Multifactorial role of mitochondria in echinocandin tolerance revealed by transcriptome analysis of drug-tolerant cells

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Running title: Mitochondria in *C. glabrata* echinocandin tolerance

Abstract (250 max)

Fungal infections cause significant mortality and morbidity worldwide, and the limited existing antifungal reservoir is further weakened by the emergence of strains resistant to echinocandins, a first line of antifungal therapy. *Candida glabrata* is an opportunistic fungal pathogen that rapidly develops mutations in the echinocandin drug target, β -1,3-glucan synthase (GS), which are associated with drug resistance and clinical failure. Although echinocandins are considered fungicidal in *Candida*, a subset of *C. glabrata* cells survive echinocandin exposure, forming a drug-tolerant cell reservoir, from which resistant mutations are thought to emerge. Despite their importance, the physiology of

29 rare drug-tolerant cells is poorly understood. We used fluorescence-activated cell
30 sorting to enrich for echinocandin-tolerant cells, followed by modified single cell RNA
31 sequencing to examine their transcriptional landscape. This analysis identified a
32 transcriptional signature distinct from the stereotypical yeast environmental stress
33 response and characterized by upregulation of pathways involved in chromosome
34 structure and DNA topology and downregulation of oxidative stress responses, the
35 latter of which was observed despite increased levels of reactive oxygen species.
36 Further analyses implicated mitochondria in echinocandin tolerance, wherein
37 inhibitors of mitochondrial complexes I and IV reduced echinocandin-mediated cell
38 killing, but mutants lacking various mitochondrial components all showed an
39 echinocandin hypo-tolerant phenotype. Finally, GS enzyme complexes purified from
40 mitochondrial mutants exhibited normal *in vitro* inhibition kinetics, indicating that
41 mitochondrial defects influence cell survival downstream of the drug-target
42 interaction. Together, these results provide new insights into the *C. glabrata* response
43 to echinocandins and reveal a multifactorial role of mitochondria in echinocandin
44 tolerance.

45

46 **Importance (150 max)**

47 Echinocandin drugs are a first line therapy to treat invasive candidiasis, which is a
48 major source of morbidity and mortality worldwide. The opportunistic fungal pathogen
49 *Candida glabrata* is a prominent bloodstream fungal pathogen and it is notable for
50 rapidly developing echinocandin-resistant strains associated with clinical failure.
51 Echinocandin resistance is thought to emerge within a small echinocandin-tolerant
52 subset of *C. glabrata* cells that are not killed by drug exposure, but mechanisms

underlying echinocandin tolerance are still unknown. Here we describe the unique transcriptional signature of echinocandin-tolerant cells and the results of follow-up analyses, which reveal a multifactorial role of mitochondria in *C. glabrata* echinocandin tolerance. In particular, although chemical inhibition of respiratory chain enzymes increased echinocandin tolerance, deletion of multiple mitochondrial components made *C. glabrata* cells hypo-tolerant to echinocandins. Together, these results provide new insights into the *C. glabrata* response to echinocandins and reveal the involvement of mitochondria in echinocandin tolerance.

INTRODUCTION

Invasive candidiasis is an emerging life-threatening infection recognized as a major cause of morbidity and mortality (1). For decades, antifungal drugs of the azole class have been used as the primary therapy to treat *Candida* infections. Azoles have excellent activity against the predominant *Candida* species, *C. albicans*, and azole resistance in this species remains relatively low. However, an epidemiological shift has been taking place worldwide toward non-*albicans* species that are inherently resistant or readily acquire azole resistance, most notably *Candida glabrata* (2). This shift has led to the widespread use of echinocandin drugs as a first line antifungal therapy to treat and prevent invasive candidiasis (3). Alarming, an increase in echinocandin-resistant *C. glabrata* isolates associated with clinical failure has been reported worldwide (4-7). However, the mechanisms enabling *C. glabrata* to develop echinocandin resistance are still very poorly understood.

