

Metabolism-induced oxidative stress and DNA damage selectively trigger genome instability in polyploid fungal cells

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Abstract

Understanding how cellular activities impact genome stability is critical to multiple biological processes including tumorigenesis and reproductive biology. The fungal pathogen *Candida albicans* displays striking genome dynamics during its parasexual cycle as tetraploid cells, but not diploid cells, exhibit genome instability and reduce their ploidy when grown on a glucose-rich “pre-sporulation” medium. Here, we reveal that *C. albicans* tetraploid cells are metabolically hyperactive on this medium with higher rates of fermentation and oxidative respiration relative to diploid cells. This heightened metabolism results in elevated levels of reactive oxygen species (ROS), activation of the ROS-responsive transcription factor Cap1, and the formation of DNA double-strand breaks. Genetic or chemical suppression of ROS levels suppresses each of these phenotypes and also protects against genome instability. These studies reveal how endogenous metabolic processes can generate sufficient ROS to trigger genome instability in polyploid *C. albicans* cells. We also discuss potential parallels with metabolism-induced instability in cancer cells and speculate that ROS-induced DNA damage could have facilitated ploidy cycling prior to a conventional meiosis in eukaryotes.

Keywords *Candida albicans*; DNA double-strand breaks; parasexual cycle; reactive oxygen species; tetraploid

Subject Categories Cell Cycle; DNA Replication & Repair; Microbiology, Virology & Host Pathogen Interaction

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Introduction

Polyploidy, a state where cells contain more than two copies of the genome, is ubiquitous among eukaryotic organisms. In *Arabidopsis*, polyploid cells can arise via gamete fusion or whole-genome

duplication (Bomblies & Madlung, 2014) and provide advantages such as heterosis, gene redundancy, and enhanced nutrient uptake due to polyploid cells being larger than diploid cells (Comai, 2005; Chao *et al.*, 2013). In humans, polyploidization is known to occur in heart muscle, liver, brain, and blood cells and is associated with both normal development and pathological conditions (Gentric & Desdouets, 2014). Polyploid cells form in uterine muscle tissue as a normal part of pregnancy and are thought to aid in embryonic implantation (Mori *et al.*, 2011; Sroga *et al.*, 2012). Cancer progression is also associated with polyploidization; aneuploid tumors often arise through formation of a tetraploid intermediate and promote malignancy by disrupting regulators of cell growth (Storchova & Pellman, 2004; Fujiwara *et al.*, 2005; Zack *et al.*, 2013).

Polyploid forms have also been characterized in diverse fungal lineages, including the budding yeast *Saccharomyces cerevisiae* which can exist in ploidy states from haploid (1n) to tetraploid (4n) (Zhu *et al.*, 2016). Higher ploidy states can arise via gamete fusion during a conventional sexual cycle or via asexual processes such as mitotic collapse. A characteristic feature of polyploid cells is increased genome instability, with tetraploid cells exhibiting rates of chromosome loss that are orders of magnitude greater than those of diploid cells (Mayer & Aguilera, 1990). Polyploidy and genome instability can facilitate evolution, with higher ploidy states undergoing adaptation faster than diploid or haploid cells due to higher mutation rates with greater fitness effects (Selmecki *et al.*, 2015). Ploidy shifts in *S. cerevisiae* cells are induced by a variety of environmental stresses, including culture in the presence of high salt or ethanol (Gerstein *et al.*, 2006; Voordeckers *et al.*, 2015).

Ploidy variation has also been described in the opportunistic fungal pathogen *Candida albicans*, a commensal species that can seed both debilitating mucosal and life-threatening systemic infections. Candidiasis is a major problem in the hospital setting and is exacerbated by the prevalence of drug-resistant *C. albicans* strains (Ksiezopolska & Gabaldon, 2018). Natural *C. albicans* isolates are diploid although rare haploid forms have been isolated, albeit with reduced fitness (Hickman *et al.*, 2013). As in *S. cerevisiae*, ploidy shifts can enable adaptation; diploid *C. albicans* cells exposed to the

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antifungal drug fluconazole form a transient tetraploid state that generates drug-resistant aneuploid progeny via aberrant divisions (Harrison *et al*, 2014).

Tetraploid *C. albicans* cells are also formed by fusion of diploid cells during mating (Hull *et al*, 2000; Magee & Magee, 2000; Bennett & Johnson, 2003). Sexual reproduction in *C. albicans* is unusual in that cells must undergo an epigenetic switch from the sterile “white” state to the mating-competent “opaque” state (Lockhart *et al*, 2002; Miller & Johnson, 2002; Bennett, 2015). In addition, rather than undergoing a conventional meiosis, tetraploid cells return to a diploid (or aneuploid) state via aberrant mitotic divisions, a process termed “concerted chromosome loss” (CCL; Bennett & Johnson, 2003; Forche *et al*, 2008; Hickman *et al*, 2015). Growth on *S. cerevisiae* “pre-sporulation” (PRE-SPO) medium at 37°C induces CCL in tetraploid cells, whereas diploid cells are stable under these culture conditions. In addition to genome instability, many tetraploid cells undergo cell death during growth on PRE-SPO medium whereas most diploid cells remain viable. However, the mechanism whereby PRE-SPO medium induces polyploid-specific responses in *C. albicans* has yet to be addressed.

In this work, we propose that a metabolic shift triggers genome instability in polyploid *C. albicans* cells. Transcriptional and metabolic profiling reveals that *C. albicans* cells grown on glucose-rich PRE-SPO medium are metabolically hyperactive, with increased rates of both fermentation and oxidative respiration. Critically, cellular metabolism is further upregulated in polyploid cells relative to diploid cells. We demonstrate that elevated metabolic flux results in the production of reactive oxygen species (ROS) and DNA damage, and that the latter is a driver of genome instability in polyploid cells. These studies therefore establish links between metabolic flux, ROS, oxidative stress, and DNA damage in *C. albicans*, and that this mechanism selectively impacts genome integrity in polyploid cells. We discuss parallels with tumorigenesis in mammalian cells and propose that metabolism-induced genome instability may have promoted primitive ploidy cycles prior to a conventional meiosis.

Results

Polyploid *Candida albicans* cells undergo CCL on PRE-SPO medium

A conventional meiosis has not been observed in *C. albicans* despite its genome containing numerous conserved meiosis genes (Tzung *et al*, 2001; Butler *et al*, 2009). Instead, tetraploid mating products reduce their ploidy via a parasexual program of CCL, during which tetraploid cells return to a diploid or aneuploid state (Bennett & Johnson, 2003; Forche *et al*, 2008). Concerted chromosome loss can be efficiently induced by incubation of tetraploid *C. albicans* cells on *S. cerevisiae* PRE-SPO medium at 37°C (Bennett & Johnson, 2003). Tetraploid cells experience extensive cell death during the first 3 days of growth on PRE-SPO medium followed by a rebound in viability, whereas diploid cells largely remain viable under these conditions (Fig 1A), as previously reported (Bennett & Johnson, 2003). We find that tetraploid cells grown on PRE-SPO medium also exhibit erratic microtubule and nuclear dynamics, indicative of aberrant nuclear divisions on this medium (Fig 1B).

To monitor genome stability, we used a tetraploid strain, RBY18, that is heterozygous for *GAL1* on chromosome 1 (*GAL1/GAL1/gal1/gal1*) (Fig 1C). Only strains that have lost *GAL1* function are viable on 2-deoxygalactose (2-DOG) medium (Gorman *et al*, 1992), providing a selection for tetraploid cells that have lost the two *GAL1* alleles and undergone a reduction in ploidy (Bennett & Johnson, 2003). Genome stability in diploid cells was monitored using strain RBY1, which is also heterozygous for *GAL1* on chromosome 1 (*GAL1/gal1*). When RBY18 was cultured on PRE-SPO medium at 37°C for 7 days, 5.8% of cells lost *GAL1* and became 2-DOG^R, an increase of 116-fold over cells grown on YPD medium (0.05% 2-DOG^R cells; Fig 1D). In contrast, there was no significant difference in *GAL1* loss in diploid RBY1 cells cultured on PRE-SPO v. YPD medium (Fig 1D). Flow cytometric analysis of DNA content in 2-DOG^R colonies recovered from PRE-SPO medium indicated that tetraploid (4n) cells had reduced their ploidy and now exhibited a DNA content between diploid (2n) and triploid (3n) (Fig 1E).

Transcriptional profiling of diploid and tetraploid cells on YPD and PRE-SPO media

RNA sequencing (RNA-seq) was used to examine gene expression of *C. albicans* cells grown on standard YPD medium (at 30°C) or on PRE-SPO medium (at 37°C). YPD cultures were performed at the lower temperature as extensive filamentation occurs during growth on YPD, but not PRE-SPO, medium at 37°C. Both diploid and tetraploid cells were analyzed on these media at 3, 6, 12, and 24 h time points.

RNA-seq analysis revealed that diploid and tetraploid cells showed similar gene expression profiles on both YPD and PRE-SPO media, despite clear differences in cell survival and genome stability on the two media (Fig 2A and Table EV1). Notably, gene ontology (GO) term analysis revealed that processes related to DNA double-strand break (DSB) formation, DNA repair, DNA recombination, and meiosis were significantly enriched among differentially expressed genes between cells grown on the two media (24 h time point, Fig 2B), with an upregulation of these processes on PRE-SPO relative to YPD medium (Fig 2C). However, no obvious ploidy-specific expression patterns of meiosis-specific genes were observed (Fig EV1). We also conducted GO term analysis on differentially expressed genes between diploid and tetraploid cells grown on PRE-SPO medium. At both 3 h and 6 h, nicotinamide binding and acetate metabolism genes were enriched among differentially expressed genes (Appendix Tables S1 and S2), while at 12 h and 24 h DNA replication, regulation of helicase activity and DNA binding genes were identified (Appendix Tables S3 and S4). These results implicated metabolism and DNA damage as potentially relevant to the distinct behavior of tetraploid cells grown on PRE-SPO v. YPD media.

Inspection of metabolic gene expression also revealed that glycolytic genes were induced earlier on PRE-SPO than on YPD medium in both diploid and tetraploid cells (compare 6 h time points, Fig 3A). In addition, oxidative stress-associated genes, including those encoding the Sod3 superoxide dismutase and the Ddr48 DNA damage response factor, were activated at earlier time points on PRE-SPO medium. These results indicate that growth on PRE-SPO medium potentially results in enhanced metabolic activity and increased activation of oxidative stress responses than that on YPD medium.

