, there is a marked difference between the levels of homologous recombination that occur during meiosis and parasex. In meiosis

I, recombination between homologous chromosomes leads to high levels of meiotic recombinants (for detailed reviews, see Petronczki et al. 2003; Keeney and Neale 2006; Miller et al. 2013). Meiotic recombination is dependent on the processing of DNA double-strand breaks introduced by the conserved endodeoxyribonuclease, Spo11 (Keeney 2008). In *S. cerevisiae*, each meiotic progeny experiences 140–170 double-strand breaks per meiosis and all chromosomes experience at least one genetic crossover (Buhler et al. 2007; Mancera et al. 2008). In contrast, the frequency of mitotic recombination during parasex is significantly lower. In *C. albicans*, only a subset of parasexual progeny showed evidence of homologous recombination, and, even in these, progeny recombination events were limited to a small number of chromosomes (Forche et al. 2008). Similarly, in *A. nidulans*, mitotic segregation and recombination occurred at a lower level than that during conventional sexual reproduction (Pontecorvo et al. 1953b).

Fifth, many of the genes mediating meiotic recombination have been defined and are generally conserved in sexually reproducing species (Keeney et al. 1997; Keeney 2001; Villeneuve and Hillers 2001; Gerton and DeRisi 2002; Ramesh et al. 2005; Richard et al. 2005; Malik et al. 2008), but they are not required for parasexual chromosome loss. Thus, chromosome loss in *C. albicans* was not inhibited by the deletion of meiosis-specific genes such as *HOP1* or *DLH1* (ortholog of *DMC1*) (Bennett and Johnson 2003). It should be noted, however, that some fungal species undergo meiosis despite the absence of orthologs considered to be a prerequisite for meiosis (Butler et al. 2009; Reedy et al. 2009). Curiously, Spo11, the conserved meiotic endodeoxyribonuclease, was shown to be required for parasexual recombination in *C. albicans*, but not for chromosome loss per se (Forche et al. 2008). The requirement of Spo11 for parasexual recombination suggests intriguing links between parasex and a conventional meiosis despite the significant mechanistic differences between these programs.

A further difference between meiosis and parasexual chromosome loss is the presence

and absence of spore formation, respectively. Meiosis and sporulation are coupled processes in sexual fungi, ultimately yielding protective spores (Neiman 2011). Sexual spores have the potential to be highly antigenic, and the resulting immune response from the host could be detrimental to the pathogen (Nielsen and Heitman 2007). Interestingly, human-associated isolates of *S. cerevisiae* (including clinical isolates from immunocompromised patients) show a reduced frequency of sexual recombination relative to isolates from the environment (Magwene et al. 2011). This reduction in sexual activity could reduce the formation of spores and is also consistent with the hypothesis that sexual reproduction is disadvantageous for pathogens, as recombination may break up combinations of genes that are optimal for growth in the host (Nielsen and Heitman 2007).

Endoreduplication and Mitotic Collapse

Increases in fungal ploidy are often the result of conjugation between mating partners, yet ploidy increases also can occur by endoreduplication. In this process, replication of the DNA is uncoupled from mitotic division, resulting in an overall doubling of genome content. Endoreduplication has been observed in several human pathogens and provides an alternative mechanism for generating ploidy change and genotypic diversity (Fig. 2C) (Lee et al. 2009; Fox and Duronio 2013).

In *C. albicans*, classical studies showed that diploid strains could undergo spontaneous increases in ploidy, including the formation of triploid and tetraploid derivatives (Suzuki et al. 1982; Wickes and Petter 1996). Changes in ploidy were associated with accompanying differences in virulence (Suzuki et al. 1989). Also, as discussed earlier, in vitro passaging of *C. albicans* haploids can result in autodiploidization, a striking form of endoreduplication, to form homozygous diploids (Hickman et al. 2013).