Although echinocandins are fungicidal drugs in yeast, a small subset of *C. glabrata* cells within a given population demonstrate drug tolerance by surviving

77 prolonged exposures to echinocandins (8, 9). Tolerance to fungicidal drugs is defined
78 as the ability of cells to survive a drug concentration that is expected to kill cells,
79 usually at or above the MIC (8), whereas resistance is manifested as the ability of the
80 cells to grow in the presence of drug and is genetically stable. Clinical resistance to
81 echinocandins is virtually always due to mutations in the hotspot regions of *FKS1* and
82 *FKS2* genes (10). These genetic mutations leading to stable drug resistance are thought
83 to emerge from the drug-tolerant cellular reservoir, making tolerance as a key
84 prerequisite to echinocandin resistance (8, 11). However, the molecular underpinnings
85 of tolerance are currently not understood. Because echinocandins target β -1,3-glucan
86 synthase, an enzyme that helps build the fungal cell wall, thus far studies of
87 echinocandin tolerance have focused on the role of cell wall integrity pathways (12-
88 14). A more unbiased examination of echinocandin tolerance can be derived from
89 exploring the transcriptional landscape of echinocandin-tolerant cells. However, this
90 approach has not been attempted thus far due to the difficulty of studying a very small
91 subset of cells. Instead, the few studies that have examined the transcriptional
92 response to echinocandins have used bulk-RNA isolated from entire echinocandin-
93 treated cultures (15-16), which are largely comprised of dead and dying cells due to
94 the drugs' quick fungicidal action (17), thus potentially obscuring the transcriptional
95 state of drug-tolerant cells.

96 In this study, we used fluorescence-activated cell sorting to highly enrich for *C.*
97 *glabrata* cells that have survived prolonged echinocandin exposure *in vitro*, followed
98 by their transcriptional analysis using a modified single cell RNA sequencing approach
99 wherein transcriptomes of groups of several surviving cells were sequenced. This
100 analysis identified several unique features of the transcriptional landscape of

101 echinocandin-tolerant cells, including a downregulation of the oxidative stress
 102 response pathway, which occurs despite an echinocandin-induced increase in reactive
 103 oxygen species (ROS) abundance. We also found that, although inhibitors of
 104 mitochondrial complexes I and IV reduced both echinocandin-induced ROS formation
 105 and echinocandin-mediated cell killing, an ROS scavenger did not alter cell killing
 106 dynamics, indicating that ROS per se do not significantly contribute to cell death upon
 107 echinocandin exposure. Moreover, we found that mutants lacking various
 108 mitochondrial components, including both respiration-proficient and respiration-
 109 deficient strains, all showed an echinocandin hypo-tolerant phenotype at sub-MIC
 110 concentrations. Finally, we partially purified the echinocandin target enzyme β -1,3-
 111 glucan synthase from mitochondrial mutants and found that the enzyme's sensitivity
 112 to echinocandins was not altered, indicating that mitochondrial status influences cell
 113 survival downstream of the echinocandin-enzyme interaction. Together, these results
 114 provide new insights into the *C. glabrata* response to echinocandins and reveal the
 115 involvement of mitochondria in echinocandin tolerance.

116

117 RESULTS

118 Using fluorescence-activated cell sorting to enrich for echinocandin-tolerant *C.*

119 *glabrata* cells for RNA sequencing (RNAseq)

120 To isolate ultra-rare echinocandin-tolerant *C. glabrata* cells for subsequent
 121 transcriptome analysis, we used fluorescence-activated cell sorting (FACS). We tested
 122 several combinations of live/dead dyes and fluorescent markers (Table S1) in cells that
 123 had been cultured in the presence of either 0.25 μ g/ml caspofungin or 0.06 μ g/ml
 124 micafungin (corresponding to 2X-MIC for strain ATCC2001) for 24 hours, followed by