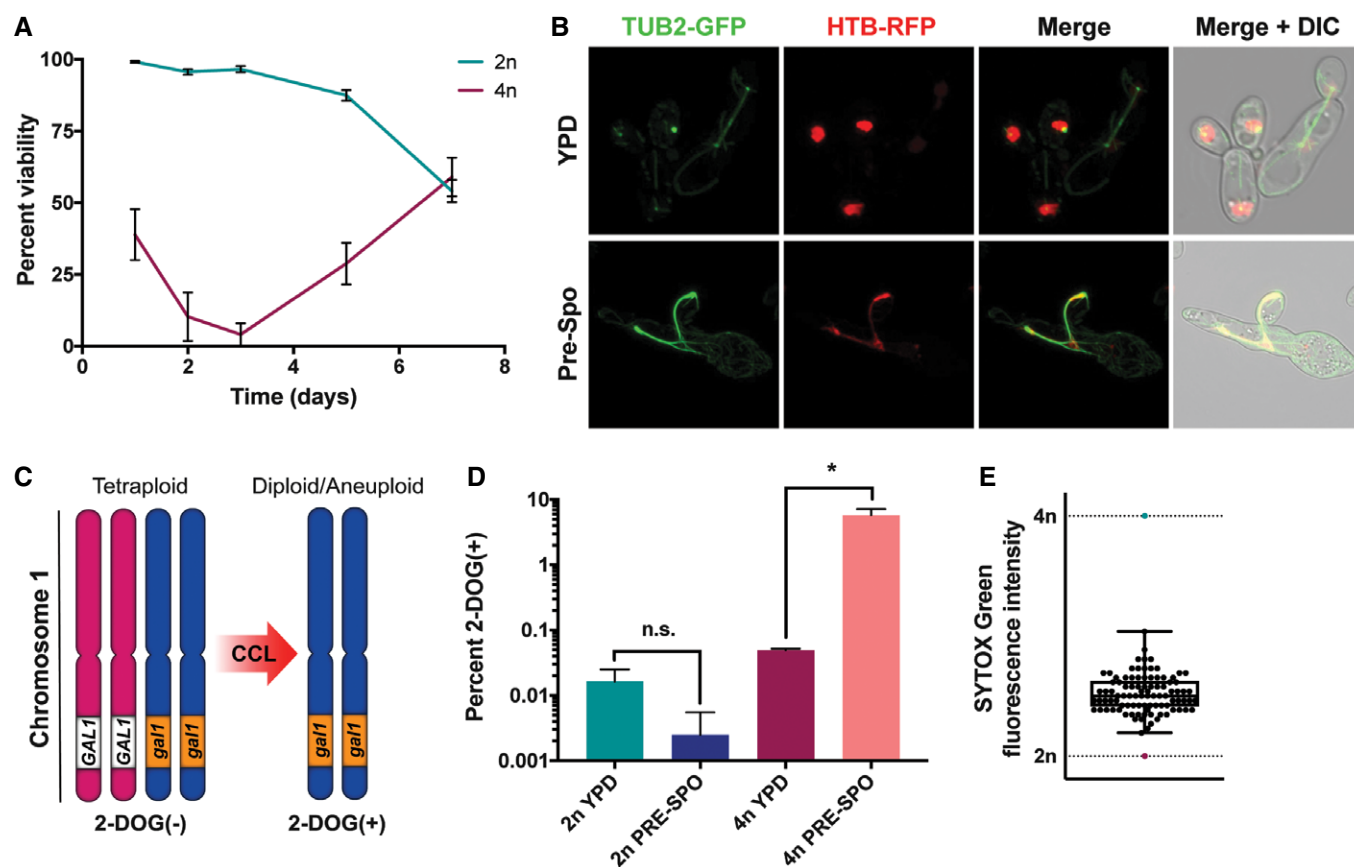


Figure 1. Tetraploid *C. albicans* cells undergo genome instability and cell death on PRE-SPO medium.

- A Viability of diploid (SC5314) and tetraploid (RBY18) cells grown on PRE-SPO medium at 37°C ($n = 5$, error bars represent SEM).
- B A tetraploid β -tubulin (TUB2)-GFP, histone H2B (HTB)-RFP strain was cultured on YPD or PRE-SPO medium at 30 or 37°C, respectively, for 24 h. Cell images indicate maximum intensity Z projections for GFP and RFP channels, and merged images of the GFP/RFP and GFP/RFP/DIC channels.
- C Assay to monitor for loss of *GAL1* in tetraploid strain RBY18. Strain RBY18 is heterozygous for the *GAL1* gene, and only cells that have lost *GAL1* function can grow on 2-DOG medium.
- D Diploid strain RBY1 and tetraploid strain RBY18 were cultured on YPD or PRE-SPO medium at 30 or 37°C, respectively, for 7 days and plated onto 2-DOG medium to monitor for *GAL1* loss. (*) denotes a statistically significant difference between the indicated sample groups ($P < 0.05$, Student's t-test, $n = 3$, error bars represent SEM).
- E 2-DOG^R progeny arising from RBY18 on PRE-SPO medium were DNA-stained and their ploidy determined via flow cytometry. Box extends from the 25th to 75th percentiles, center line represents the median value, and upper and lower whiskers represent the maximum and minimum values from the analyzed progeny ($n = 94$).

Candida albicans tetraploid cells are metabolically hyperactive on PRE-SPO medium

Given that *C. albicans* cells show earlier activation of glycolytic and oxidative stress genes on PRE-SPO versus YPD medium, we compared the metabolic activity of diploid and tetraploid cells on these media using several complementary approaches. First, a 2,3,5-triphenyltetrazolium chloride (TTC) overlay technique was performed as this method can assess activity of the electron transport chain in microbial populations (Ogur *et al*, 1957; Rich *et al*, 2001; Morales *et al*, 2013). TTC is white and can passively diffuse through the plasma membrane to interact with mitochondria, where it is converted to red 1,3,5-triphenylformazan (TPF). We grew equivalent numbers of diploid and tetraploid *C. albicans* cells on YPD and PRE-SPO media at 30 and 37°C, respectively, and after 24 h, TTC was applied to each plate. We found that diploid and

tetraploid colonies generated greater reddening on PRE-SPO medium than on YPD medium, with tetraploid cells also producing more color than diploid cells suggestive of heightened respiratory activity (Fig 3B).

Next, we examined media acidification which is indicative of the formation of acidic fermentation end products such as acetate (Morales *et al*, 2013). YPD and PRE-SPO media were supplemented with the pH indicator bromocresol purple, which is yellow at pH values below 5.2 and purple at pH values above 6.8. Equal numbers of diploid and tetraploid cells were inoculated onto YPD and PRE-SPO media and grown for 24 h at 30 and 37°C, respectively. Diploid and tetraploid cells both acidified PRE-SPO medium whereas the pH was largely unchanged with cells grown on YPD medium (Fig 3C), suggesting increased production of acidic fermentation end products during growth on PRE-SPO medium.

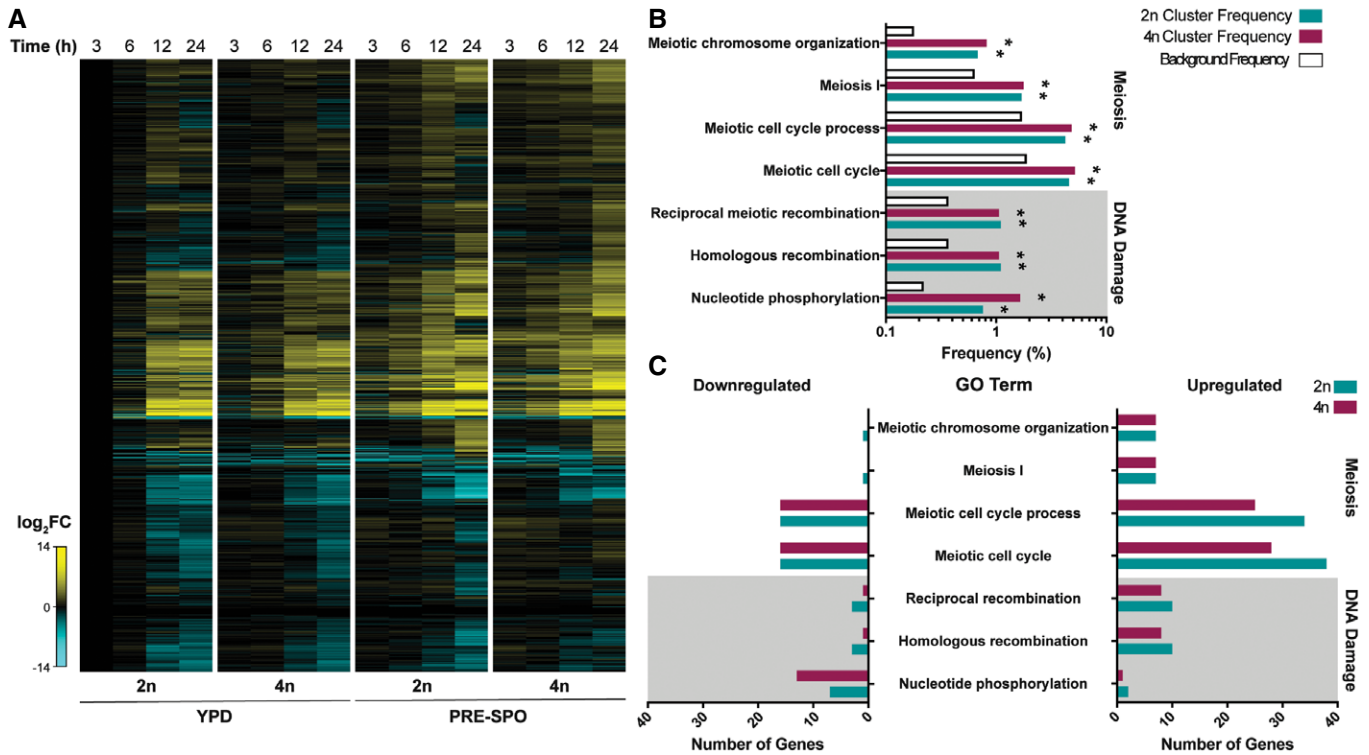


Figure 2. Transcriptional profiling of *Candida albicans* diploid and tetraploid cells on YPD and PRE-SPO medium.

A Heatmap of RNA-seq data showing global gene expression changes in diploid (SC5314, 2n) and tetraploid (RBY18, 4n) cells on YPD and PRE-SPO medium over 24 h. **B** Results of gene ontology (GO) term enrichment analysis of differentially expressed genes between YPD and PRE-SPO media at 24 h in diploid (2n) and tetraploid (4n) cells. Background frequency represents the total number of genes annotated to each GO term in the *C. albicans* genome divided by the total number of genes in the genome. (*) denotes a statistically significant enrichment of genes annotated to the indicated GO term ($P < 0.05$, Fisher's exact test). **C** Number of genes in each category that are upregulated or downregulated on PRE-SPO medium v. YPD medium in diploid (2n) and tetraploid (4n) cells for the indicated GO terms.

