Ploidy variation in multinucleate cells changes under stress

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Abstract

Ploidy variation is found in contexts as diverse as solid tumors, drug resistance in fungal infection, and normal development. Altering chromosome or genome copy number supports adaptation to fluctuating environments but is also associated with fitness defects attributed to protein imbalances. Both aneuploidy and polyploidy can arise from multinucleate states after failed cytokinesis or cell fusion. The consequences of ploidy variation in syncytia are difficult to predict because protein imbalances are theoretically buffered by a common cytoplasm. We examined ploidy in a naturally multinucleate fungus, Ashbya gossypii. Using integrated lac operator arrays, we found that chromosome number varies substantially among nuclei sharing a common cytoplasm. Populations of nuclei range from 1N to >4N, with different polyploidies in the same cell and low levels of aneuploidy. The degree of ploidy variation increases as cells age. In response to cellular stress, polyploid nuclei diminish and haploid nuclei predominate. These data suggest that mixed ploidy is tolerated in these syncytia however there may be costs associated with variation as stress homogenizes the genome content of nuclei. Furthermore, the results suggest that sharing of gene products is limited and thus there is incomplete buffering of ploidy variation despite a common cytosol.

Introduction

Variation in ploidy within an organism can be a defining feature of either pathologies or normal developmental programs. Understanding both the utility and the deleterious consequences of varying DNA copy number relates to problems in fields as diverse as cancer biology, microbial pathogenesis and ecology, and plant development. Copy-number variation can be considered from the scale of small insertions or deletions that impact a single gene to amplifications of whole chromosomes (aneuploidy) or the entire genome (polyploidy). DNA copy number changes can lead to changes in the levels of the
mRNA and proteins encoded in the amplified regions (Torres et al., 2007; Pavelka et al., 2010). Expression of altered copy-number regions thus has the potential to dramatically impact the physiology of cells.

The consequences of aneuploidy on cell function are highly variable and context dependent. The majority of human solid tumors display aneuploid karyotypes and are also composed of rapidly proliferating cells (Weaver and Cleveland, 2006). Robust tumor growth is paradoxical in light of certain model aneuploid cells, which can be observed to grow slowly (Torres et al., 2007). The mechanisms by which tumors tolerate aneuploidy are still under study. However, one known function of key tumor suppressors is to monitor and arrest growth when cells display chromosome instability (CIN), a context in which aneuploidies arise (Li et al., 2010; Thompson and Compton, 2010). These programs are dismantled with inactivation of the tumor suppressors coincident with tumor progression. Work in model aneuploid yeast and fibroblast cells with engineered extra chromosomes also indicate that one major cause of growth defects is proteotoxic stress. This stress emerges from the attempt to degrade excess proteins and presumably tumor cells adapt to this stress (Torres et al., 2007; Pavelka et al., 2010; Oromendia et al., 2012).

As in tumors, aneuploidy is also commonly observed in diverse fungal species in natural, clinical, and lab environments (Morrow and Fraser, 2013; Bennett et al., 2014). For example, in the fungal pathogen Candida albicans, aneuploid cells readily emerge through transient tetraploidization and in response to antifungals (Forche et al., 2008; Selmecki et al., 2010; Harrison et al., 2014). This capacity to remodel the genome is beneficial to the pathogen during infection and enables the development of drug resistance. Variation in ploidy is also seen during the course of sexual development and infection with the human pathogen Cryptococcus neoformans (Idnurm, 2010; Semighini et al., 2011). Additionally, under some stress conditions aneuploid S. cerevisiae cells are more fit than euploid strains indicating that ploidy variation may be highly adaptive depending on the environmental conditions (Pavelka et al., 2010; Yona et al., 2012; Zhu et al., 2012). Furthermore, industrial Saccharomyces yeast strains developed for brewing and baking are largely polyploid and/or aneuploid (Querol and Bond, 2009). Thus, the capacity to induce and tolerate large genome changes can provide an adaptive advantage for diverse fungi in the context of pathogenesis and environmental stress.

Aneuploidy can arise through multiple, distinct molecular mechanisms. Current known routes to aneuploidy include failure in the spindle assembly checkpoint (SAC), altered error-correction of misattached chromosomes, and transient polyploidization through cytokinesis failure or cell fusion (Burds et al., 2005; Lu and Kang, 2009; Krajcovic et al., 2011; Bakhoum and Compton, 2012). There is evidence that polyploidy can increase the probability of many cells, including budding yeast, of becoming aneuploid (Storchova et al., 2006). Similarly, aneuploid liver cells can arise from a polyploid starting population (Duncan et al.,
While polyploidy has long been appreciated for functional relevance in plants and specific animal tissues, recent work in fungi has started to focus attention to polyploidy as an engine of evolution (Albertin and Marullo, 2012). Thus, polyploidy could serve as a reservoir to generate functional aneuploidies under specific stresses or be useful unto itself for specific metabolic needs in a given tissue or environment.