In *C. neoformans*, a remarkable example of ploidy change via endoreduplication has been established. Populations of cells recovered from lung lavages included “titan” cells that were up

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to 100 μm in diameter, far larger than the typical *C. neoformans* cell (Fig. 2C) (Zaragoza et al. 2010; Okagaki et al. 2011; Okagaki and Nielsen 2012). Titan cells also formed during coculture with macrophages or following treatment with host phospholipids, forming tetraploids, octaploids, and even higher ploidy cells with as many as 64 copies of the genome (Zaragoza et al. 2010; Chrisman et al. 2011; Garcia-Rodas et al. 2011; Okagaki et al. 2011; Okagaki and Nielsen 2012; Zaragoza and Nielsen 2013). The large size of *C. neoformans* titan cells precludes their phagocytosis by host immune cells, and these cells also are resistant to oxidative and nitrosative stress. Titan cells may promote the establishment of latent lung infections; subsequently, the generation of smaller cells with lower ploidy could facilitate dissemination to the central nervous system (Crabtree et al. 2011; Zaragoza and Nielsen 2013). Titan-cell-derived daughter cells may include those with altered karyotypes, thereby further increasing the diversity of isolates that can disseminate through the body (Fig. 2C) (Morrow and Fraser 2013).

C. neoformans haploid cells can also form homotypic diploid cells by autodiploidization (Lin et al. 2005, 2009; Desnos-Ollivier et al. 2010). Autodiploidization can occur via either endoreduplication of the genome or self-mating of haploid cells (Lin et al. 2005). Although both processes are likely to occur in nature, one study showed that homotypic diploids formed during infection of a mouse were generated solely by endoreduplication and not by self-fusion (Desnos-Ollivier et al. 2010). Whether autodiploidization observed during in vivo infection models is related to the much more striking ploidy changes observed in titan cells remains to be determined.

ANEUPLOIDY

Aneuploidy Is Driven by Rapid Gains and Losses of Chromosomes

Aneuploidy, the presence of unequal numbers of chromosomes, is a common occurrence in fungi and plays an important role in adapta-

tion to environmental stress. Diploid organisms can undergo chromosome gains (trisomy or tetrasomy) as well as chromosome losses (monosomy) (Fig. 1). Haploid organisms become aneuploid by acquiring extra chromosomes (disomy) because all chromosomes carry essential genes, and, thus, chromosome loss events are lethal. Aneuploidy is thought to arise through chromosome missegregation caused by nondisjunction events, although endoreduplication of individual chromosomes also can give rise to aneuploidy.

Aneuploidy is surprisingly frequent in fungal species and can arise both in vitro and in vivo (Bouchonville et al. 2009; Forche et al. 2009). For example, many commonly used *C. albicans* strains, such as CAI-4 and WO-1, contain supernumerary chromosomes (SNCs) (Chibana et al. 2000; Chen et al. 2004; Selmecki et al. 2005; Rustchenko 2007). Aneuploidy can result in phenotypic changes in *C. albicans* (Suzuki et al. 1989; Rustchenko-Bulgac et al. 1990), as well as in natural *S. cerevisiae* isolates (Tan et al. 2013). Furthermore, whole-genome sequencing of clinical isolates revealed that a number of *C. albicans* strains contain SNCs (C Cuomo and RJ Bennett, pers. comm.), and in vivo passaging of strains through a mouse also induced aneuploid formation (Forche et al. 2009). A survey of fluconazole-resistant isolates found at least one aneuploid chromosome in 50% of the isolates (Selmecki et al. 2006).

As a general rule, the acquisition of extra chromosomes in diploid fungi is far more frequent than chromosome loss, presumably because a 33% increase in gene dosage (from two copies to three copies) is better tolerated than a 50% decrease in gene dosage (from two copies to one copy) (Gresham et al. 2008; Pavelka et al. 2010; Tan et al. 2013). However, monosomy also is seen under selective growth conditions. In a classic example, growth of diploid *C. albicans* cells on sorbose medium results in the loss of one copy of chromosome 5 (Chr5) (Janbon et al. 1998, 1999). Subsequent growth on nutrient-rich medium results in spontaneous endoreduplication of Chr5, indicating that removal from the selective environment results in reestablishment of the diploid state of Chr5. Perhaps of

more relevance to the clinic, Chr5 monosomy also affected the response to several classes of antifungal drugs (Yang et al. 2013).