To further characterize the metabolic activities of diploid and tetraploid cells, aerobic respiratory and fermentative rates were assayed by measuring the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR), respectively. *C. albicans* cells were grown on YPD and PRE-SPO media for 24 h at 30 and 37°C, respectively, and transferred to a Seahorse instrument to measure metabolic rates. Interestingly, we found that metabolic activities were considerably higher in polyploid cells than in diploid cells; both OCR and ECAR were ~4-fold higher (YPD medium) or ~8-fold higher (PRE-SPO medium) in tetraploid cells compared to diploid cells (Fig 3D). We also found that cells grown on PRE-SPO medium exhibited significantly higher OCR and ECAR values relative to the same cells grown on YPD medium. Thus, tetraploid cells showed a 2.9-fold increase in OCR and a 2.1-fold increase in ECAR when grown on PRE-SPO relative to YPD, while diploid cells exhibited a 1.5-fold increase in OCR and 1.9-fold increase in ECAR between the two media (Fig 3D). The OCRs that we observed were due to mitochondrial respiration and not other oxygen-consuming processes as these were blocked by addition of the electron transport chain inhibitors antimycin A and oligomycin together with the alternative oxidase inhibitor salicylhydroxamic acid (SHAM) (Fig EV2A). ECARs were not inhibited by this treatment (Fig EV2B). Together, these results establish that both cell ploidy and culture media impact

C. albicans metabolism; growth on PRE-SPO medium enhances metabolic activity relative to that on YPD medium, and tetraploid cells are also considerably more active than diploid cells.

Given that tetraploid cells exhibited a higher OCR than diploid cells, we sought to probe the relationship between ploidy and mitochondrial content. Diploid and tetraploid cells were grown on YPD medium for 24 h at 30°C and stained with the mitochondrial marker MitoTracker Green. Cells were analyzed by flow cytometry to determine mitochondrial content and by microscopy to examine cell size. Tetraploid cells had a cell volume ~2.2-fold greater than diploid cells, and this correlated with an increased MitoTracker Green signal indicating an increased mitochondrial content (Fig 3E). These results indicate that *C. albicans* tetraploid cells are more metabolically active than diploid cells due, at least in part, to their larger size and increased mitochondrial content.

***Candida albicans* cells experience elevated oxidative stress on PRE-SPO medium**

Transcriptional profiling showed that oxidative stress genes were upregulated more rapidly in *C. albicans* cells grown on PRE-SPO than on YPD medium (Fig 3A), and we therefore directly evaluated oxidative stress responses on the two media. To determine whether

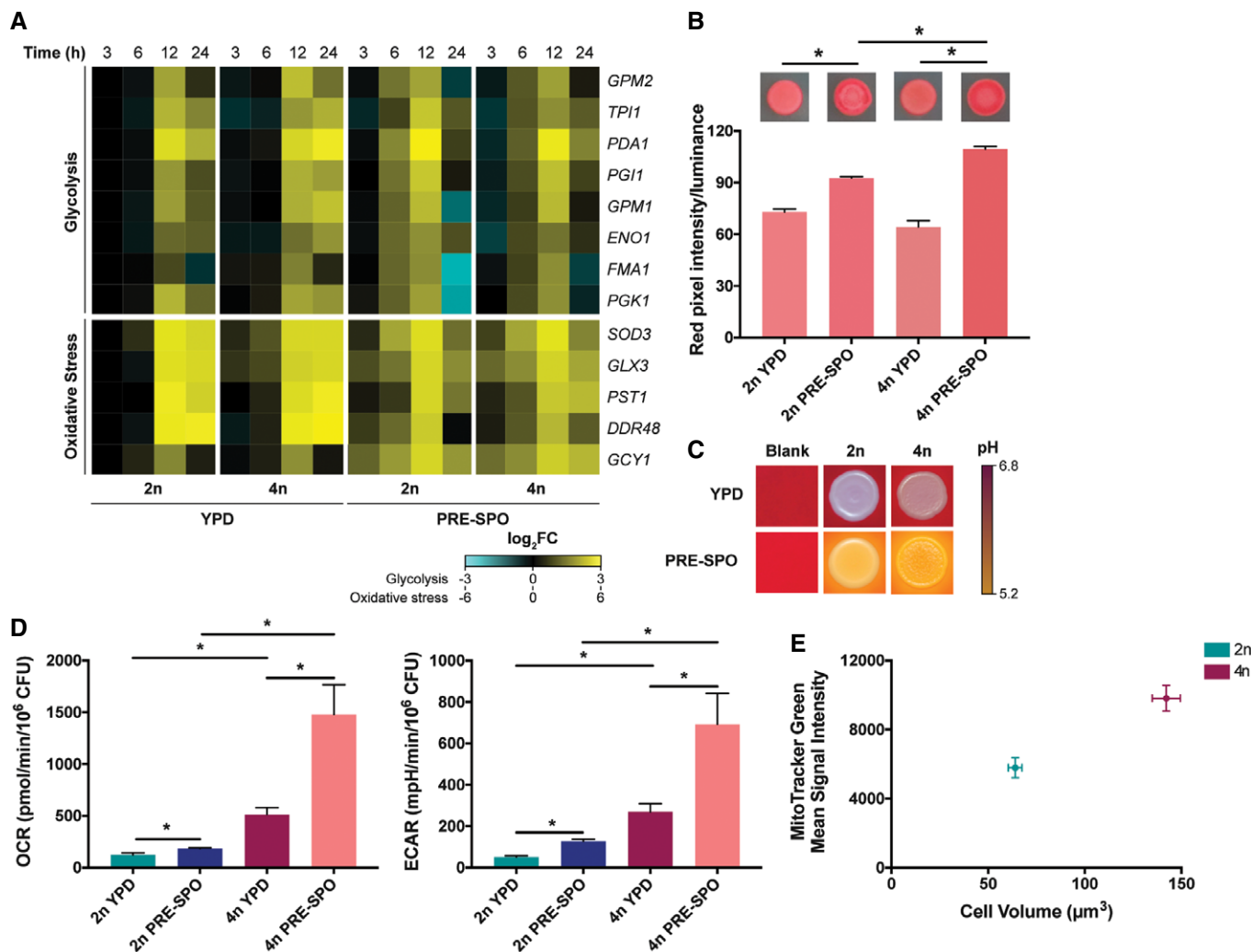


Figure 3. Analysis of metabolic and oxidative stress responses in diploid and tetraploid cells grown on YPD and PRE-SPO medium.

- A Heatmap of RNA-seq data for glycolysis and oxidative stress genes in diploid (2n) and tetraploid (4n) cells grown on YPD or PRE-SPO medium over 24 h.
- B TTC assay results in diploid and tetraploid cells grown on YPD or PRE-SPO medium at 30 or 37°C, respectively. Cells were grown for 24 h at which point a TTC overlay solution was applied and color allowed to develop for 15 min. Calculated values represent the total amount of reddening. (*) denotes a significant difference between the indicated groups ($P < 0.05$, Student's *t*-test, $n = 5$, error bars represent SEM).
- C Diploid and tetraploid strains were spot-inoculated onto YPD or PRE-SPO medium supplemented with the pH indicator bromocresol purple and cultured for 24 h at 30 or 37°C, respectively.
- D Analysis of metabolic flux in diploid and tetraploid cells following growth for 24 h on YPD or PRE-SPO medium (30 or 37°C, respectively), using a Seahorse instrument. OCR values represent rates of aerobic respiration, while ECAR values represent rates of fermentation. (*) denotes a significant difference between the indicated experimental groups ($P < 0.05$, Student's *t*-test, $n = 5$, error bars represent SEM).
- E Cell size and mean MitoTracker Green signal intensity in diploid and tetraploid cells grown on YPD medium for 24 h at 30°C ($n = 100$ and $n = 5$ for cell volume and MitoTracker Green measurements, respectively, error bars represent SEM).

reactive oxygen species (ROS) were present, diploid and tetraploid cells were grown for 24 h on YPD and PRE-SPO media at 30 and 37°C, respectively, and stained with CellROX Green. This is a membrane permeant dye that fluoresces upon oxidation by ROS and DNA binding. No observable fluorescent signal was present in cells grown on YPD medium when examined via microscopy, whereas both diploid and tetraploid cells grown on PRE-SPO medium stained positively indicating that ROS are generated on this medium (Fig 4A). Flow cytometric analysis of CellROX green-stained cells corroborated cell microscopic images, with an increased

fluorescence signal in diploid/tetraploid cells on PRE-SPO relative to YPD medium (Fig 4B). Notably, tetraploid cells grown on PRE-SPO medium exhibited the strongest signal, with 2.0-fold higher fluorescence levels than diploid cells on this medium (Fig 4B).

Previous work showed that *C. albicans* also produces ROS during hyphal morphogenesis (Rossi *et al*, 2017). Consistent with this finding, cells grown on YPD medium at 37°C, a growth condition which induces filamentation, stained positively with CellROX Green (Fig EV3A). However, a fluorescence signal was absent in cells grown on SCD medium at 37°C (Fig EV4A), a growth condition

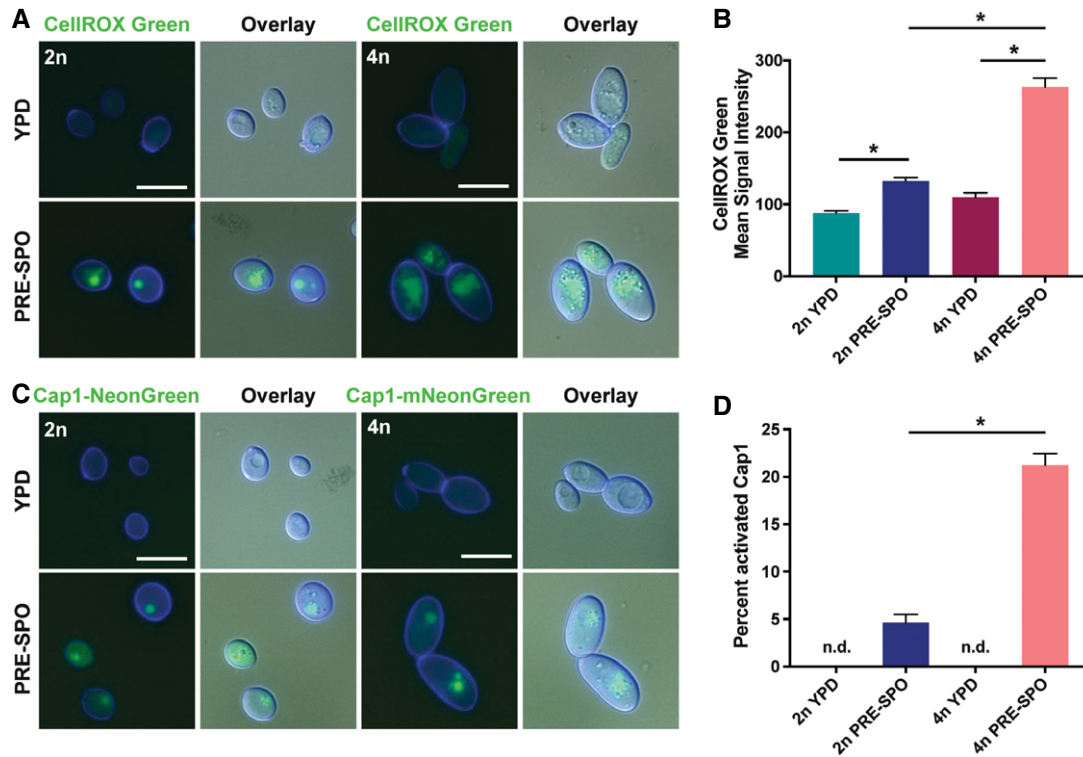


Figure 4. *Candida albicans* cells accumulate ROS and activate oxidative stress responses when cultured on PRE-SPO medium.