Most filamentous fungi exist in syncytial states where many nuclei share a common cytoplasm during mycelial growth. Furthermore, it is clear that in many fungi different genomes can coexist, cooperating and/or competing in a single cell (Roper et al., 2011). However it is less clear how genomes that differ due to chromosome imbalances may function in fungal syncytia. Syncytia theoretically may be a highly permissive environment for aneuploidy as imbalances may be complemented across multiple nuclei in the same cytoplasm. This would require that gene products are well mixed so that the cytosol buffers protein imbalances. In this case, syncytial cells may harbor high frequencies of aneuploidy and suffer fewer fitness consequences than uninucleate cells with a chromosome imbalance. This idea is supported by the multinucleate states seen in tumors that are also aneuploid (Lu and Kang, 2009). Alternatively, if the cytosol is compartmentalized in large multinucleate cells such that gene products are not shared between nuclei, then ploidy may be more tightly controlled due to functionally heterogeneous cytosol.

We have monitored ploidy in the multinucleate fungus Ashbya gossypii to examine the variability of chromosome content of individual nuclei within a single cell. Previous work in Ashbya has suggested that the euploid state of the system is haploid in multinucleate mycelia and uninucleate haploid spores are produced by asexual sporulation (Dietrich et al., 2004). In Ashbya nuclei have highly variable division cycle durations so that nuclei divide out of sync with their neighbors (Gladfelter et al., 2006). One possible source of timing variation between nuclei in a single cell is variable DNA content. In this study, we tested the hypotheses that there is ploidy variation between nuclei in the same cell and that aneuploidy is tolerated in syncytia.

Results

DNA content varies among nuclei in a single cell

Ashbya cells expressing GFP-labeled histone-4 (Hhf1-GFP) were filmed and the fluorescence intensity of the histone signal in single nuclei was measured at birth (pre-DNA replication) and moments before division (post-DNA replication). If all nuclei are haploid, we would predict two discrete peaks of histone intensities corresponding to 1N ploidy at birth and 2N ploidy before mitosis (Figure 1A). Surprisingly, nuclei showed a wide range of intensities at birth and immediately preceding mitosis (Figure 1B, N=47 nuclei). The two distributions of intensities show substantial overlap although the mean intensity at mitosis is modestly but significantly higher than at birth (birth mean=12720 ± 3970 a.u.; mitosis
mean=15030 ± 5623 a.u.; 2-sample t-test, p<0.03). The signal variation is not
due to differential photobleaching as the Hhf1-GFP intensity of nuclei is not
correlated with the timepoint in the image acquisition (Supplemental Figure 1).
This suggests that nuclei are born with and divide with variable amounts of DNA
in the same cell.

To ensure that histone intensities increase with cell cycle progression and DNA
replication, we plotted the histone intensity for individual nuclei and see that for
all nuclei the signal increases with progression towards mitosis. The time from
minimum to maximum signal intensity ranged from 30 to 134 minutes (Figure 1C,
mean=83 min, SD=21 min, N=47), with an average 1.5-fold change in the histone
intensity (Figure 1D). This change may not be 2-fold due to a soluble pool of
histones that is present in the nucleus at birth and used to build nucleosomes
during replication or because not all the DNA is replicated. These data suggest
that the duration of S-phase is likely highly variable and this could be due to
variable DNA content, heterogeneous import of Hhf1-GFP, or other factors
controlling DNA replication being limiting. Therefore, as a second measure of
DNA content through the cell cycle, we measured the incorporation of the DNA
dye, DAPI, into nuclei that were scored for cell cycle stage based on the state of
the spindle pole body (SPB). As seen in live cells, nuclei in the same SPB state,
and presumably the same cell cycle stage, show a broad distribution of DAPI
intensities supporting that there may be substantial variation in the DNA content
among nuclei in the same cell (Figure 1E).

Nuclei vary in the number of copies of individual chromosomes

Given the heterogeneity of histone and DAPI signals, we next investigated the
numbers of individual chromosomes by integrating 32 lac operator repeats into
intergenic regions of either Chromosome I (smallest in size) or Chromosome VI
(largest in size) in a strain also expressing GFP-LacI-NLS (Figure 2, A and B).
Nuclei had between 0 and >4 spots of GFP-LacI signals for each of the
chromosomes, the largest number of spots detected was 6 (Figure 2, A-C,
N>300 nuclei for each chromosome, cells scored at a stage of >100 nuclei). At
very low frequency, nuclei could be seen without a LacI signal suggesting rare
loss of the chromosome. Work using similarly marked chromosomes in S.
cerevisae showed that LacI signals on sister chromosomes are not resolvable
until anaphase as long as the repeat sequences are far from centromeres
(Straight et al., 1997; Pearson et al., 2001). This suggests that the Ashbya nuclei
with 2 or more spots in fact have extra chromosomes rather than having finished
S-phase. Nevertheless, we assessed the SPB state of nuclei along with LacI
signals and see that 40% of nuclei with a single SPB have >1 LacI spot
suggesting that the presence of additional LacI spots is not simply due to
replication (Figure 2D). As an alternative measure of chromosome number, we
employed fluorescence in situ hybridization (F.I.S.H.) to visualize Chromosome
VI at the CLN1/2 locus. Specifically, we localized CLN1/2 mRNA to identify sites
of active transcription and determine chromosome number within nuclei. Using
this approach, 32% of nuclei have two or more copies of Chromosome VI (Figure
Chromosome number increases as cells age
We next looked at how the frequency of altered chromosome number changes as cells age. *Ashbya* spores do not germinate synchronously so we use the number of nuclei within a cell as a measure of age. This is because the number of nuclei scales with growth, which is generally similar among individuals of a given age. We defined “young” cells as those with fewer than 40 nuclei and “old” cells as those with greater than 40 nuclei. Interestingly, Chromosome I counts per nucleus increased with the age of a mycelia with a shift from a mode of 1 in very young cells and a mode of 2 in older cells. This suggests that deviations from a haploid complement of chromosomes accumulate with cell age (Figure 3A).