125 plating of the sorted fractions on drug-free medium and counting the colony forming
 126 units. We found that sorting cells that did not stain with propidium iodide (PI) (Figure
 127 1A) resulted in the strongest enrichment for cells capable of producing colonies, from
 128 less than one in 1000 events to one in 20-25 events. Thus, the PI-negative cell subset,
 129 which contained <1% of all cells, was designated as containing echinocandin-tolerant
 130 cells. Next, the sorted cells were analyzed using a modified single cell RNA sequencing
 131 approach as follows. Based on pilot experiments comprised of FACS followed by
 132 plating and CFU counts, we calculated the number of total events that had to be sorted
 133 to obtain approximately ten viable *C. glabrata* cells per well (in order to ensure a
 134 robust level of RNA for sequencing). This was done both for echinocandin treated cell
 135 samples as well as for untreated controls. Thus, for untreated cells, the wells would
 136 contain the same number of viable cells as for echinocandin-treated cells. Although for
 137 the latter the wells would also contain some cell debris and inviable cells, viable cells
 138 would be significantly enriched in these wells relative to “bulk” RNAseq (see below).
 139 Because the echinocandin-tolerant cells were on the one hand expected to be non-
 140 growing but on the other hand present in a rich nutritional environment, we used two
 141 kinds of “no drug” controls: cells in either logarithmic (mimicking the nutrient-rich
 142 environment) or stationary phase (mimicking the non-growing state), stained with PI
 143 and sorted in the same fashion (Figure 1A).

144 In addition to performing RNAseq on samples enriched for tolerant cells, we
 145 also performed “bulk” RNAseq on unsorted echinocandin-treated cells as follows. 0.06
 146 µg/ml of micafungin was added to 10^7 *C. glabrata* cells/ml, which were then cultured
 147 for 24h, and their RNA was isolated and analyzed by RNAseq. Cells grown for 24h in the
 148 absence of micafungin (i.e., stationary phase cells) were used as the control.

149

150 **Tolerant cell-enriched RNAseq identified a unique transcriptional signature of**
 151 **echinocandin-tolerant cells**

152 The sorted PI-negative cells were briefly treated with zymolyase to digest the fungal
 153 cell walls, followed by analysis using the single cell RNAseq pipeline at the Columbia
 154 University Genome Center. As is evident from the resulting UMAP schematic, the
 155 transcriptional profiles of caspofungin-tolerant cells and micafungin-tolerant cells were
 156 highly similar to each other but distinct from either stationary or logarithmic controls
 157 (Figure 1B). Therefore, for all following analyses, transcriptional data from
 158 caspofungin- and micafungin-tolerant cells were grouped together. Next, raw tolerant
 159 cell-enriched RNAseq data were normalized using the SCTransform package and Seurat
 160 was used to generate a dataset of individual genes' expression changes in
 161 echinocandin-tolerant cells relative to either stationary or logarithmic control cells
 162 (Dataset 1). We defined differentially expressed genes (DEGs) as showing at least one
 163 log₂ unit (two-fold) expression difference in echinocandin-tolerant cells relative to no
 164 drug controls. DEGs were also defined in the "bulk" RNAseq dataset using the same
 165 criteria.

166 A comparison of "bulk" and "enriched" RNAseq datasets revealed a number of
 167 similarities and differences (Figure 2A, Figure S1A, B). Functional enrichment analysis
 168 (FUNGAT) showed that downregulated genes in all three datasets ("bulk" relative to
 169 stationary phase controls, "enriched" relative to stationary controls, and "enriched"
 170 relative to log phase controls) belonged to multiple functional categories involved in
 171 energy production and metabolism, particularly those related to mitochondrial
 172 functions (e.g., electron transport, respiration, and oxidative stress response) (Figure

173 S1A-C). In contrast, there was no overlap between the FUNCAT categories of
 174 upregulated genes in “bulk” vs “enriched” RNAseq datasets (Figure S1A vs S1B, C). The
 175 “bulk” RNAseq dataset contained only a few upregulated FUNCAT categories, all of
 176 which related to cell wall metabolism (Figure S1A), consistent with the role of
 177 echinocandins in inhibiting cell wall biogenesis. In contrast, both “enriched” RNAseq
 178 datasets contained a larger number of upregulated FUNCAT gene categories, including
 179 ATP binding, DNA binding, DNA repair, DNA topology, and modification of
 180 chromosome structure (Figures S1B, C). Interestingly, although several individual cell
 181 wall maintenance genes were upregulated in the “enriched” datasets (see below), the
 182 cell wall maintenance FUNCAT categories were not identified in these datasets,
 183 suggesting that enriching for surviving cells does not necessarily enrich for cells with
 184 transcriptionally upregulated cell wall integrity pathways.