- A Diploid and tetraploid cells were grown on YPD or PRE-SPO medium at 30 or 37°C, respectively, for 24 h and stained with CellROX Green. Cell images indicate calcofluor white staining (cell wall; blue), GFP, and a merged image of GFP/DAPI/DIC channels. Scale bar = 10 μ m.
- B Diploid and tetraploid cells were grown on YPD or PRE-SPO medium at 30 or 37°C, respectively, for 24 h, stained with CellROX Green, and signal analyzed via flow cytometry. (*) denotes a significant difference between the indicated groups ($P < 0.05$, Student's *t*-test, $n = 5$, error bars represent SEM).
- C Diploid and tetraploid cells containing a fluorescently labeled version of the transcription factor Cap1 (Cap1-mNeonGreen) were grown on YPD or PRE-SPO medium at 30 or 37°C, respectively, for 24 h. Cell images indicate calcofluor white staining (cell wall; blue), GFP, and a merged image of GFP/DAPI/DIC channels. Scale bar = 10 μ m.
- D Quantitation of the percentage of diploid and tetraploid cells with activated Cap1 on YPD or PRE-SPO medium. "n.d." indicates that no cells with activated Cap1 were detected. (*) denotes a significant difference between the indicated groups ($P < 0.05$, Student's *t*-test, $n = 5$, error bars represent SEM).

where filamentation does not occur, indicating that growth at 37°C is not sufficient for ROS production by *C. albicans* cells.

We next examined oxidative stress responses using a fluorescently tagged Cap1 protein, a transcription factor that regulates multiple oxidative stress response pathways in *C. albicans* including antioxidant defense, protein degradation, and drug resistance (Wang *et al.*, 2006). Cap1 is distributed homogeneously between the nucleus and the cytoplasm under non-stress conditions, but accumulates in the nucleus in response to oxidative stress to activate antioxidant gene expression (Patterson *et al.*, 2013). To assay for Cap1 activation, diploid and tetraploid strains were generated expressing fluorescently labeled Cap1-mNeonGreen. These reporter strains were grown for 24 h on YPD and PRE-SPO media at 30 and 37°C, respectively, and imaged to examine Cap1 localization. Diploid and tetraploid cells grown on PRE-SPO medium exhibited a clear nuclear Cap1 signal, whereas cells grown on YPD exhibited only a diffuse signal (Fig 4C). Growth at 37°C was not solely responsible for the observed phenotypes on PRE-SPO medium, as tetraploid cells grown on YPD medium at 37°C also did not exhibit a nuclear Cap1 signal (Fig EV3B). Quantitation of viable cells revealed that more

tetraploid cells than diploid cells activated Cap1 during PRE-SPO culture (Fig 4D). These results establish that tetraploid cells experience higher levels of oxidative stress than diploid cells when cultured on this medium.

Suppression of ROS protects cells from oxidative stress and instability

To determine whether oxidative stress contributes to decreased tetraploid cell viability, PRE-SPO medium was supplemented with varying levels of the antioxidants ascorbic acid, dithiothreitol (DTT), or glutathione. Each of these antioxidants significantly increased viability on PRE-SPO medium in a dose-dependent manner, with higher concentrations providing greater protection (Fig 5A). Antioxidants were also tested for their ability to mitigate genome instability in tetraploid cells grown on PRE-SPO medium. All of the tested antioxidants significantly reduced genome instability, with higher concentrations again providing a greater protective effect (Fig 5B). Finally, antioxidants were examined at the concentrations that provided the greatest protective effect against cell death and

genome instability to determine whether oxidative stress responses were reduced. At these optimized concentrations (glutathione, 1 mM; DTT, 1 mM; ascorbic acid, 20 mM), each antioxidant significantly reduced Cap1 activation in cells grown on PRE-SPO medium (Fig 5C).

We also assessed the ability of endogenous enzymatic activities to protect against genome instability on PRE-SPO medium. Tetraploid strains were constructed containing a doxycycline repressible “Tet-OFF” cassette to regulate overexpression (O/E) of antioxidant genes including *SOD1* and *SOD3*, encoding two cytosolic superoxide dismutases (Lamarre et al, 2001; Hwang et al, 2002), *SOD2*, a predicted mitochondrial superoxide dismutase (Rhie et al, 1999), *TRX1*, encoding the major thioredoxin (da Silva Dantas et al, 2010), and *GCS1*, encoding a key enzyme in glutathione biosynthesis (Baek et al, 2004). While each of these genes is linked to antioxidant

responses, we note that *SOD1* and *SOD3* have also been recently shown to minimize glucose uptake in *C. albicans* (Broxton et al, 2018). Ectopic expression of each of the tested enzymes except *SOD2* rescued genome instability of tetraploid cells (Fig 5D). Together, these results indicate that oxidative stress contributes to both cell death and chromosome instability of tetraploid *C. albicans* cells grown on PRE-SPO medium.

DNA double-strand breaks are generated by growth on PRE-SPO medium

A key mechanism by which ROS induces cellular toxicity is through structural changes to DNA including base modifications, DNA cleavage events, and DNA–protein cross-links (Jena, 2012). Given that detectable levels of ROS accrue in cells during growth on PRE-SPO

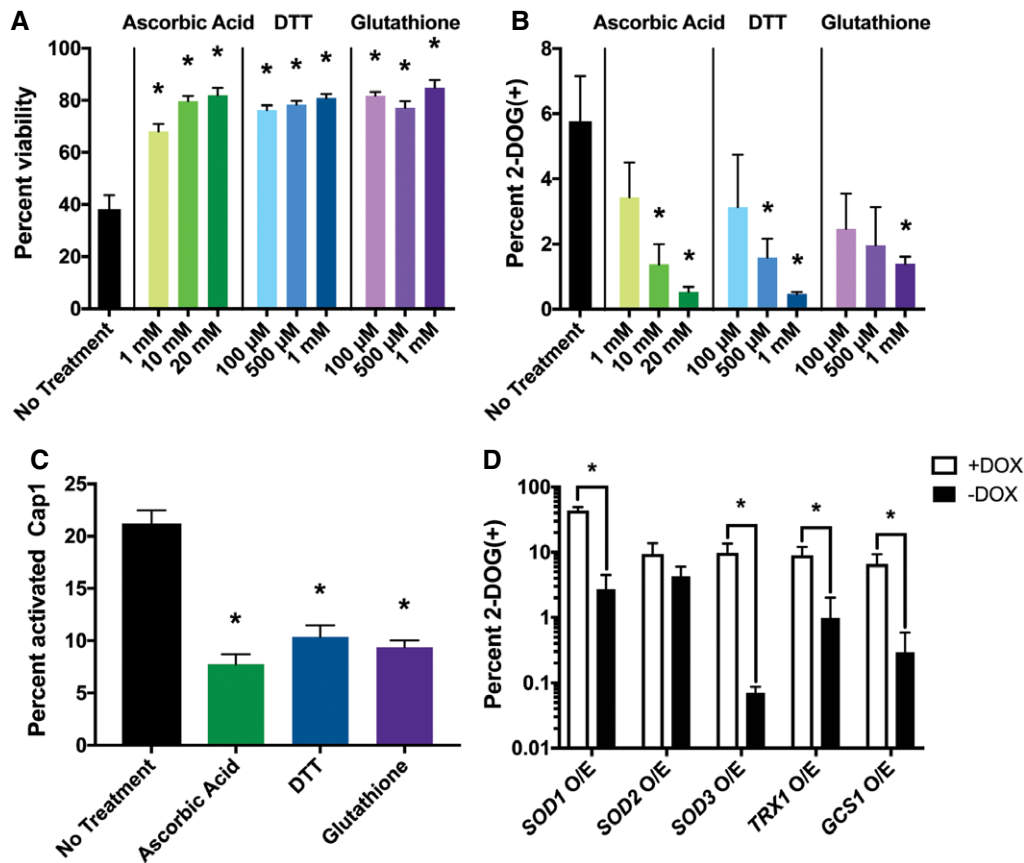


Figure 5. Antioxidants protect against cell death and genome instability in tetraploid cells grown on PRE-SPO medium.

- A Analysis of cell viability in tetraploid cells after culture on PRE-SPO medium (37°C for 24 h) when supplemented with ascorbic acid, DTT, or glutathione at the indicated concentrations. (*) denotes a significant difference in viability between cells with and without antioxidant ($P < 0.05$, Student's t -test, $n = 5$, error bars represent SEM).
- B Analysis of *GAL1* loss (2-DOG resistant colonies) in tetraploid strain RBY18 after growth on PRE-SPO medium with or without antioxidants for 7 days at 37°C. (*) denotes a significant difference between the percentage of 2-DOG(+) colonies with and without antioxidant ($P < 0.05$, Student's t -test, $n = 3$, error bars represent SEM).
- C Tetraploid cells expressing Cap1-mNeonGreen were grown on PRE-SPO medium supplemented with 20 mM ascorbic acid, 1 mM DTT, or 1 mM glutathione for 24 h at 37°C. Cell images were analyzed to determine the percentage of viable cells with activated Cap1. (*) denotes a significant difference in Cap1 activation between cells with and without antioxidant ($P < 0.05$, Student's t -test, $n = 5$, error bars represent SEM).
- D Assay to monitor for loss of *GAL1* from tetraploid RBY18 overexpressing the indicated genes. Cells were grown for 7 days at 37°C on PRE-SPO medium in the presence (gene OFF) or absence (gene ON) of doxycycline (DOX). (*) denotes a significant difference between + and – DOX conditions ($P < 0.05$, Student's t -test, $n = 3$, error bars represent SEM).

medium and that these cells exhibit activation of oxidative stress responses, we examined whether DNA double-strand breaks (DSBs) are formed.