Nuclei with increased chromosome content are positioned throughout the cell
The increase in chromosome copy number with cell age prompted us to examine the position of the nuclei with extra DNA. Nuclei in *Ashbya* cells have been shown to move and intermix within the mycelium (Anderson et al., 2013). Therefore, we hypothesized that nuclei with many copies of individual chromosomes may be positioned further away from the actively growing tips of *Ashbya* cells. This would ensure that haploid nuclei would predominantly populate the most active portions of the cell, while those nuclei with altered chromosome numbers reside in the more interior portions of the cell. Surprisingly, however, there is no apparent bias in position for specific ploidies as nuclei with each chromosome count were found both close to and distant from growing cell tips (Figure 3B, ANOVA, F=1.56, p>0.20). Thus, nuclei of variable ploidies can be found throughout the cell indicating either the altered ploidy nuclei are functional or at least tolerated even in actively growing areas.

Chromosomes are faithfully segregated at mitosis
Given the variation in the copy number of Chromosomes I and VI and the increase as cells age, we hypothesized that *Ashbya* nuclei were missegregating chromosomes during mitosis. We filmed cells with labeled Chromosome I and remarkably found no evidence of missegregation as determined by the equal inheritance of LacI-GFP spots (Figure 4A, Movie S1 and S2, N=36 mitoses). Regardless of whether the observed nucleus started with 1 or 2 LacI spots, all the marked chromosomes were accurately segregated. Consistent with faithful segregation, most sister nuclei were born with comparable levels of Hhf1-GFP intensities, which would be unlikely if there were large chromosome imbalances (Figure 4B-C, N=15 sister pairs). The similarities between sisters were seen regardless of the intensity of the mother nucleus. These data support that chromosome copy number variation does not arise from sloppy chromosome segregation in mitosis. Thus, even in a syncytial cell, where more genome copy
number variability theoretically could be tolerated, there are controls of chromosome instability (CIN).

**Populations of nuclei are polyploid with limited aneuploidy**
The faithful segregation of chromosomes in *Ashbya* suggests that many nuclei may be polyploid rather than aneuploid. Unfortunately due to limited selectable markers available in *Ashbya*, we are unable to make use of two different marked chromosome reporters such lacO and tetO together. Therefore, to assess aneuploidy and polyploidy frequencies, we generated a strain with both Chromosome I and VI marked with lacO repeats in the same nuclei (Figure 5A). If nuclei are polyploid, nuclei should have LacI spots in multiples of two such as 2, 4, or 8. However, if nuclei are aneuploid, then odd numbers should be observed at some frequency as by chance Chromosome I or VI would be in excess or missing (Figure 5B). When scored in very young cells, the majority of nuclei in the strain with two marked chromosomes contain 2 LacI spots consistent with the idea that nuclei are haploid at a young age (<40 nuclei). As cells grow (>40 nuclei) the distribution shifts to a modal number of 4 spots, indicative of 2 copies of each chromosome (Figure 5C, N=118 (>40 nuclei), N=253 (<40 nuclei)). Coincident with aging, there is also an increase in nuclei with odd numbers of chromosomes, which reflects modest levels (~25%) of aneuploidy. These data are consistent with the observed faithful segregation of chromosomes at mitosis in young cells (Figure 4A, Movie S1 and S2) and supports that nuclei within the syncytium can exist with chromosomal aneuploidies but are predominantly polyploid. We speculate that as the volume of cytosol increases with age, low levels of aneuploidy become tolerable. Thus, nuclei vary in ploidy in the same cell but the majority of the variation likely involves amplification of whole genomes rather than individual chromosomes.

**Spindle checkpoint protein Mad2 is required for normal growth**
Given that the frequency of aneuploidy is small in young cells, we hypothesized that the spindle assembly checkpoint (SAC) is critical for keeping aneuploidy in check. Activity of the checkpoint may then decrease as the cells age when aneuploid nuclei appear. We generated mutants lacking Mad2, part of the conserved spindle assembly checkpoint machinery and found that mad2Δ cells grow slowly and had highly irregular borders, which reflects unequal growth across multiple hyphae (Figure 6). Such variable growth edges would be expected if there is stochastic loss of genome stability and therefore localized cell death or senescence in certain branches leading to their inability to grow while others maintain growth. This observed growth phenotype is considered “haploinsufficient” as these are heterokaryons and there is still wild-type Mad2 expressed from a subset of non-transformed nuclei. Therefore, in areas where there are many transformed mad2Δ nuclei, presumably growth cannot occur, while those branches with enough Mad2-expressing nuclei continue to grow. Alternatively, the overall dose of Mad2 is insufficient for each nucleus to trigger a checkpoint when needed or nuclei residing in the same cell are not generally sharing checkpoint components. We hypothesize that the loss of Mad2 in
branches causes higher levels of aneuploidy than can be tolerated and localized growth arrest. Heterokaryon cells lacking Mad2 also fail to produce asexual spores making it impossible to generate homokaryon, null mutants. This requirement for the spindle checkpoint suggests that aneuploidy in young cells is not well-tolerated and is likely kept in check by the SAC. It also suggests that chromosome imbalances are not compensated for even though there are multiple neighboring nuclei capable of supplying missing or imbalanced gene products. The SAC is likely active and required even in non-perturbed conditions indicating that there may be generally high levels of errors in chromosome attachment-perhaps due to the presence of extra chromosomes in polyploid nuclei and insufficient spindle microtubules.