185 For the following analyses we focused on the RNAseq data obtained from
 186 samples enriched for echinocandin-tolerant cells. Comparisons of echinocandin-
 187 tolerant cells to either log phase or stationary phase controls resulted in similar but not
 188 identical expression patterns and FUNCAT categories (Figure 2B, Figures S1B, C), as is
 189 also evident from U-map analysis (Figure 1B). We were particularly interested in the
 190 genes that were upregulated or downregulated relative to both no drug controls
 191 (Dataset S1). The upregulated gene set was enriched for functional categories of ATP
 192 binding, DNA conformation modification and topology, and chromosome organization
 193 (Figure 2C). Consistent with echinocandins targeting β -1,3-glucan synthase (GS), an
 194 enzyme essential for maintaining cell wall structure, several genes involved in cell wall
 195 biosynthesis were upregulated in echinocandin-tolerant cells, including *FKS1* and *FKS2*,
 196 which encode GS, as well as *KRE5*, which encodes a protein required for β -1,6 glucan

197 biosynthesis. Interestingly, several genes encoding multidrug ABC transporters (*CDR1*
198 and *YOR1*) were also among the upregulated group, even though multidrug
199 transporters are not thought to mediate resistance to echinocandins (18, 19). Genes
200 downregulated relative to both no drug controls were enriched for functional
201 categories of oxidative stress response, NAD/NADH binding, and oxygen and radical
202 detoxification (Figure 2C). Of note, this is distinct from the stereotypical yeast
203 environmental stress response, where these functional gene categories are typically
204 upregulated (20, 21), underscoring that the transcriptional status of echinocandin-
205 tolerant cells is unique and distinct from other types of stressed cells.

206

207 **Mitochondrial inhibitors but not topoisomerase inhibitors increase echinocandin** 208 **tolerance in *C. glabrata***

209 Based on the functional categories of upregulated and downregulated genes, we
210 selected different types of chemical inhibitors to modulate the most significantly up- or
211 downregulated pathways and ask whether this modulation altered *C. glabrata*
212 echinocandin tolerance. We note that, because caspofungin- and micafungin-tolerant
213 cells had similar transcriptional profiles (Figure 1B), the experiments described below,
214 especially the most laborious ones, were performed using only caspofungin. First, we
215 treated *C. glabrata* with inhibitors of topoisomerase I (topotecan) or II (doxorubicin
216 and etoposide) together with caspofungin for 24 hours. Etoposide and doxorubicin
217 were used at concentrations corresponding to one third and one quarter of their
218 minimal inhibitory concentration (MIC) in *C. glabrata*, respectively. Topotecan has
219 been described to have no antifungal effect and not to affect viability, so we used 100
220 μ M as did previous studies (22, 23) (Table S2). We found that addition of these

221 topoisomerase inhibitors to cells cultured in the presence of caspofungin did not alter
222 the *C. glabrata* tolerance phenotype (Figure 3A).

223 Next, we asked whether inhibitors of mitochondrial enzymes that alter reactive
224 oxygen species (ROS) production would alter tolerance to caspofungin. We used
225 inhibitors of mitochondrial complex I (rotenone and diphenyleneiodonium chloride
226 (DPI)) and complex IV (sodium azide) at concentrations corresponding to one third of
227 their MIC in *C. glabrata* for DPI and sodium azide. The rotenone concentration used
228 (0.3 mM) was determined by the highest concentration at which it was soluble in YPD
229 medium (Table S2). We observed that these inhibitors increased the fraction of cells
230 surviving after 24 hours of treatment by 10 to 100-fold (Figure 3B). Both complex I
231 inhibitors (rotenone and DPI) improved survival more than complex IV inhibitor sodium
232 azide.