To track formation of DSBs in *C. albicans*, we generated a reporter using a viral Gam protein that binds irreversibly to sites of DSBs. Fluorescent labeling of Gam has been shown to enable the visualization of DSBs in live mammalian and bacterial cells (Shee *et al*, 2013; Belenky *et al*, 2015). As a control, a tetraploid *C. albicans* strain containing a doxycycline-inducible (Tet-ON) Gam-GFP construct was treated with methyl methanesulfonate (MMS) or hydroxyurea (HU), and both DNA-damaging agents resulted in a strong nuclear signal that was also detected by flow cytometry (Fig EV4A and B).

To assay for DSB formation on different media, diploid and tetraploid Gam-GFP reporter strains were grown for 24 h on YPD and PRE-SPO media at 30 and 37°C, respectively, and examined by fluorescence microscopy and flow cytometry. An intense nuclear signal was detected in both diploid and tetraploid cells cultured on PRE-SPO medium, whereas no signal was observed on YPD medium (Fig 6A). This finding was corroborated by flow cytometry where 57% of tetraploid cells became Gam-GFP positive after 24 h on PRE-SPO medium versus 0.2% on YPD medium (see +DOX cells, Fig 6B). Growth at 37°C was not solely responsible for DSBs on PRE-SPO medium as tetraploid cells cultured on YPD medium at 37°C did not exhibit a nuclear Gam-GFP signal and the percentage of Gam-GFP-positive cells increased minimally, albeit significantly, relative to growth on YPD medium at 30°C (Fig EV3C and D).

We next examined if protection from oxidative stress would reduce DSB formation in tetraploid cells grown on PRE-SPO medium. The tetraploid Gam-GFP reporter strain was cultured with and without antioxidants on PRE-SPO medium for 24 h and cells examined using fluorescence microscopy and flow cytometry. Interestingly, all antioxidants significantly reduced the formation of DSBs on PRE-SPO medium, with ascorbic acid blocking most DSB formation (Fig 6C). These results demonstrate that *C. albicans* tetraploid cells experience extensive DSB formation when cultured on PRE-SPO medium and that chemical agents that diminish ROS levels can reduce these high levels of DNA damage.

Tetraploid cells exposed to DNA-damaging agents undergo ploidy reduction

DNA damage (including DSBs) can impact genome integrity (Jackson & Bartek, 2009; Mehta & Haber, 2014), and we therefore tested the effect of hydroxyurea (HU) and methyl methanesulfonate (MMS) on *C. albicans* cell viability and genome stability. As shown above, both DNA-damaging agents promote DSB formation in tetraploid *C. albicans* cells using the Gam-GFP reporter (Fig EV3A and B). When assayed for sensitivity to MMS and HU at 30 and 37°C, tetraploid cells were more sensitive to both compounds compared to diploid cells, and this was particularly evident at 37°C (Fig 7A). To examine whether DNA damage promotes chromosome instability, the tetraploid RBY18 strain was exposed to HU, MMS, or ultraviolet (UV) radiation and cells assayed for those that had lost *GAL1* and become 2-DOG^R. Exposure of cells to each of these genotypic stresses caused significantly elevated frequencies of 2-DOG^R colonies (Fig 7B), indicating that DNA damage led to increased chromosome instability in tetraploid cells.

Evidence that oxidative stress induces genome instability in tetraploid cells

The experiments described above indicate that endogenous ROS are associated with DNA damage and that this can induce genome instability in tetraploid *C. albicans* cells. We next sought to characterize if exogenous oxidative stress could recapitulate the phenotypes seen on PRE-SPO medium and cause chromosome instability. Tetraploid *C. albicans* cells were exposed to hydrogen peroxide (H₂O₂), as well as the oxidative stress-inducing agents paraquat (PQT) and piperlongumine (PL). PQT generates superoxide anions within the mitochondrial respiratory chain (Cocheme & Murphy, 2008), whereas PL is a potential anticancer compound that increases ROS and selectively kills cancer cells (Karki *et al*, 2017). When exposed to H₂O₂, PQT, or PL for 24 h at 37°C, tetraploid cells stained positively for ROS and Cap1 protein translocated into the nucleus, consistent with cells experiencing increased oxidative stress (Fig 8A and B). A nuclear Gam-GFP signal was also observed in tetraploid cells exposed to each of these agents, indicative of DSB formation in the presence of these stressors (Fig 8C and D). Moreover, analysis of *GAL1* marker stability revealed that H₂O₂, PQT, and PL induced significantly elevated levels of genome instability in tetraploid cells (Fig 8E). Together, these results establish that exposure to oxidative stress can induce DSB formation and chromosome instability in tetraploid cells in a manner that parallels that observed during growth on PRE-SPO medium.

Discussion

Ploidy change is a common occurrence in eukaryotic cells being integral to sexual reproduction, tumorigenesis, and the development of certain somatic tissues (Storchova & Pellman, 2004; Bennett *et al*, 2014; Todd *et al*, 2017). A striking example of ploidy change occurs in *C. albicans* where diploid cells mate to form tetraploid cells that then reduce their ploidy via aberrant mitotic divisions (Bennett & Johnson, 2003; Forche *et al*, 2008; Bennett, 2015). Ploidy reduction is promoted by growth of tetraploid cells on PRE-SPO medium whereas diploid cells are stable under these conditions. In this work, we reveal that nutritional cues trigger both genomic instability and cell death in tetraploid cells and identify causal links to metabolism, ROS production, and DNA double-strand break (DSB) formation.

The elevated metabolism of polyploid cells enhances ROS levels

Candida albicans tetraploid cells are relatively stable during standard culture although long-term passaging, as in *S. cerevisiae*, causes cells to reduce their ploidy toward the diploid state (Gerstein *et al*, 2006; Hickman *et al*, 2015; Selmecki *et al*, 2015). Growth on PRE-SPO medium influences genome instability in a ploidy-specific manner; tetraploid cells, but not diploid cells, show reduced viability and increased chromosomal instability when grown at 37°C on this medium. Expression profiling revealed limited differences between diploid and tetraploid *C. albicans* cells, consistent with studies in *S. cerevisiae* where ploidy and cell size have only a modest effect on the transcriptome (Galitski *et al*, 1999; Wu *et al*,

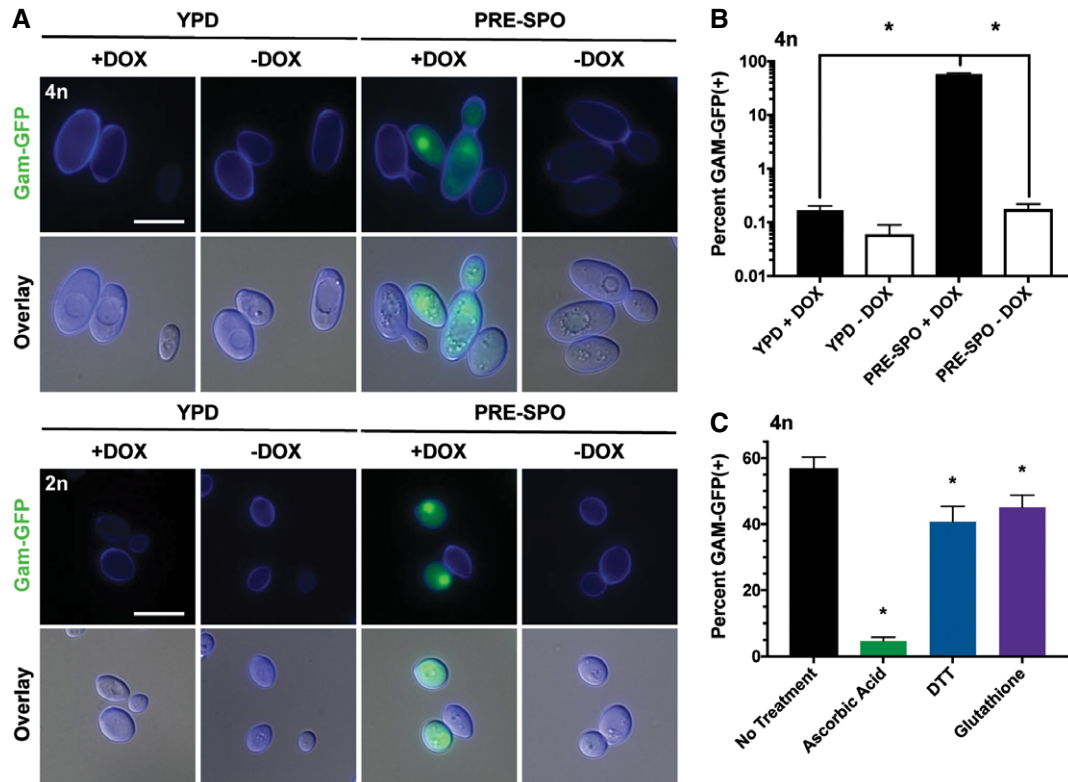


Figure 6. Oxidative stress contributes to DNA double-strand break formation during *Candida albicans* growth on PRE-SPO medium.

- A Diploid (2n) and tetraploid (4n) strains expressing a Gam-GFP reporter were cultured on YPD or PRE-SPO medium at 30 or 37°C, respectively, in the presence (Gam-GFP ON) or absence (Gam-GFP OFF) of doxycycline (DOX) for 24 h. Cell images indicate calcofluor white staining (cell wall; blue), GFP, and a merged image of GFP/DAPI/DIC channels. Scale bar = 10 μm.
- B Flow cytometric analysis of the tetraploid Gam-GFP reporter strain grown on YPD or PRE-SPO medium at 30 or 37°C, respectively, in the presence or absence of doxycycline for 24 h. (*) denotes a significant difference in the percentage of Gam-GFP(+) cells between experimental groups ($P < 0.05$, Student's *t*-test, $n = 3$, error bars represent SEM).
- C Flow cytometric analysis of the tetraploid Gam-GFP reporter strain grown on PRE-SPO medium with or without 20 mM ascorbic acid, 1 mM DTT, or 1 mM glutathione at 37°C for 24 h. (*) denotes a significant difference between Gam-GFP cells with and without antioxidant ($P < 0.05$, Student's *t*-test, $n = 3$, error bars represent SEM).

2010). The accelerated induction of glycolytic and oxidative stress response genes, however, suggested that increased metabolic activity could contribute to the phenotypes observed on PRE-SPO medium.