**Stress promotes a redistribution of nuclei to a haploid state**

In previous studies it has been seen or postulated that variation in ploidy, primarily aneuploidy, may be adaptive under stressful or fluctuating conditions. In fact, experimental evolution experiments have shown that aneuploidy can be one of the fastest ways yeast cells adapt to fluctuating conditions (Yona et al., 2012). If so, we predict that growing cells under stresses may promote even more extreme ploidy differences and potentially increase the level of chromosome variation among nuclei. We subjected *Ashbya* strains with a lacO marked Chromosome I to a panel of stress conditions including cell wall stress (2mM caffeine), osmotic stress (200mM NaCl), excess zinc (10mM ZnSO4), the ergosterol synthesis inhibitor and clinical antifungal fluconazole (325nM), and temperature (37°C). Remarkably, in all stress conditions except caffeine, the ploidy status shifted significantly to a euploid state rather than a more polyploid state such that the majority of nuclei now contained a single detectable LacI-GFP spot (Figure 7, Z-test p<0.05, N>85 nuclei each condition). This supports that *Ashbya* cells can vary ploidy in response to external signals but remarkably stress drives the cells to a more euploid state rather than more variable ploidy.

**Discussion**

Ploidy variation has been shown to be advantageous or deleterious depending on the biological context. For example, aneuploidy is a common feature of solid tumors and is associated with infection and drug resistance in human fungal pathogens yet when artificially engineered into cells, aneuploidy can be a fitness disadvantage. Although many aneuploidies arise from cells that undergo a multinucleate stage, the functional consequences of having variable ploidies in one cytosol have not been examined. Given that in multinucleate cells there is the potential for mixing and sharing of gene products between nuclei with different genotypes, it is unclear whether aneuploidy would be deleterious, beneficial, or neutral. Here, we analyzed natural ploidy variation in the multinucleate fungus *Ashbya* where nuclei transit the division cycle asynchronously (Gladfelter et al., 2006; Nair et al., 2010). Thus, the goal of this work was to understand both how ploidy may vary in syncytia and to see if ploidy could be a source of differences among nuclei. Recent work has implicated
aneuploidy in delaying cell cycle progression (Thorburn et al., 2013) and we speculated that ploidy variation may be tolerated in a shared cytoplasm and be a source of division timing variation among nuclei.

We observed in Ashbya that nuclei have variable numbers of chromosomes, ranging from one to greater than four copies of individually tagged chromosomes. Nuclei are able to faithfully segregate chromosomes and appear to be frequently polyploid and less frequently aneuploid. Deletion of MAD2 is deleterious suggesting a functional spindle assembly checkpoint is necessary for normal growth in Ashbya. Notably, growth under stress causes ploidy to decrease to near euploid suggesting that chromosome count variability is not maintained under stress. Thus, polyploidy and to a certain degree aneuploidy is tolerated in Ashbya under optimal growth conditions. Electron tomography analysis of a small number of nuclei in young Ashbya cells showed the mitotic spindle contains a similar number of kMTs as budding yeast, yet haploid Ashbya gossypii has less than half the number of chromosomes (7 chromosomes in Ashbya vs. 16 in S. cerevisiae (Gibeaux et al., 2012)). Therefore, it is probable that in diploid nuclei, sufficient kinetochore microtubules are made for correct chromosome attachments at mitosis however as ploidies increase with cell age kinetochore microtubules become limiting and/or improper attachments are more common.

In addition to polyploidy, there is also a degree of aneuploidy among Ashbya nuclei suggesting some level of chromosome loss. In the pathogenic fungus, Candida albicans, parasexual cycles result in tetraploid nuclei that undergo concerted chromosome loss to reach near diploidy (Hull et al., 2000; Bennett, 2003; Forche et al., 2008). Chromosome loss after polyploidization has also been observed in S. cerevisiae where the frequency of loss increases with increased ploidy (Mayer and Aguilera, 1990; Gerstein et al., 2006; 2008). Similarly, in Drosophila rectal cells, polyploid cells produce high levels of aneuploidy (Schoenfelder et al., 2014). It is possible that predominantly polyploid nuclei infrequently undergo chromosome loss resulting in the low levels of aneuploidy we observe in Ashbya. Consistent with this hypothesis, we see increased levels of aneuploidy in older cells, suggesting that the increased ploidy variation in more mature cells may be leading to increased chromosome loss.