An important feature of aneuploidy is its potential transience. Extra chromosomes can arise rapidly and be lost rapidly, even within a single mitotic division. In general, cells that are aneuploid show reduced genome stability and are therefore more prone to further chromosomal changes, at least under replete growth conditions (Torres et al. 2008, 2010). As a result, aneuploidy is often lost and cells return to the euploid state if there is no selective pressure to maintain the aneuploidy (Selmecki et al. 2006, 2009a). An attractive model is that aneuploidy provides a rapid, albeit imprecise, solution for adaptation to stress, thereby allowing cells to evolve more elegant solutions with a lower cost-to-benefit ratio. This was recently shown in a series of evolution experiments using chronic temperature stress in haploid *S. cerevisiae* cells (Yona et al. 2012). In this case, Chr3 disomy initially conferred improved growth at elevated temperatures, although point mutations eventually accumulated and aneuploidy was lost, presumably because the fitness cost of aneuploidy became higher than the associated advantage. Thus, under stress conditions, aneuploidy can provide a transient fix that promotes survival until a more refined (and lower cost) solution can be acquired.

Aneuploidy has the potential not only to diversify population phenotypes and promote adaptation, but also to drive additional genomic change, especially in cells exposed to stresses that threaten survival (Rancati et al. 2008). In haploid *S. cerevisiae* strains, aneuploidy increased rates of chromosome loss, genetic mutation, and microsatellite instability (Sheltzer and Amon 2011; Sheltzer et al. 2011). Importantly, although aneuploid cells generally showed elevated rates of genomic change, not all aneuploidies were equal, and elevated levels of noncoding DNA did not cause the same changes. This implies that excess copies of specific proteins, especially those in protein complexes sensitive to imbalanced stoichiometry, are primarily responsible for driving genotypic instability (Torres et al. 2008).

Mechanisms that Generate Aneuploidy

Both conventional sexual and parasexual cycles can generate extensive karyotypic diversity, including aneuploidy. In *C. neoformans*, Heitman and coworkers recently established that meiotic products from either bisexual α - α mating or unisexual α - α mating showed a wide range of phenotypes, and these phenotypes correlated with aneuploid chromosomes (Ni et al. 2013). Specifically, in unisexual α - α matings, six out of 90 meiotic progeny ($\sim 7\%$) showed phenotypic changes, with four of the six progeny being aneuploid, each harboring an extra copy of chromosomes 9, 10, or 13 (Ni et al. 2013). Loss of the aneuploid chromosome restored euploidy and the parental phenotype. These experiments establish that unisexual mating between identical cells can generate phenotypic and genotypic diversity *de novo*, and this variation is often a direct consequence of karyotypic change (Ni et al. 2013).

Both *C. neoformans* and *Cryptococcus gattii* are typically haploid species that include multiple serotypes; *C. neoformans* includes serotypes A and D, whereas *C. gattii* consists of serotypes B and C. In addition, a variety of natural hybrids have been reported, including both intervarietal hybrids (AA and AD) and interspecies hybrids (AB and BD) (Tanaka et al. 1996; Cogliati et al. 2001; Lengeler et al. 2001; Bovers et al. 2006, 2008a,b; Lin et al. 2009). Most AD hybrids are diploid, whereas others show ploidies intermediate between 1N and 2N (Cogliati et al. 2001). Aneuploidy in AD hybrids is thought to be the consequence of missegregation during meiosis attributable to sequence differences (10%–15% at the nucleotide level) between A and D strains (Lengeler et al. 2001; Kavanaugh et al. 2006). CGH analysis of the genomes of A, D, and AD strains showed extensive variation, including regions of divergence, deletions, and amplifications. This included the unexpected amplification of Chr1 in two serotype A haploid strains (Hu et al. 2008; Sun and Xu 2009). In addition, AD hybrids were generally less virulent than either A or D haploid strains (Cogliati et al. 2012). Together, these observations establish that the *C. neoformans* genome exists in a

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number of different configurations, including different aneuploid states.

In the emerging pathogen *C. lusitaniae*, a haploid–diploid–haploid sexual cycle culminates in meiosis and sporulation, yet ~22% of the meiotic progeny remain diploid and 6% are aneuploid (Butler et al. 2009; Reedy et al. 2009). The level of aneuploidy and unconventional divisions seen in fungi may be more common in higher organisms than originally thought. For example, 7%–35% of human oocytes show aneuploidy (Hunt and Hassold 2008). In this case, aneuploidy is associated with detrimental consequences, including miscarriage and birth defects. In contrast, human liver and nerve cells often become polyploid or aneuploid without negative consequences. Presumably, this difference is explained by the fact that the process of embryonic development is exquisitely sensitive to imbalances in gene products, whereas certain somatic tissues can benefit from the genetic diversity and adaptive potential of aneuploidy (Duncan 2013).