233

234 **Caspofungin treatment leads to an increase in reactive oxygen species production** 235 **but a downregulation of the oxidative stress response**

236 Increased ROS have been observed as a consequence of drug treatment both in fungi
237 and bacteria and have been proposed as a key mediator of drug-induced killing in
238 some of these systems (24-26). This, together with the observation that mitochondrial
239 inhibitors improve echinocandin tolerance, led us to examine ROS levels and ROS
240 responses in caspofungin-treated cells more closely. A typical method to detect ROS is
241 by using ROS-sensitive dyes followed by flow cytometry analysis. We used two
242 different dyes, 2',7'-dichlorodihydrofluorescein diacetate (CFDA) and dihydroethidium
243 (DHE), which are thought to detect specific ROS: hydrogen peroxide and superoxide,
244 respectively (27). However, the exact nature of the species detected by these dyes,

245 especially CFDA, is still obscure (28). In these experiments, *C. glabrata* cells were
 246 treated with the indicated agents for 2, 6, or 24 hours, followed by staining with PI or
 247 Sytox Green to gate out dead cells and CFDA or DHE to detect ROS (PI was used with
 248 CFDA and Sytox Green was used with DHE due to color compatibility). We used
 249 treatment with 50 mM H₂O₂ as a positive control and an echinocandin-resistant *fks1*
 250 mutant as a negative control. As expected, we detected a strong increase in both CFDA
 251 and DHE staining induced by H₂O₂ treatment, albeit with different dynamics: CFDA
 252 staining peaked at 2 hours of exposure and then declined over 24 hours, whereas DHE
 253 staining gradually increased over 24 hours (Figure 4A). Importantly, we observed that
 254 0.25 µg/ml caspofungin also induced a strong increase in both CFDA and DHE staining,
 255 with staining intensity of both dyes increasing over the 24-hour period. This was
 256 entirely dependent on the presence of wild type GS enzyme, as no increase was
 257 observed in the *fks1* mutant (Figure 4A). Thus, we concluded that caspofungin-
 258 mediated inhibition of GS activity resulted in an increase in cellular ROS levels.

259 The observed increase in ROS but a decrease in the expression of genes
 260 involved in oxidative stress responses (Figure 2C, Figure S1) by RNA-seq was surprising,
 261 so we probed it further by examining the transcriptional levels of several individual
 262 genes during caspofungin or micafungin treatment by quantitative reverse
 263 transcriptase PCR (qRT-PCR). These experiments were performed by harvesting “bulk”
 264 RNA from echinocandin-treated or untreated cultured, followed by qPCR analysis. We
 265 chose genes with well-established roles in the oxidative stress response (OSR), such as
 266 mitochondrial manganese superoxide dismutase (*SOD2*, *CAGL0E04356g*), a catalase A
 267 (*CTA1*, *CAGL0K10868g*) and a glutathione oxidoreductase (*GRX2*, *CAGL0K05813g*), as
 268 well as three transcription factors that regulate the OSR pathway (*YAP1*,

269 *CAGLOH04631g*; *MSN2*, *CAGLOF05995g*; and *MSN4*, *CAGLOM13189g*) (29). The relative
 270 expression of each gene was normalized to a control locus (*RDN5.8*) and calculated
 271 relative to that of untreated stationary phase cells. H_2O_2 treatment was used as a
 272 control. Consistent with the RNAseq results (Figure 2C), we observed that transcript
 273 levels of all these genes were downregulated by caspofungin and micafungin
 274 treatment, with *SOD2*, *CTA1*, and *MSN4* showing the greatest reduction in RNA
 275 abundance (up to 4 log₂ units, or 16-fold, Figure 4B). This transcriptional response was
 276 highly distinct from that induced by H_2O_2 , which predominantly resulted either in an
 277 increase or no change in these genes' transcript abundance (Figure 4B). The log₂ ratios
 278 (treatment/no treatment) obtained for these genes by qPCR showed good agreement
 279 with "bulk" and "enriched" RNAseq results (Figure 4C).