While limited aneuploidies are observed, they are likely not well tolerated in Ashbya. Deletion of MAD2 results in decreased colony growth and produces phenotypes even when some nuclei express Mad2. This indicates both that aneuploidy is likely not tolerated and that checkpoints may be nuclear intrinsic in action, which has been seen in the context of binucleate yeast and marsupial cells (Rieder et al., 1997; Demeter et al., 2000). Why is aneuploidy not tolerated when gene products can theoretically be shared between nuclei in a syncytium? In fact, the cytosol may not be as functionally homogeneous as might be expected. These results are consistent with recent work in Ashbya showing that nuclei can create functional compartments and neighboring nuclei do not completely intermix their surrounding cytosol. These nuclear territories require
cytoplasmic microtubules and functional protein aggregates that localize mRNAs (Anderson et al., 2013; Lee et al., 2013). The regionalization of cytosol around individual nuclei may decrease any hypothetical buffering capacity of the syncytium. In a context where the cytosol is regionalized rather than homogenized, nuclei with polyploid complements of chromosomes create functional territories. Aneuploid nuclei may not be functionally complemented by neighboring nuclei and therefore may not be maintained.

The existence of functionally distinct regions of cytosol with limited exchange may help explain why external cell stress reduces ploidy variation. It is well established that for proteins that form larger complexes to function (for example Tubulin heterodimers) an imbalance in the expression of one of the components is deleterious (Weinstein and Solomon, 1990). Additionally, it has been shown that excess proteins that arise from a chromosome amplification create a proteotoxic burden on cells as they degrade excess proteins to achieve optimum stoichiometries in protein complexes (Oromendia et al., 2012). In Ashbya, where a haploid, diploid, and tetraploid nucleus may all be neighbors, there is the possibility for some local imbalances in levels of proteins in complexes at the boundaries between nuclear territories and this may present some low baseline of proteotoxic stress. This stress may be outweighed in normal conditions by having some nuclei at enhanced biosynthetic capacity by housing more DNA. Under stress, perhaps the burden of proteotoxic stress is too great and haploid nuclei take over as seen in the majority of stress conditions analyzed. Interestingly, caffeine did not lead to a ploidy reduction potentially due to a different response to this specific condition or the level of stress was insufficient to generate proteotoxic strain. In any case, this work reveals another example in which ploidy changes under stress and shows that the number of nuclei in the cell may be an important factor in how ploidy state may change in stress. This is important in light of the observation of multinucleate cells in tumors.

How are ploidy differences established in Ashbya? In the filming of individual nuclei with marked chromosomes, which revealed no missegregation, there were unfortunately no hints of how nuclei become polyploid. We found no evidence of nuclear fusion, failed anaphases, or clear endoreduplication but we predict there must be some frequency of these events that potentially are suppressed during live cell imaging and evade our detection. While the direct mechanism of establishing ploidy variation remains elusive, the striking shift we observed from an apparently haploid state in very young cells to polyploid states with age suggests that germlings emerge from haploid spores. This is also supported by extensive application of reverse genetics in the system where clean gene replacement strains are usually able to be generated from single spores of a heterokaryon transformant (Wendland et al., 2000).

How do haploid spores arise out of mature polyploid/aneuploid mycelia? Ashbya cells form asexual spores only after colonies have matured for multiple days on an agar plate. The specific mechanisms of spore formation in Ashbya are poorly
understood and it is not currently known if there is a meiotic component to sporulation. There are many conserved components for meiosis and it has been speculated that nuclei fuse and then undergo meiotic-like reductions to make spores (Wasserstrom et al., 2013). However, the lack of mechanistic evidence for meiotic-like reductions suggests that all spores examined in the lab are asexual. To generate haploid spores from the variable ploidy nuclei of mature mycelia means there is a likely a reduction in ploidy and correction of the low levels of aneuploidy during spore formation. Alternatively, the population of asexual spores formed may have variable ploidy and be a bet-hedging strategy for germination in different environments, which would be especially useful source of genetic diversity in an asexually reproducing population. We have seen that only half of wild-type Ashbya spores germinate (N>200, unpublished observations). This low germination frequency suggest that it is likely that spores of many ploidies are produced but only haploids are able to germinate under lab conditions. Furthermore, spores from mad2∆ strains show even lower germination rates of 33%, suggesting that altering the SAC and presumably increasing ploidy variation further decreased spore viability in lab conditions. In S. cerevisiae, greater than 90% of spores undergo germination and are viable (Klapholz et al., 1985; Diaz et al., 2002). However, many fungi that under certain conditions have been shown to be aneuploid, including some strains of S. cerevisiae, Neurospora crassa, Sordaria macrospora, and the basidiomycetes Cryptococcus neoformans and Coprinus cinereus, have decreased spore viability (Klapholz et al., 1985; Celerin et al., 2000; Diaz et al., 2002; Storlazzi et al., 2003; Bowring et al., 2006). It is possible that differences in genome copy number, even if balanced as in polyploidy, are more detrimental in spores with just a single nucleus, while ploidy variation is more tolerated in mature cells.

The high degree of ploidy variation in Ashbya is striking and may be a mechanism by which nuclei cycle asynchronously through the cell cycle. The connection between ploidy, cytoplasmic compartmentalization, and adaptive fitness is fascinating and further study will ideally link these traits to cell cycle timing variation within a common cytoplasm.