High levels of aneuploidy also can arise as a consequence of the parasexual cycle. In *C. albicans*, parasexual progeny frequently show aneuploidy, including strains carrying multiple SNCs. Thus, even when selected to be disomic for one chromosome, approximately two-thirds of progeny were aneuploid (Forche et al. 2008). Extensive phenotypic diversity also was observed, which is consistent with the idea that aneuploidy drives large-scale phenotypic changes (Rustchenko 2007; Forche et al. 2008; Rancati et al. 2008).

Finally, aneuploidy in fungi, as well as in somatic mammalian cells, can arise through defects in mitosis. In *C. albicans*, recent work suggests that exposure to fluconazole causes cells to uncouple nuclear and cell division, yielding an unusual cell type termed a “trimera.” Trimers are composed of three interconnected cells: mother, daughter, and granddaughter, with associated cell-cycle defects (Harrison et al. 2014). During trimera formation, two nuclei (from mother and daughter) replicate and divide, yielding four daughter nuclei within three cell compartments. One of the cells inherits two spindle pole bodies and two nuclei, becoming

tetraploid, but with an extra mitotic spindle. The resulting multinucleate cells are often unstable and yield aneuploid progeny via defective chromosome segregation. This novel cell cycle occurs with high frequency in the presence of fluconazole (~22% of cells) and may occur at lower frequency under other growth conditions. This phenomenon is reminiscent of the “ploidy conveyor” mechanism used by normal liver cells to cycle between diploid, polyploid, and aneuploid states (Duncan 2013), and a similar process may be responsible for aneuploidy in cancer cells (Ganem et al. 2007; Davoli et al. 2010; Davoli and de Lange 2011).

In summary, it is evident that high levels of genetic diversity can be introduced into a population via aneuploidy generated by mitosis, sexual reproduction, or the parasexual life cycle. This can occur even in the absence of interbreeding between different parent cells or homologous recombination between chromosomes. Therefore, we propose that aneuploid formation is the norm, rather than the exception, for fungal populations, with frequent karyotypic changes occurring in a subpopulation of cells. Furthermore, aneuploidy is likely to be common in certain mammalian somatic tissues as well.

The Consequences of Aneuploidy

In many species, aneuploidy is directly associated with marked phenotypic changes, and experiments have begun to address the molecular mechanisms underlying these differences. Aneuploid strains often, but not always, show slower growth, and this can be caused by the unbalanced protein stoichiometry resulting from gene expression from the aneuploid chromosome. Unlike sex chromosomes in which gene dosage compensation mechanisms regulate expression across the chromosome (Straub and Becker 2007), expression of most (but not necessarily all) encoded proteins typically scales with their gene copy number, at least in *S. cerevisiae* (Pavelka et al. 2010; Springer et al. 2010; Torres et al. 2010). This imbalance could be particularly critical for proteins that are part of multisubunit complexes, in which stoichiome-

try is important for proper complex assembly and/or function (Torres et al. 2010). The resulting “aneuploidy stress response” can cause cells to attempt to restore normal protein stoichiometries by changes in protein degradation (Torres et al. 2008). In support of this model, mutations in the Ubp6 deubiquitinating enzyme improved the growth rates of some aneuploid strains because of increased clearance of excess proteins (Torres et al. 2010). Not all aneuploidies cause a reduction in growth rate (Selmecki et al. 2008, 2009a; Torres et al. 2008; Zhu et al. 2012), suggesting that fitness defects in aneuploids are not caused by the aneuploid state per se, but rather are a result of stoichiometric changes in specific proteins that are out of balance. Other factors likely to contribute to fitness defects include the higher demands associated with DNA replication and difficulty in segregating aneuploid chromosomes. This has been shown by “ploidy-specific lethality,” as defects in factors associated with DNA repair, sister chromatid cohesion, or mitotic spindle function lead to lethality in *S. cerevisiae* tetraploids, but not in cells of lower ploidy (Storchova et al. 2006).