280

281 ROS reduction per se does not increase echinocandin tolerance in *C. glabrata*

282 As mentioned above, ROS are generally viewed as cell damaging agents and have been
 283 shown to mediate the killing effects of certain antimicrobial drugs. To investigate
 284 whether this is the case for echinocandins in *C. glabrata*, we turned our focus again to
 285 the mitochondrial inhibitors. First, we examined their effects on caspofungin-induced
 286 killing of *C. glabrata* over a wide range of caspofungin concentrations (Figure 5A). We
 287 believe such killing assay is a better indicator of drug tolerance than the standard
 288 minimum inhibitory concentration (MIC) determination, which measures growth but
 289 not survival in the presence of drug (8). The caspofungin MIC-value obtained for
 290 ATCC2001 was 0.12 μ g/ml, and it was not affected by any of the mitochondrial
 291 inhibitors (Table S2). Nevertheless, survival after 24 hours in caspofungin was rescued
 292 by rotenone, DPI and sodium azide at several different caspofungin concentrations

(Figure 5A). Next, we examined how ROS production was affected by these mitochondrial inhibitors and found that they reduced ROS levels in caspofungin-treated cells (Figure 5B). This was especially evident when ROS was detected by CFDA staining but less so when DHE staining was used, suggesting that these compounds reduce the abundance of some ROS species more than others.

The result above was consistent with the hypothesis that elevated ROS may contribute to echinocandin-induced cell death in *C. glabrata*. To test this hypothesis directly, we used ROS scavengers. We tested several compounds with ROS scavenging activities (thiourea, dimethylthiourea, sodium dithionite, catalase, α -tocopherol and ascorbic acid), but found that only ascorbic acid significantly reduced the levels of ROS in caspofungin-treated cells (Figure 5C). Interestingly, when we measured cell survival of caspofungin treatment in the presence of ascorbic acid, we found no difference between cells cultured in the absence or presence of this ROS scavenger (Figure 5D). This result indicated that it was not ROS per se that was responsible for cell death upon caspofungin treatment.

ROS can damage different cellular biomolecules, which is thought to contribute to their involvement in cell death in other contexts (30). Thus, we examined whether elevated ROS levels in caspofungin-treated cells were associated with detectable damage of two types of biomolecules: lipids and DNA. To measure lipid peroxidation, a fluorometric assay was performed using diphenyl-1-pyrenylphosphine (DPPP), a compound that reacts with lipid hydroperoxides to produce fluorescent DPPP oxide. To measure DNA damage, we examined the abundance of histone H2A phosphorylated at serine 129 (γ H2A.X), which is a universal marker of double-strand breaks (31). In both cases, H_2O_2 was used as a positive control. Interestingly, whereas H_2O_2 -treated cells

showed robust induction of lipid peroxidation and γ H2A.X levels, echinocandin-treated cells did not show evidence of either lipid peroxidation or DNA damage relative to non-treated controls (Figure 5E, F). This result was consistent with the conclusion that despite an increased abundance of ROS in echinocandin-treated cells, these ROS did not significantly contribute to cellular damage or lethality.

Deletion mutants lacking components of the mitochondrial respiratory chain show a caspofungin hypo-tolerant phenotype

The observations that mitochondrial inhibitors altered echinocandin tolerance but that ROS per se were not involved made us examine the role of mitochondria in echinocandin tolerance more closely. To this end, we generated a number of different mitochondria-deficient strains (Table S3). First, we created deletions of five genes encoding components of the mitochondrial respiratory chain: *NDI1* (*CAGL0B02431g*), whose *Saccharomyces cerevisiae* ortholog has NADH dehydrogenase activity and is a component of the fungal equivalent of mitochondrial complex I; *COX4* (*CAGL0L06160g*), whose ortholog has the cytochrome-c oxidase activity of mitochondrial complex IV, and three components of the mitochondrial complex V ATP synthase – the F1 alpha subunit of the F1F0-ATPase complex encoded by *ATP1* (*CAGL0M09581g*), the F1 beta subunit encoded by *ATP2* (*CAGL0H00506g*), and the assembly factor for the F0 sector of mitochondrial F1F0 ATP synthase encoded by *ATP10* (*CAGL0C02651g*). All these deletion mutants, with the exception of *ndi1Δ*, showed a petite phenotype characterized by slow growth and inability to use a non-fermentable carbon source (Figure S2). We also isolated several respiration-deficient (a.k.a. petite) mutants that spontaneously but rarely (approximately one in 3000-5000