Materials and Methods

Growth conditions and strain construction
Ashbya gossypii media, culturing, and transformation conditions were performed as described previously (Ayad-Durieux et al., 2000; Wendland et al., 2000). The strains in this study are described in Table S1. The plasmids used in this study are listed in Table S2. The oligonucleotide primers used in this study are listed in Table S3.

lacO plasmid construction
To generate plasmids AGB245/246, pAKH37 and AGB21 were digested with Kpnl and Ndel (NEB). The ~1600bp band (pAKH37), and the 4214bp (AGB21) band were gel extracted and ligated using T4 Ligase (NEB). Resulting plasmids
were verified by digest with KpnI and NdeI and sequencing. To generate plasmids AGB264/265, AGB246 and pRS416 were digested with NdeI and SbfI. The 3343bp band from AGB246 was gel extracted and ligated with digested pRS416 using T4 Ligase (NEB). Resulting plasmids were verified with SacI digests and sequencing with AGO37.

**Chromosome I 32-lacO::Gen3**

Chromosome I homology was engineered using gap-repair. Approximately 250bp of 5' homology to Chromosome I was amplified from ΔlΔt genomic DNA using AGO638/639. AGB264/265 were digested with StuI and were co-transformed into yeast with 5' Chromosome I homology to generate AGB266/267, which were verified by BglI digestion and sequencing with AGO640. Approximately 250bp of 3' homology to Chromosome I was amplified from ΔlΔt genomic DNA using AGO641/642. AGB266/267 were digested with NdeI and were co-transformed into yeast with 3' Chromosome I homology to generate AGB268/269, which were verified by digestion with SpeI/StuI and sequencing with AGO640 and AGO678. A 4260bp fragment was gel purified from AGB268/269 after digestion with SpeI/StuI and was transformed into AG302 to generate AG459.1-4. Primary transformants were picked onto G418 plates (200μg/ml) and single spores were picked to generate the homokaryon AG460. Strains were verified using oligo pairs AGO721/AGO761, AGO723/AGO759, and AGO721/AGO723.

**Chromosome VI 32-lacO::Gen3**

Approximately 200bp of 5' homology to Chromosome VI was amplified from ΔlΔt genomic DNA using AGO753/754. AGB264/265 were digested with StuI and were co-transformed into yeast with 5' Chromosome VI homology to generate AGB309/310, which were verified by digestion with XhoI and sequencing with AGO640. Approximately 250bp of 3' homology to Chromosome VI was amplified from ΔlΔt genomic DNA using AGO765/766. AGB309/310 were digested with NdeI and were co-transformed into yeast with 3' Chromosome I homology to generate AGB322/323, which were verified by digestion with Xmnl/XhoI and sequencing with AGO678. AGB322/323 were digested using SpeI/StuI and were transformed into AG302 to generate AG479.1-3. Primary transformants were picked onto G418 plates (200μg/ml) and single spores were picked to generate the homokaryon AG480. Strains were verified using oligo pairs AGO757/AGO758, AGO98/AGO760, and AGO757/AGO760.

**Chromosome I 32-lacO::Gen3 / Chromosome VI 32-lacO::Nat1**

To make strain AG514/515, AGB268/269 was digested using Spel/StuI and was transformed into AKH26.2. Primary transformants were picked onto G418 plates (200μg/ml) and single spores were picked to generate the homokaryon AG517/518. Strains were verified using oligo pairs AGO721/AGO761, AGO723/AGO759, and AGO721/AGO723. To make a Chromosome VI homology Nat1 plasmid, a 1817bp fragments was gel extracted from AGB09 digested with SapI and NdeI. Using this band, Nat1 was PCR amplified using
AGO1050/968 and was co-transformed with AGB322/323 to generate plasmid AGB403.1/403.2. Plasmids were verified using KpnI digests and sequencing with AGO234/235. AGB403.1 was transformed into AG517 to generate strain AG720. Strains were verified using oligo pairs AGO757/AGO758, AGO760/AGO234, and AGO757/AGO760.

**mad2Δ::Nat1**
To generate strain AG600/601, AGB9 was cut with PvuII-HiFi. NAT1 was amplified off this cut plasmid using AGO1101/AGO1102. PCR products were pooled and directly integrated into *Ashbya*. Primary transformants were picked onto Clonat plates. Strains were verified using oligo pairs AGO1103/AGO235, AGO234/AGO1104, and AGO1103/AGO1105.

**Microscope setup and imaging conditions**
A Zeiss Axioimage-M1 upright light microscope (Carl Zeiss, Jena, Germany) equipped with a Plan-Apochromat 63X/1.4NA oil objective was used. To visualize the fluorescence signals, an Exfo X-Cite 120 lamp was used in conjunction with Zeiss 38HE (GFP), Chroma 41002B (TAMRA), Zeiss 49 (Hoechst/DAPI), and Chroma 41043 (mCherry). Images were acquired on an Orca-AG charge-coupled device camera (C4742-80-12AG; Hamamatsu, Bridgewater, NJ) driven by Volocity (Improvision, Lexington, MA).