Although the detrimental effects of aneuploidy on fungal growth are often evident under replete culture conditions, aneuploid chromosomes can provide significant fitness advantages under suboptimal growth conditions (Fig. 1) (Rancati et al. 2008; Pavelka et al. 2010; Ni et al. 2013). Early studies in *Candida glabrata* showed that a fluconazole-resistant strain carried an extra copy of the chromosome containing *ERG11*, which encodes the target of fluconazole (vanden Bossche et al. 1992; Marichal et al. 1997). Aneuploidy also provided a growth advantage to *C. albicans* cells on sorbose medium, as Chr5 monosomy conferred improved growth on this medium and Chr5 reduplication reversed this phenotype (Janbon et al. 1998, 1999). In this case, sorbose use requires expression of the *C. albicans* *SOU1* gene on Chr4, and it appears that multiple negative regulators of *SOU1* expression are spaced along Chr5 (Kabir et al. 2005). Thus, loss of Chr5 facilitates *SOU1* expression and provides a remarkable example in which, essentially, a whole chromosome is acting as a single regulatory unit.

An association between aneuploidy and fluconazole resistance also was suggested by studies in *C. albicans*, in which high levels of the drug yielded strains with different aneuploidies (Perepnikhatka et al. 1999). Analysis of multiple clinical isolates by CGH revealed a direct connection between drug resistance and the presence of isochromosome 5L (i(5L)) (Selmecki et al. 2006). The isochromosome consisted of the two left arms of Chr5, and contained the *ERG11* and *TAC1* genes, encoding the target of fluconazole and a transcriptional regulator of drug efflux pumps, respectively. Loss of i(5L) during nonselective growth led to loss of the drug resistance phenotype, showing that the isochromosome was responsible for resistance (Selmecki et al. 2006). Furthermore, the increased copy number of *ERG11* and *TAC1* was sufficient to explain the levels of azole resistance conferred by i(5L) (Selmecki et al. 2006, 2008, 2009b).

In *C. neoformans*, a similar mechanism of drug resistance has been uncovered. Here, aneuploid chromosomes were associated with the phenomenon of “heteroresistance,” in which a subpopulation of cells acquired reversible resistance to fluconazole (Mondon et al. 1999; Ngamskulrungron et al. 2012a,b). Treatment of haploid *C. neoformans* cells with fluconazole resulted in disomy of up to four chromosomes (Chrs 1, 4, 10, and 14) (Sionov et al. 2009, 2010). Chr1 disomy was common to all resistant isolates and included the genes encoding Erg11 and the major azole transporter, Afr1 (Sionov et al. 2010). Genes on Chr4 also were linked to drug resistance. These encode a GTPase and two GTPase-activating proteins that are involved in the regulation of endoplasmic reticulum morphogenesis and trafficking (Ngamskulrungron et al. 2012b). The endoplasmic reticulum is a site for sterol synthesis, but the mechanism(s) by which these genes increase drug resistance is not known.

In *S. cerevisiae*, haploid strains have been engineered to be aneuploid by either the introduction of specific disomic chromosomes (Torres et al. 2010) or induction of meiosis in triploid cells (Pavelka et al. 2010). Importantly, when exposed to a range of stress conditions, most aneuploid isolates showed a growth advantage under at least one stress condition (Pavelka

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et al. 2010; Chen et al. 2012; Zhu et al. 2012). These studies concluded that different combinations of chromosomes could provide a selective advantage in the face of conditions that stress euploid cells. A study of natural *S. cerevisiae* isolates found that aneuploidy could result in a semistable colony phenotype involving filamentous growth (Tan et al. 2013). Interestingly, several different chromosomes, when aneuploid, could confer a similar colony phenotype (Tan et al. 2013).

A recent study uncovered an interesting link between apoptosis and the tolerance of aneuploid chromosomes. In *C. neoformans*, the conserved *AIF1* gene encodes apoptosis-inducing factor and is involved in promoting apoptosis-like cell death (Hamann et al. 2008). Deletion of *AIF1* potentiated adaptation to fluconazole caused by decreased apoptosis and increased tolerance of aneuploid chromosomes (Semighini et al. 2011). Significantly, clinical *C. neoformans* isolates were found to show varied *AIF1* expression. For example, one isolate showed high levels of heteroresistance to fluconazole and stable Chr1 aneuploidy, and these characteristics were a consequence of decreased *AIF1* expression (Semighini et al. 2011). Thus, regulation of apoptosis-like pathways can modulate aneuploid formation and associated drug resistance in pathogens such as *C. neoformans*.