A direct assessment of metabolism revealed that PRE-SPO-cultivated *C. albicans* cells showed increased fermentative and aerobic respiratory rates compared to those on standard YPD medium. We also found that tetraploid cells exhibited metabolic activities that were fourfold to eightfold higher than diploid cells, while cell size and mitochondrial content were doubled in tetraploid cells relative to diploid cells. In line with increased metabolic flux and induction of oxidative stress, cells grown on PRE-SPO showed higher ROS levels and activation of the ROS-responsive transcription factor Cap1 compared to cells grown on YPD. Importantly, supplementation of PRE-SPO medium with antioxidants or overexpression of endogenous antioxidant enzymes suppressed both ROS levels and the oxidative stress response. The addition of antioxidants may protect against oxidative stress, in part, by lowering the overall redox potential as shown for *S. cerevisiae* cells (Bednarska et al, 2008).

Endogenous ROS induces DNA damage and chromosome instability in tetraploid cells

Reactive oxygen species production is known to promote DNA damage in multiple cell types (Storr et al, 2013; Tafani et al, 2016). In line with this, we observed increased ROS-induced DSB formation in *C. albicans* cells grown on PRE-SPO medium. Supplementation of this medium with antioxidants mitigated both DSB formation and genetic instability in tetraploid cells. This suggests a model whereby a hyperactive metabolism on PRE-SPO medium generates sufficient ROS to cause DSB formation, which in turn results in genome instability and cell death in polyploid *C. albicans* cells.

We also note that *C. albicans* tetraploid cells were more susceptible to treatment with DNA-damaging agents than diploid cells. Thus, tetraploid cells showed increased cell death and chromosomal instability when treated with MMS or HU than diploid cells. This result mirrors studies in *S. cerevisiae* where tetraploid cells were more sensitive to DSB-inducing agents than diploid cells and tetraploid cells were also more reliant on homologous recombination for DNA repair, particularly at elevated temperatures

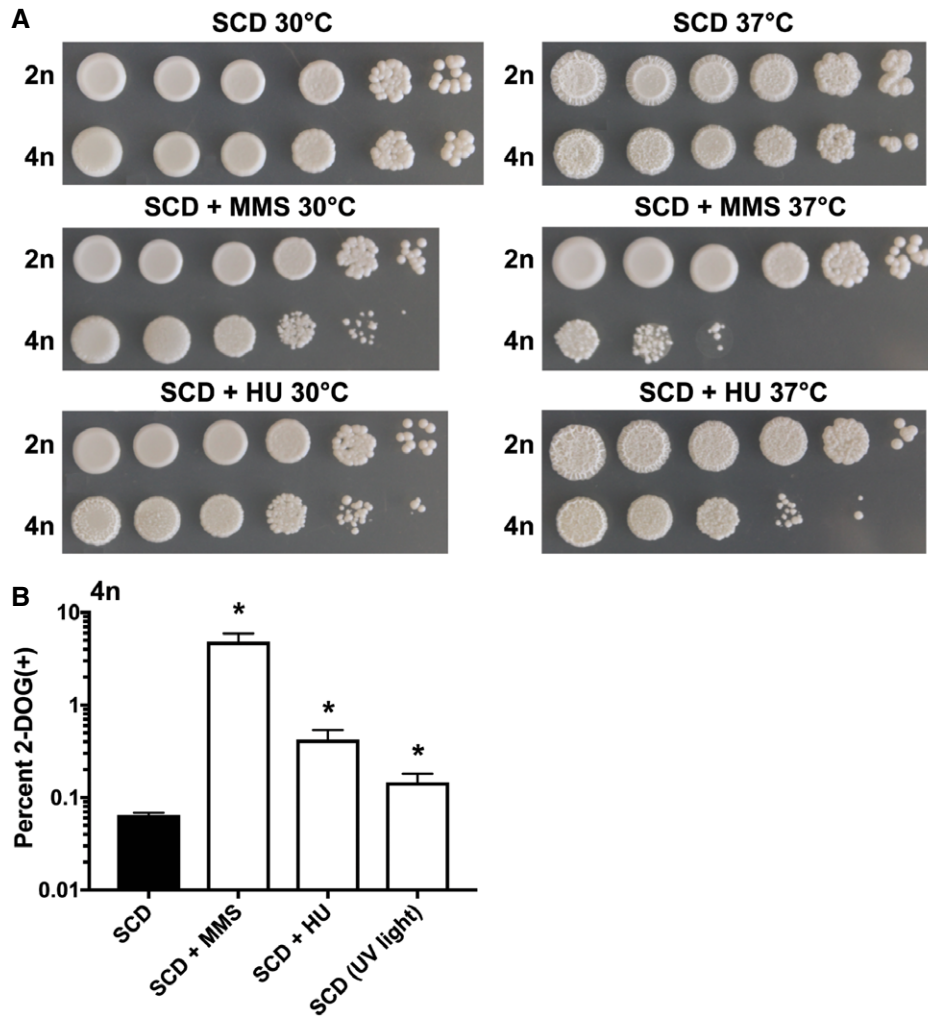


Figure 7. DNA damage induces chromosome instability in tetraploid *Candida albicans* cells.

A 10-fold dilutions of diploid and tetraploid cells were spot-inoculated onto SCD medium with or without the DNA-damaging agent hydroxyurea (HU, 20 mM) or methyl methanesulfonate (MMS, 0.01%). Cells were grown for 48 h at 30 or 37°C and the plates imaged.

B Assay to monitor for loss of *GAL1* (2-DOG resistant cells) arising from tetraploid strain RBY18 after culture at 37°C on SCD medium alone, SCD supplemented with HU (20 mM) or MMS (0.01%), or SCD medium with exposure to UV light for 5 s a day for 7 days. (*) denotes a significant difference between control SCD medium and the indicated treatment ($P < 0.05$, Student's *t*-test, $n = 3$, error bars represent SEM).

(Storchova *et al*, 2006; Storchova, 2014). Cells of higher ploidy may be more susceptible to DNA damage and chromosome mis-segregation due to the altered geometry of the mitotic spindle as cell size increases (Storchova *et al*, 2006; Storchova, 2014). Treatment of *C. albicans* cells with exogenous sources of oxidative stress also recapitulated the phenotypes on PRE-SPO medium. Treatment of tetraploid cells with these agents therefore resulted in elevated levels of ROS, DSB formation, and chromosome instability. This reveals similarities to studies in *S. cerevisiae* where deletion of DNA repair genes was found to promote chromosome rearrangements due to increased oxidative DNA damage (Degtyareva *et al*, 2008).

Taken together, these results suggest that polyploid *C. albicans* cells, but not diploid cells, experience genome instability and cell death on PRE-SPO medium due to two main factors. First,

tetraploid cells are more metabolically active on this medium than diploid cells, thereby generating higher levels of ROS and DNA damage. Second, tetraploid cells are also markedly more sensitive to DNA damage than diploid cells, so that these cells are more prone to damage-induced instability and cell killing. Culture on PRE-SPO medium therefore produces sufficient ROS/DNA damage to cause extensive genome instability in tetraploid cells, whereas ROS levels do not reach the threshold necessary for instability in diploid cells.

Polyploidy, cancer, and ROS in mammalian cells

Cancer progression is a complex process and yet several parallels exist between the processes supporting tumorigenesis and those observed in polyploid fungal cells. Strikingly, ~90% of all solid

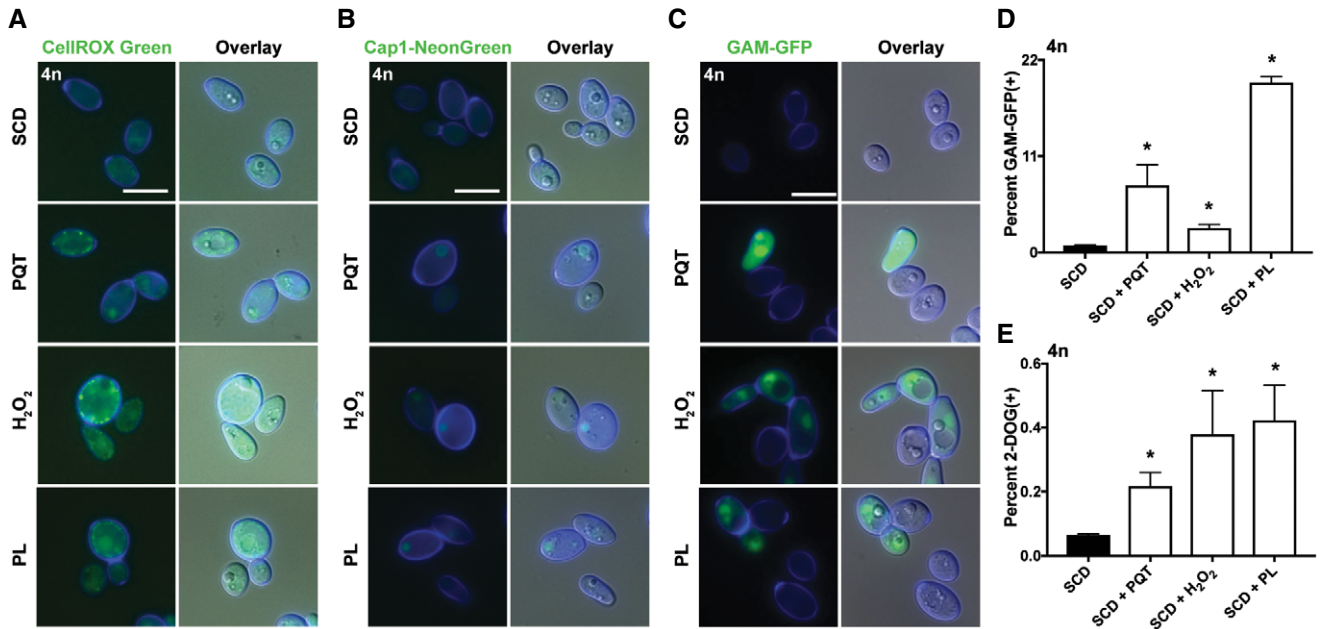


Figure 8. Tetraploid cells exposed to exogenous oxidative stress experience increased DSB formation and chromosome loss.