To image LacI chromosome spots, cells were grown for 16 hours and were imaged on thin gelpads containing 2% agarose and 100% 2X low fluorescence minimal media. Z stacks were acquired with 0.5μm slices spanning the hyphae. The images were exposed for 50-100ms at 100% transmission. All images were processed by iterative deconvolution (100 iterations) using calculated point spread functions in Volocity (Improvision, Lexington, MA) and nuclei were visually scored for number of LacI-GFP labeled chromosomes.

To image chromosome segregations, cells were imaged on an OMX microscope equipped with a Hamamatsu EMCCD. A 488nm solid-state laser was used for GFP illumination, using 1.08% laser power. Cells were imaged every minute through a 10μm z-depth (21 slices at 0.5μm).

**Hhf1-GFP intensity quantification**
Timelapse movies and previous nuclear tracking were used to quantify Hhf1-GFP intensity over time (Anderson *et al.*, 2013). Timelapse images and nuclear coordinates were imported into MATLAB and the nuclei around the central coordinates was found using thresholding. Photobleaching correction was applied to individual nuclei over time. Histone intensity was measured as the sum GFP intensity for each nucleus. Fold change for each nucleus was calculated using the maximum and minimum Hhf1-GFP signal throughout the tracking. S-phase duration was the time between maximum and minimum GFP intensity.
**Spindle pole body (SPB) scoring**

Cell cycle stages were determined using spindle pole bodies (SPBs) as described previously (Nair et al., 2010). Briefly, nuclei with a single SPB were assigned to G1, nuclei with either a single SPB that was twice the intensity and size as a G1 SPB or had two adjacent SPBs that were less than 0.5 μm apart were assigned to S/G2, and nuclei with 2 SPBs that were more than 0.5 μm apart on opposite sides of the nucleus were assigned to M.

**F.I.S.H.**

A single molecule RNA FISH protocol was used to visualize chromosome expressing the *CLN1/2* gene (Lee et al., 2013). This transcript is expressed in over 90% of nuclei and the site of expression of gene is readily detected as spots that are generally brighter than the intensity of single mature transcripts in the cytosol. As all attempts at DNA FISH were unsuccessful and a DNA FISH protocol has never been established for *Ashbya*, we used RNA FISH of transcription as a proxy for chromosome number. Cells were grown for 12-16 hours at 30°C while shaking and were fixed in 3.7% formaldehyde for 1 hour. Cells were washed into Buffer B and were spheroplasted using zymolyase (15μg/mL) and incubate at 37°C until phase dark. Cells were washed twice with wash buffer and were mixed with hybridization solution (100μl hybridization buffer, 1.5μl probe). Cells were incubated at 92°C for 3 minutes and then overnight at 37°C. Cells were washed twice with wash buffer and were incubated for 10 minutes at room temperature in 500μl wash buffer + 1μl Hoechst. Cells were washed twice more, were mounted in Prolong Gold mounting media (Life Technologies), and were imaged.

**Germination and colony growth assays**

For germination assays, spores were spread onto AFM (*Ashbya Full Media*) plates with appropriate selection and were allowed to germinate at 30°C for 8 hours. Plates were imaged and scored in ImageJ (NIH) for spore germination. For colony growth assays, 10 μl of spores were plated in the center of AFM plates with appropriate selection. Colonies grew at 30°C and plates were imaged daily for up to 10 days using a Bio-Rad ChemiDoc XR5 molecular imager with ImageLab software. Colony area and perimeter were measured using a macro written in ImageJ (NIH), which “autothresholded” images and determined colony area and perimeter.

**Cellular stress tests**

*Ashbya gossypii* spores were germinated in liquid AFM in the presence of ampicillin (100μg/mL), G418 (200μg/mL), and Clonat (50μg/mL) for 10 hours while shaking in baffled flasks at 30°C. Cells were transferred to fresh media with selection containing 2mM caffeine (Sigma-Aldrich), 200mM NaCl (Fisher Scientific), 10mM ZnSO₄ (Sigma-Aldrich), or 325nM fluconazole (Sigma-Aldrich) and returned to 30°C with shaking for an additional six hours. The appropriate fluconazole concentration was determined by comparing radial growth rates on AFM plates with ampicillin (100μg/mL) and 1.5% agarose, supplemented with
varying concentrations of fluconazole (Sigma-Aldrich). Control and heat-stressed cells were also transferred to fresh media after 10 hours and returned to 30°C or 37°C, respectively, for an additional six hours. Cells were spun down at 300rpm for 5 minutes, washed with 5 mL of 2X low fluorescence minimal media, and spun as before. 10μL of cells were taken directly from the pellet and plated onto glass slides for imaging.