Aneuploid formation in fungi also can be influenced by molecular chaperone activities. Inhibition of Hsp90 resulted in increased chromosome instability and aneuploid formation in *S. cerevisiae* (Chen et al. 2012). Furthermore, the karyotypic changes induced by Hsp90 inhibition increased adaptation to other unrelated forms of cellular stress. This is consistent with the stress-induced mutagenesis model (Galhardo et al. 2007), in which aneuploidy promotes formation of a pool of karyotypic variants that then have the potential to enhance adaptation to additional, diverse perturbations (Rancati et al. 2008; Chen et al. 2012).

Loss of Heterozygosity

LOH has emerged as a major mechanism to generate genetic diversity in populations of

heterozygous organisms. This is relevant for *C. albicans*, which is normally found as a highly heterozygous diploid (Butler et al. 2009), as well as for hybrid forms of *Cryptococcus* (Cogliati et al. 2001; Lengeler et al. 2001; Lin et al. 2008; Li et al. 2012; Ni et al. 2013). Homozygosis of short genomic regions (usually <2 kb) can arise by recombination via gene conversion or double-chromosome crossovers. Long-range LOH is attributable to either single crossovers with segregation of opposite alleles away from each other, or break-induced replication (BIR), in which a DNA break is repaired by strand invasion and replication fork progression (Fig. 3). As a result, long-range LOH events are homozygous from the site of the DNA break to the end of the chromosome (for reviews of BIR, see Kraus et al. 2001; Llorente et al. 2008). Whole-chromosome LOH can result from chromosome loss caused by nondisjunction followed by reduplication of the remaining homolog (Fig. 3). Alternatively, whole-chromosome LOH can result from chromosome gain followed by loss of the heterozygous homolog (e.g., nondisjunction could first yield a segregant with a trisomic “aab” configuration, and subsequent loss of homolog b would generate a homozygous “aa” cell, as shown in Fig. 3).

Similar to ploidy shifts, LOH can arise rapidly in a single cell cycle. However, once LOH occurs, it is irreversible, unless the cell outcrosses to regain the lost heterozygous alleles. In *C. albicans*, the opportunity for outcrossing while in the host is likely to be limited (Ramírez-Zavala et al. 2008; Morschhäuser 2010; Xie et al. 2013). Thus, tracts of LOH are often shared among related progeny within a given host, and they also can be traced in laboratory-derived strain lineages (Abbey et al. 2011). A detailed analysis of LOH showed that most *C. albicans* isolates have at least one LOH tract, usually of the “long-range LOH” type (Jones et al. 2004; Butler et al. 2009; C Cuomo and RJ Bennett, pers. comm.). For example, strain SC5314 is homozygous for both distal ends of ChrR, most of Chr3R, as well as large regions of both arms of Chr7 (Jones et al. 2004), whereas strain WO-1 has at least one long-range LOH tract on each chromosome (Butler et al. 2009).

Figure 3. LOH via recombination and chromosome nondisjunction in a heterozygous diploid organism. (A) Recombination can generate short-range LOH events by gene conversion or double crossovers. Long-range LOH events are caused by BIR or single crossovers. (B) Nondisjunction/chromosome missegregation events can generate whole-chromosome LOH. Nondisjunction can yield trisomic or monosomic progeny. Whole-chromosome LOH then arises by either missegregation of the heterozygous homolog in the trisomic individual or endoreduplication of the hemizygous homolog (hom).

Multiple processes can affect LOH rates and types in *C. albicans*. For example, the frequency of LOH is enhanced by passage through a murine host (Forche et al. 2009), indicating that it occurs in response to in vivo signals. In vitro studies show that environmental stress also increases LOH; exposure to oxidative stress, febrile temperature, or an antifungal drug all increased LOH in derivatives of strain SC5314 (Forche et al. 2011). Oxidative stress increased the proportion of short-range LOH, whereas high temperatures or exposure to fluconazole increased the proportion of whole-chromosome LOH. This presumably reflects the nature of different stresses; oxidative stress introduces DNA breaks that must be resolved via recombination, whereas heat shock and fluconazole presumably show a stronger effect on chromosome-segregation mechanisms. Whole-chromosome LOH also was prevalent in parasexual progeny (Bennett and Johnson 2003; Forche et al. 2008) and could be a consequence of the stress conditions used to induce concerted chromosome loss.