- A Tetraploid cells were grown on SCD medium with or without the oxidative stress-inducing agents paraquat (PQT; 600 $\mu\text{g/ml}$), hydrogen peroxide (H_2O_2 ; 2 mM), or piperlongumine (PL; 100 $\mu\text{g/ml}$) for 24 h at 37°C and stained with CellROX Green. Cell images indicate calcofluor white staining (cell wall; blue), GFP, and a merged image of GFP/DAPI/DIC channels. Scale bar = 10 μm .
- B Tetraploid cells expressing Cap1-mNeonGreen were grown on SCD medium with or without PQT (600 $\mu\text{g/ml}$), H_2O_2 (2 mM), or PL (100 $\mu\text{g/ml}$) for 24 h at 37°C. Cell images as in (A). Scale bar = 10 μm .
- C Tetraploid cells expressing a Gam-GFP reporter were cultured on SCD medium with or without PQT (600 $\mu\text{g/ml}$), H_2O_2 (2 mM), or PL (100 $\mu\text{g/ml}$) for 24 h at 37°C and analyzed via fluorescence microscopy. Cell images as in (A). Scale bar = 10 μm .
- D Flow cytometric analysis of the tetraploid Gam-GFP reporter strain grown on SCD with or without PQT (600 $\mu\text{g/ml}$), H_2O_2 (2 mM), or PL (100 $\mu\text{g/ml}$) for 24 h at 37°C. (*) denotes a significant difference in Gam-GFP values between control and test agent ($P < 0.05$, Student's t -test, $n = 3$, error bars represent SEM).
- E 2-DOG assay to monitor for loss of *GAL1* function from tetraploid strain RBY18 after culture on SCD medium with or without PQT (600 $\mu\text{g/ml}$), H_2O_2 (2 mM), or PL (100 $\mu\text{g/ml}$) at 37°C for 7 days. (*) denotes a significant difference between 2-DOG resistant colonies between control and the indicated agent ($P < 0.05$, Student's t -test, $n = 3$, error bars represent SEM).

tumors are aneuploid and many of these forms arise due to the inherent genome instability of polyploid cells (Coward & Harding, 2014). Both polyploid and aneuploid forms facilitate karyotypic variation, tumor progression, and the emergence of drug resistance. Polyploid *C. albicans* cells similarly exhibit increased genome instability, and the resulting karyotypic diversity can promote adaptation and resistance to antifungal drugs (Bennett & Johnson, 2003; Harrison et al, 2014; Hirakawa et al, 2017).

While links between ploidy and genome instability are well recognized both in yeast and in mammalian cells (Storchova & Kuffer, 2008), those between ploidy and metabolism are considerably more complex. Cancer polyploid cells are larger than diploid cells and can display a higher metabolic rate commensurate with their size (Coward & Harding, 2014; Donovan et al, 2014). It also appears that this increase in metabolism promotes genome instability in certain cancer cells. For example, polyploid prostate and mammary epithelial cells display higher ROS levels than diploid cells due to their increased mitochondrial content (Roh et al, 2012). Increased ROS levels can, in turn, induce DNA damage and genome instability (Samper et al, 2003; Radisky et al, 2005), while expression of a mitochondrial superoxide dismutase suppressed genome instability and tumor formation caused by ROS (van de Wetering et al, 2008). These

results suggest that, as in *C. albicans*, connections exist between endogenous ROS and genome instability in certain cancer cells.

It should be noted, however, that eukaryotic cells do not exhibit a universal correlation between cell size and metabolic activity. For example, varying the size of hepatocytes led to a nonlinear relationship with mitochondrial respiration, with optimal rates of oxidative phosphorylation observed at intermediate cell sizes (Miettinen et al, 2014). In the case of hepatocytes, this may be a functional adaptation that limits cellular ROS accumulation in the toxic liver environment (Schoenfelder & Fox, 2015).

It is therefore apparent that metabolic cues are implicated in ROS production, DNA damage, and chromosomal instability in both fungal and animal cells. Studies of unicellular eukaryotes could therefore provide additional mechanistic insights into links between metabolism, ROS, and tumorigenesis.

A model for metabolism-induced ROS in the evolution of eukaryotic ploidy cycles?

A central unresolved question in biology is how did sexual reproduction evolve in eukaryotes? The emergence of sex would have enhanced DNA repair yet came with significant costs including the

twofold cost of sex (de Visser & Elena, 2007; Bernstein *et al*, 2011; Goddard, 2016; McDonald *et al*, 2016). Intriguingly, sex may have been especially advantageous in dealing with oxidative stress from the endosymbiont that formed the pre-mitochondrion, as this aerobic bacterium would have generated high levels of ROS (Blackstone, 1995; Bernstein *et al*, 2011; Horandl & Speijer, 2018). Sex would have been particularly effective in repairing subsequent ROS-induced DNA damage via homologous recombination. In support of a connection between ROS and sex, oxidative stress still promotes sexual reproduction in multiple extant eukaryotes (Reeves & Jackson, 1974; Bernstein & Johns, 1989; Nedelcu *et al*, 2004).

The current work suggests that ROS could have facilitated ploidy cycling as a forerunner to meiosis in the pre-eukaryote. High levels of ROS would have been particularly detrimental to cells of higher ploidy and, thus, if haploid cells generated diploid or polyploid cells (e.g., by cell fusion or endoreduplication), then the resulting cells may have been forced to revert to a lower ploidy state. In this model, ploidy reduction would have been a natural consequence of the high levels of ROS produced by early eukaryotes (Horandl & Speijer, 2018), and a primitive ploidy cycle could have occurred without meiosis.

This model, while speculative, aligns with other aspects of meiosis in extant eukaryotes. For example, DSBs are essential for meiotic recombination with their production regulated by the Spo11 endonuclease (Lam & Keeney, 2014). Spo11 is dispensable for meiosis, however, if DSBs are generated by an alternative mechanism such as γ radiation (Dernburg *et al*, 1998). It is therefore possible that ROS-induced DSBs were the causal agents of ploidy reduction in pre-eukaryotes, and that this step subsequently came under the control of Spo11 to allow for tighter regulation of potentially lethal DNA breaks.

Conclusions

In this study, we establish connections between metabolism, endogenous ROS, DNA damage, and genome instability, and establish how polyploid cells can be selectively induced to reduce their ploidy even in the absence of a conventional meiosis. These studies parallel those in certain cancer cells where polyploidy, ROS, and chromosome instability are intertwined, indicating that similar mechanisms may impact genome stability in diverse eukaryotes. Our results also provide a speculative model for how metabolism-induced processes could have enabled a rudimentary ploidy cycle in evolution. Further studies are necessary to more precisely define the relationship between metabolism, ROS, and genome integrity, as well as their impact in multiple eukaryotic species.

Materials and Methods

Media and chemicals

Yeast extract peptone dextrose (YPD) and synthetic complete dextrose (SCD) media were prepared as described (Guthrie & Fink, 1991). *Saccharomyces cerevisiae* pre-sporulation ("PRE-SPO") medium contained 0.8% yeast extract, 0.3% peptone, 10% glucose (added prior to autoclaving), and 2% agar. Quercetin hydrate (Acros Organics, #AC174070100), curcumin (Acros Organics, #AC218580100),

L-ascorbic acid (Acros Organics, #AC352680050), L-glutathione (Sigma-Aldrich, Cat. No. G4251-10G), dithiothreitol (Fisher Scientific, #BP172-25), paraquat dichloride hydrate (Sigma-Aldrich, #36541-100MG), hydrogen peroxide (Sigma-Aldrich, #H1009-100ML), piperlongumine (Cayman Chemical, #20069-09-4), methyl methanesulfonate (Acros Organics, #AC156890050), and hydroxyurea (MP Biomedicals, #0210202310-10 g) were added to the indicated media once the media had cooled to approximately 40°C, at which point the media was poured and plates were used for assays within 24 h.

Strain construction

A *CAP1*-mNeonGreen cassette was amplified from pRB895 (*pSFS2A-mNeonGreen*) (Frazer *et al*, 2019) using oligonucleotides 4564 and 4565 (see Appendix Table S5), which contain homology to the *CAP1* gene and the *mNeonGreen* plasmid. This cassette was transformed into the diploid strain SC5314 and the tetraploid strain RBY18 to create strains CAY9331 (diploid) and CAY9332 (tetraploid).

The plasmid *pRS11* was generated by first synthesizing a *C. albicans* codon-optimized *Gam* gene flanked by two *Sall* restriction sites, which was then digested with *Sall* and cloned into the *pNIM1* vector (Park & Morschhauser, 2005). *pRS11* was linearized with restriction enzymes *SacII* and *ApaI* and transformed into the diploid strain SC5314 and the tetraploid strain RBY18 to generate strains CAY7504 (diploid) and CAY7571 (tetraploid).

The plasmid *pLC605* contains a *Tet-OFF* promoter sequence which can be fused to genes of interest to generate overexpression (O/E) cassettes (Veri *et al*, 2018). Tet-OFF O/E cassettes were created for the antioxidant enzymes encoded by *SOD1*, *SOD2*, *SOD3*, *TRX1*, and *GCS1* by PCR amplification of pLC605 using oligos 5547/5548, 4823/4824, 4702/4703, 4813/4814, and 4817/4818, respectively (see Appendix Table S5). These cassettes were transformed into RBY18 to generate strains CAY10665 (*SOD1 O/E*), CAY9516 (*SOD2 O/E*), CAY9388 (*SOD3 O/E*), CAY9501 (*TRX1 O/E*), and CAY9507 (*GCS1 O/E*).

Quantification of chromosome loss

Tetraploid stain RBY18 was cultured in quadrants on media of interest and incubated for 7 days at 37°C. Cells were then scraped off each plate and suspended in sterile PBS. Two successive 1:100 dilutions were made in sterile PBS. 100 μ l of the undiluted and 1:100 dilution cell suspensions was plated onto SC 2-DOG medium, and 100 μ l of the 1:100 dilution and 1:10,000 dilution cell suspensions was plated onto YPD medium. These plates were incubated at 30°C for 48 h, at which point the plates were collected and the total number of colonies were counted on each plate. Each experiment was performed in biological triplicate.

Viability assays

Strains SC5314 and RBY18 were collected from YPD medium, and approximately 6×10^7 cells from each strain were top-spread onto YPD and PRE-SPO media and incubated at 30 and 37°C, respectively. At 1 and 5 days, cells were collected, stained with 0.5 mg/ml propidium iodide, and imaged using a fluorescence microscope (Zeiss Z1 Axio Observer, Zeiss filter set 45, 250 ms exposures). Images were then viewed with Fiji image processing software, and

~300 cells were scored for viability, with PI-positive cells representing dead yeast cells (Dudgeon *et al*, 2008). Each experiment was performed in biological quintuplicate.