**Statistical analysis of cellular stress populations**
A two sample Z test for proportions was applied to each population compared to the control population. For $p<0.05$, the Z value must be greater than 1.96 or less than -1.96. Strains were considered significantly different than the control if two or more categories were $p<0.05$.

\[
\frac{(\bar{p}_1 - \bar{p}_2)}{\sqrt{p (1-p) \left( \frac{1}{n_1} + \frac{1}{n_2} \right)}}
\]

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**References**


Figure 1 – DNA content varies among Ashbya nuclei
A) Schematic of predicted relative Hhf1-GFP intensity changes pre-replication
and post-replication. B) Hhf1-GFP intensity at birth and mitosis. Background-corrected GFP intensity was measured by thresholding around the central nuclear coordinate that was manually recorded immediately before and after mitosis (Anderson et al., 2013). N=47 nuclei. C) Background corrected Hhf1-GFP intensity summed for each nucleus over time. Each line represents one nucleus. N=47 nuclei. D) Fold change in Hhf1-GFP intensity over time for each nucleus tracked. Fold change was defined as the maximum Hhf1-GFP intensity normalized to the starting Hhf1-GFP intensity for each nucleus. N=47 nuclei. E) Histogram of DAPI intensity for all cell cycle phases. Cell cycle stage was scored by spindle pole body (SPB) state and GFP intensity was normalized to the 1SPB population mean. Black bars represent 1SPB (G1, N=126), green bars represent 2SPB (S/G2, N=117), and blue bars represent 2SPB that are bioriented (M, N=19).
Figure 2 – Individual chromosomes are variable between nuclei
A,B) Schematic of Ashbya chromosomes. All 7 chromosomes and the
mitochondrial DNA are shown to scale. Red ovals indicate centromeres, green bars indicate the location of the 32lacO::Gen3 integration onto Chromosome I (A) and Chromosome VI (B). The Chromosome I and IV lacO integrations are 126KB and 56KB away from the centromere, respectively. Still images of Ashbya nuclei containing 0-4 copies of both Chromosome I and Chromosome VI are shown beside each schematic. C) Quantification of chromosome counts in Ashbya nuclei. Chromosome I counts are indicated in black and Chromosome VI counts are indicated in grey (ChI, N=350; ChVI, N=364). D) Chromosome I counts are independent of cell cycle phase as indicated by spindle pole body (SPB) state. Dark blue bars represent Chromosome I distribution for 1SPB (G1) and light blue bars represent the Chromosome I distribution for 2SPB (S/G2/M). 1SPB; N=112, 2SPB; N=66. E) RNA F.I.S.H. of individual nuclei using CLN1/2-TAMRA probes that hybridize to mRNA. Signals in nucleus represent sites of gene expression and >90% nuclei express CLN1/2 transcript. Images of DNA (Hoechst), chromosomes, and a merge image show that individual nuclei containing 1 or 2 signals indicating sites where CLN1/2 is expressed in a single nucleus.
Figure 3 – *Ashbya* chromosome variation is independent of location in the cell and increases as cells age.
A) Chromosome I counts and cell age. Black bars represent the Chromosome I distribution for young cells (<40 nuclei) and grey bars represent the Chromosome I distribution for older cells (>40 nuclei). Young cells; N=178, Old cells; N=350. B) Cumulative distribution plot of Chromosome I counts and distance from cell tip (ANOVA, F=1.56, p>0.20).
Figure 4 – Ashbya faithfully segregates chromosomes at mitosis
A) Still images of chromosome segregation at mitosis. Top panels are frames
from Movie S1 showing 1 copy of Chromosome I being faithfully segregated. Arrowheads point to single chromosome spots. Bottom panels are frames from Movie S2 showing 2 copies of Chromosome I being segregated. In these panels, an asterisk (*) is centered between the two copies of Chromosome I in each nucleus. B) Sister Hhf1-GFP intensity at birth. The sum Hhf1-GFP intensity of the brighter sister is plotted in black with the dimmer sister overlaid in grey (N=15 pairs of sisters). C) K-S test plot of observed sister Hhf1-GFP intensities. The observed difference in sister Hhf1-GFP intensity at birth (immediately after mitosis) displayed as a cumulative distribution plot in black. A randomized difference was calculated for two different populations of observed nuclei; the lowest 10 observed Hhf1-GFP intensities were used as a distribution for 1N, and the highest 10 observed Hhf1-GFP intensities were used as a distribution for 2N. No difference is observed between the difference in sister Hhf1-GFP intensity and randomly pairing nuclei in these two sub-populations (N=15 sister pairs; compared to 1N (red line) - D=0.26, p=0.26; compared to 2N (blue line) - D=0.19, p=0.65).
Figure 5 – Ashbya nuclei are predominantly polyploid
A) Schematic of Ashbya chromosomes. All 7 chromosomes and the
mitochondrial DNA are shown to scale. Red ovals indicate centromeres, green bars indicate the location of the 32lacO::Gen3 integration onto Chromosome I and Chromosome VI. B) Schematic of ChI 32lacO::Gen3/ChVI 32-lacO::Nat1 results. Nuclei with 2, 4, or 8 resolvable LacI spots are polyploid. All other LacI spot counts would be evidence for aneuploidy. C) Quantification of chromosome counts in ChI 32-lacO::Gen3/ChVI 32-lacO::Nat1 Ashbya nuclei. Black bars represent the distribution for young cells (N<40 nuclei, N=118) and grey bars represent the distribution for older cells (N>40 nuclei, N=253).
Figure 6 – *mad2Δ* cells have decreased viability in *Ashbya*
Colony growth of wild-type (-ade2) and *mad2Δ::NAT1* heterokaryon strains.
Bars=SD, N=3 plates per strain. Images represent colony growth after 5 days.
Figure 7 – Chromosome number variation decreases in response to various cellular stresses.
Quantification of Chromosome I counts in *Ashbya* nuclei under normal growth and in the presence of various cellular stresses (N>85 nuclei per condition).