LOH represents the loss of genetic information, yet it can lead to phenotypic variation at the population level attributable to differences between heterozygous alleles. Phenotypes resulting from LOH can have a profound effect on clinically relevant traits, including increased drug resistance. In *C. albicans*, resistance can be altered because of LOH of *TAC1*, which encodes a transcription factor that up-regulates the ABC-transporter genes *CDR1* and *CDR2* (Coste et al. 2006), and *MRR1*, which encodes a transcription factor that regulates expression of the Mdr1 multidrug-resistance transporter (Dunkel et al. 2008). For both *TAC1* and *MRR1*, hyperactive mutant alleles that increase expression of the corresponding transporters exist, and homozygosis of these alleles, therefore, increases drug resistance. LOH after the acquisition of a point mutation also was detected at *ERG11* (White 1997), which is the direct target of fluconazole, and *UPC2*, which encodes a transcription factor that up-regulates *ERG11* expression (Heilmann et al. 2010). Thus, LOH

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of mutant alleles appears to be a common mechanism by which strains can acquire stable drug resistance.

As discussed earlier, the diploid state of *C. albicans* was originally thought to be obligate because of recessive lethal alleles dispersed across the heterozygous genome. However, cells containing homozygous chromosomes (Bennett and Johnson 2003; Forche et al. 2008; Zörgö et al. 2013), and the subsequent identification of true haploid forms (Hickman et al. 2013), showed that *C. albicans* cells could tolerate homozygosity on all eight chromosomes. Nonetheless, a conspicuous bias for homozygosity was observed for several homologs, suggesting that lethal recessive alleles do exist on some homologs (i.e., alleles on homologs that were not recovered). Haploid isolates all showed reduced fitness, implying that alleles that result in suboptimal growth are present on the recovered homologs (Hickman et al. 2013). Consistent with this, the fitness of diploid strains decreased in proportion to their homozygosity, with strains carrying longer LOH tracts generally having slower growth rates (Abbey et al. 2011). Furthermore, the level of heterozygosity in clinical isolates of *C. albicans* correlates with the ability to cause disease in an invertebrate host (C Cuomo and RJ Bennett, pers. comm.). Therefore, it is likely that heterozygosity, at least when averaged across the whole genome, is an important virulence trait in this species.

In theory, different alleles can confer distinct phenotypes because of amino acid differences in the encoded protein, as seen with *TAC1* and *MRR1*, or because of changes in expression levels between alleles (Staib et al. 2002; Holmes et al. 2006; Sanz et al. 2007). A recent study used a phased diploid sequence assembly to analyze *C. albicans* RNAseq data (Bruno et al. 2010) and found that alleles were differentially expressed in 3.6% of the genes (Muzzey et al. 2013). Thus, at least for *C. albicans*, LOH can result in altered phenotypes based on changes in gene expression levels as well as changes in the encoded proteins.

LOH has been similarly documented in AD hybrids of *C. neoformans*, which are highly dy-

namic and undergo extensive chromosome loss, often followed by reduplication of the remaining homolog (Li et al. 2012). AD hybrids showed hybrid vigor (heterosis), adapting to changing environments better than either A or D isolates (Li et al. 2012). Furthermore, extensive LOH in AD hybrid strains resulted in preferential retention of the serotype A homolog of Chr1 (Hu et al. 2008), which may contribute to a selective advantage in the mammalian host (Kwon-Chung and Bennett 1984; Irokanulo and Akueshi 1995; Lin et al. 2008). This provides an example of how LOH has the potential to confer adaptive benefits under selective pressure.

SUMMARY

In general, human fungal pathogens show considerable genome plasticity and can generate diversity through unconventional mechanisms. Many possess specialized sexual or parasexual life cycles that are unusual when compared with model species. These differences include the phenomena of white-opaque switching in *C. albicans*, and modes of same-sex mating in both *C. albicans* and *C. neoformans*. Mitosis can also drive large-scale karyotypic changes, including endoreduplication, trimera cell formation, concerted chromosome loss, and LOH. The consequences of these genomic changes are nontrivial and include the frequent emergence of drug-resistant strains in the clinic. Whether it is the stress encountered during growth in a mammalian host, or other factors that led to these unusual mechanisms of genome diversification, remains to be seen. What is clear, however, is that analogous mechanisms of genome change occur in mammalian tissues, suggesting that these processes are more widespread than previously considered. Advances in our understanding of genome plasticity will be key to the appropriate understanding, treatment, and prevention of fungal infections.

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