RNA sequencing

Strains SC5314 and RBY18 were collected from YPD medium; approximately 6×10^7 cells from each strain were top-spread onto YPD and PRE-SPO media and incubated at 30 and 37°C, respectively. Cells were collected at 3, 6, 12, and 24 h, flash-frozen in liquid nitrogen, and stored at -80°C . Each experiment was performed in biological duplicate. RNA was purified using the Ambion RiboPure Yeast RNA Purification Kit (ThermoFisher, #AM1926) and RNA integrity assessed using an Agilent Bioanalyzer 2100. mRNA libraries were prepared using the Illumina TruSeq Stranded mRNA Library Preparation Kit (Illumina, #20020595). Libraries were then pooled and sequenced on an Illumina HiSeq 4000 platform generating 100-bp paired-end reads (at The Ohio State University Comprehensive Cancer Center, Columbus, OH). Reads were aligned to the *C. albicans* SC5314 reference genome (Assembly 21) using the Spliced Transcripts Alignment to a Reference (STAR) aligner (Dobin *et al*, 2013). Differential expression analysis was conducted using edgeR. Gene ontology enrichment analysis was conducted using the Gene Ontology Consortium enrichment analysis tool (Mi *et al*, 2017).

2,3,5-Triphenyltetrazolium chloride (TTC) reduction assay

To assess metabolic activity of *C. albicans* cells on YPD and PRE-SPO media, a TTC overlay technique was used (Ogur *et al*, 1957). Approximately 2×10^7 cells of SC5314 and RBY18 strains were spotted onto YPD and PRE-SPO media and incubated for 24 h at 30 and 37°C, respectively. Prior to applying the TTC overlay solution, each plate was imaged for a 0 min reference. A TTC overlay agar solution (0.067 M potassium phosphate buffer pH 7.0, 1.5% agar, 0.1% TTC) was warmed to 55°C and 10 ml added onto the plates containing the spot-inoculated colonies. The flooded plates were imaged 15 min after the addition of the overlay solution. Each experiment was performed in biological quintuplicate.

For image quantitation, collected images were viewed with Fiji image processing software. Spot colonies were selected, and mean RGB breakdowns of pixel values for each colony were determined using the color histogram tool. The relative luminance (Y) of each colony was calculated by plugging the RGB values into the formula $Y = 0.2126R + 0.7152G + 0.0722B$, and the percent of red light contributing to the overall luminance was calculated using the formula $(0.2126R/Y) \times 100$. The percent red light of overall luminance was calculated for both the 0 min and 15 min images, and the 15 min value was normalized to the 0 min value for a final red pixel intensity value.

Aerobic respiratory and fermentative rate assays

Strains SC5314 and RBY18 were collected from YPD medium, and approximately 6×10^7 cells from each strain were top-spread onto YPD and PRE-SPO media and incubated at 37°C for 24 h. An Agilent Seahorse XFe96 sensor cartridge (Agilent Technologies, Cat. No. 102416-100) was hydrated in a utility plate with each well

containing 200 μl of sterile water overnight at 37°C. On the following day, the sensor cartridge was moved into a utility plate containing 200 μl of XF Calibrant (Agilent Technologies, Cat. No. 100840-000) in each well and incubated for one hour at 37°C. Cells were harvested from each plate and suspended in sterile water to a concentration of approximately 5.0×10^7 CFU/ml. 10 μl of each cell suspension was seeded into wells containing 200 μl of pre-warmed Seahorse XF assay medium (supplemented with 10 mM glucose, 10 mM sodium pyruvate) in an Agilent Seahorse XFe96 cell culture microplate (Agilent Technologies, #102416-100). Where appropriate, drug injection ports for each well were loaded with 20 μl of 50 μM antimycin A, 50 μM rotenone, and 5 mM salicylhydroxamic acid (SHAM) diluted in Seahorse XF assay medium, representing a final concentration of 5 μM antimycin A, 5 μM rotenone, and 500 μM SHAM after injection. The cell plate was placed into the Seahorse XFe96 instrument and the assay run for fourteen total cycles, with each cycle consisting of one minute of mixing, zero minutes waiting, and three minutes measuring. The first seven measurements were used to collect baseline OCR and ECAR measurements, at which point the antimycin A, rotenone, and SHAM cocktail were injected into each well and seven measurements were recorded to collect treated OCR and ECAR measurements. Upon completion of assay, the exact number of cells in each cell suspension was assessed via hemocytometer counts and oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) measurements were normalized to both the total number of cells seeded into each well and the viability of cells under each growth condition (calculated in the viability assays detailed above). These normalized measurements were then multiplied by 10^6 for final OCR measurements in the units of pmol/min/ 10^6 CFU and ECAR measurements in the units of mpH/min/ 10^6 CFU. Each experiment was performed in biological quintuplicate.

Antioxidant enzyme overexpression assays

PRE-SPO medium plates containing 5 $\mu\text{g/ml}$ doxycycline (DOX) were prepared and used the same day for each assay. Strains CAY10665 (*SOD1 O/E*), CAY9516 (*SOD2 O/E*), CAY9388 (*SOD3 O/E*), CAY9501 (*TRX1 O/E*), and CAY9507 (*GCS1 O/E*) were grown on YPD medium and then cultured in quadrants on PRE-SPO medium with or without DOX and incubated for 7 days at 37°C, at which point cells were harvested and a 2-DOG assay was conducted as detailed above. Each experiment was performed in biological triplicate.

Stress assays

SC5314 and RBY18 cells were grown overnight in liquid YPD medium at 30°C. The following day, the cultures were diluted 1:50 and grown to mid-logarithmic phase (approximately 4–5 h). Cell density was determined by hemocytometer count, and cells were diluted to a density of 2×10^8 CFU/ml. Five successive 1:10 serial dilutions were made in sterile phosphate-buffered saline (PBS) and 5 μl of each cell suspension spotted onto freshly prepared SCD, SCD + 20 mM hydroxyurea, and SCD + 0.01% methyl methanesulfonate media. Plates were incubated for 48 h at 30 and 37°C at which point plates were imaged.

CellROX Green staining

SC5314 and RBY18 were cultured in quadrants on YPD and PRE-SPO media and incubated for 24 h at 30 and 37°C, respectively. Cells were harvested and washed with sterile PBS. PBS-washed cells were then suspended in 5 µM CellROX Green Reagent (ThermoFisher Scientific, #C10444) and incubated for 30 min at 37°C. The staining solution was then removed from cells, and cells were washed three times with PBS. CellROX green-stained cells were then co-stained with calcofluor white at a final concentration of 0.1 mg/ml to visualize the cell wall. For microscopy, cells were imaged using a fluorescence microscope (Zeiss Z1 Axio Observer; CellROX Green: Zeiss filter set 38, 500 ms exposure; calcofluor white: Zeiss filter set 49, 25 ms exposure), and images viewed and processed with Fiji image processing software. For flow cytometry, a total of 100,000 cells were analyzed on a FACSAria (BD Biosciences), and data were analyzed via FlowJo (TreeStar, Ashland, OR). Each experiment was performed in biological quintuplicate.

MitoTracker Green staining

SC5314 and RBY18 were cultured in quadrants on YPD medium and incubated for 24 h at 30°C. Cells were harvested and washed with sterile PBS. PBS-washed cells were then suspended in 1 µM MitoTracker Green Reagent (ThermoFisher Scientific, #M7514) and incubated for 30 min at 30°C. The staining solution was then removed, and cells were washed once with PBS. For flow cytometry, a total of 500,000 cells were analyzed on a FACSAria (BD Biosciences) and data were analyzed via FlowJo (TreeStar, Ashland, OR). Each experiment was performed in biological triplicate.

Gam-GFP assays

A Gam-GFP reporter strain was used to detect the presence of DSBs in tetraploid *C. albicans* cells. This strain was grown on the indicated growth medium in the presence of doxycycline (+DOX; 12.5 µg/ml) to induce the Gam-GFP protein. Plates without doxycycline (−DOX) were included as controls where Gam-GFP was not induced. CAY7504 (diploid) and CAY7571 (tetraploid) cells containing Gam-GFP were inoculated into quadrants on the indicated medium and incubated for 24 h at the indicated temperature. Cells were recovered from each plate and suspended in 1 ml of sterile PBS. A total of 500,000 cells from each sample were run on a BD FACSAria (Becton Dickinson Biosciences) and data analyzed using FlowJo software (Tree Star, Ashland, OR). Each experiment was performed in biological triplicate. For microscopy, cells were co-stained with calcofluor white at a final concentration of 0.1 mg/ml. Cells were imaged using a fluorescence microscope (Zeiss Z1 Axio Observer; Gam-GFP: Zeiss filter set 38, 250 ms exposure; calcofluor white: Zeiss filter set 49, 25 ms exposures), and images were viewed and processed with Fiji image processing software.

Cap1-mNeonGreen assays

Strains CAY9331 (diploid) and CAY9332 (tetraploid) expressing Cap1-mNeonGreen were inoculated into quadrants on the

indicated medium. Cells were recovered from each plate and suspended in sterile PBS. Cells were co-stained with calcofluor white at a final concentration of 0.1 mg/ml. Cells were imaged using a fluorescence microscope (Zeiss Z1 Axio Observer; Cap1-NeonGreen: Zeiss filter set 38, 250 ms exposure; calcofluor white: Zeiss filter set 49, 25 ms exposure), and images were viewed and processed with Fiji processing software. For quantitation of Cap1 activation, brightness on the GFP channel was adjusted to the maximum observed brightness in the data set and cells that exhibited a robust nuclear signal were counted as Cap1-activated. Each experiment was performed in biological quintuplicate.

DNA staining for ploidy analysis

Candida albicans cells were grown overnight at 30°C in liquid YPD medium, at which point cells were collected, fixed with 70% ethanol, and incubated for 1 h at 4°C. Cells were then washed twice with 50 mM Tris-Cl, pH 8.0, 5 mM EDTA, resuspended in 2 mg/ml RNase A (Sigma-Aldrich, #R5503-500MG) in 50 mM Tris-Cl, pH 8.0, 5 mM EDTA, and incubated for 2 h at 37°C. RNase-treated cells were pelleted and resuspended in 5 mg/ml pepsin (Sigma-Aldrich, #P7000-500G) in 55 mM HCl and incubated for 1 h at 37°C. Cells were then washed twice with 50 mM Tris-Cl, pH 7.5, 5 mM EDTA and suspended in 1 µM SYTOX Green Nucleic Acid Stain (Thermo Fisher Scientific, #S7020) in 50 mM Tris-Cl, pH 7.5, 5 mM EDTA and incubated overnight at 4°C. For ploidy analysis, 100,000 cells were run on an Attune NxT Flow Cytometer (Thermo Fisher Scientific) and data were analyzed via FlowJo (TreeStar, Ashland, OR).

Data availability

All RNA-seq data associated with this project have been uploaded to NCBI Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>), with accession code PRJNA507025.

Expanded View for this article is available online.

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Author contributions

Conceptualization, GJT and RJB; Methodology, GJT, CH, NA, and RJB; Formal Analysis, GJT; Resources, RSS and PB; Writing—Original Draft, GJT and RJB; Supervision, GJT and RJB; Funding Acquisition, RJB.

Conflict of interest

The authors declare that they have no conflict of interest.

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