colonies) emerged after 24 hours of 0.25 µg/ml caspofungin treatment. Finally, we generated several petite mutants by treatment with ethidium bromide (32). All isolated petite mutants were respiration-deficient and slow growing; however, all of them stained to various degrees by MitoTracker Green, indicating that they were not fully lacking mitochondria (Figure S3A). As expected, most mitochondrial mutants, including several gene deletions (*atp1Δ*, *atp2Δ*, and *atp10Δ*) and all petite mutants, showed elevated MICs to fluconazole (Table S3), consistent with previous reports that mitochondrial dysfunction in *C. glabrata* induces *PDR1* expression and promotes azole resistance (33, 34).

All mitochondria-deficient mutants generated above were examined in the caspofungin killing assay. Interestingly, and contrary to the expectation formed based on the phenotype of mitochondrial inhibitors, all deletion mutants (including *ndi1Δ*, which was not petite) showed a caspofungin hypo-tolerant phenotype at one or more concentrations (Figure 6A, B). The same hypo-tolerant phenotype was observed in all examined petite mutants (Figure 6C and Figure S3B). Because this hypo-tolerance was different from the phenotype caused by inhibitors of the mitochondrial respiratory chain, we wondered whether it may be due not to a lack of respiratory genes per se but to general mitochondrial defects. To test this, we also deleted three genes encoding mitochondrial proteins not directly involved in the respiratory chain: *PET9* (*CAGL0F04213g*), encoding major ADP/ATP carrier of the mitochondrial inner membrane; *TIM18* (*CAGL0A03784g*), encoding a translocase of the inner mitochondrial membrane; and *YME1* (*CAGL0K05093g*), encoding a catalytic subunit of i-AAA protease complex responsible for degradation of misfolded mitochondrial gene products. We found that each of these mutants was likewise hypo-tolerant to caspofungin, indicating

365 that this hypo-tolerance may be a general feature of *C. glabrata* strains with
366 mitochondrial defects (Figure S3C).

367 Interestingly, the hypo-tolerance of mitochondrial mutants to caspofungin was
368 rescued by rotenone in every tested case (Figure 6D, E, F), indicating that rotenone's
369 effect on echinocandin tolerance is exerted whether cells have functional
370 mitochondria or not. In contrast, DPI did not improved survival in any examined
371 mitochondrial mutants, indicating that its effect was mediated by mitochondria (Figure
372 S4A, S4B). Furthermore, we examined the effect of sodium azide on the *cox4Δ* mutant
373 because complex IV is the target of this mitochondrial inhibitor. No rescue of survival
374 was observed, indicating that a functional complex IV is necessary to mediate the
375 effect of sodium azide (Figure S4C).

376 To test whether the hypo-tolerance to caspofungin was due to a general cell
377 wall integrity defect, we assayed the sensitivity of mitochondrial mutants to two
378 different cell wall damaging agents: Congo red and calcofluor white. We found that
379 none of the examined mutants were more sensitive to these agents than the parental
380 wild type strains (Figure S2), indicating that the hypo-tolerance effect was specific to
381 echinocandin-mediated cell killing.

382 Because the effect of mitochondrial inhibitors (which increased tolerance) was
383 opposite of that of mitochondrial mutants (which reduced tolerance), we asked
384 whether one important difference between the two was the timing of the onset of
385 mitochondrial dysfunction. In the experiments using mitochondrial inhibitors, the
386 inhibitors had been added to cells with intact mitochondria at the same time as the
387 echinocandins. However, in the mutants, the mitochondria had been compromised by
388 mutation prior to addition of echinocandins. Therefore, we asked whether inhibition of

mitochondria prior to echinocandin exposure would elicit different effects relative to mitochondrial inhibition induced at the same time as echinocandin exposure. We cultured *C. glabrata* for 18 hours in the presence of rotenone or DPI, then we collected the cells and cultured in fresh YPD media with the mitochondrial inhibitor and caspofungin for another 24 hours, as in the standard tolerance assay (thus, rotenone or DPI were present in the culture both before and after caspofungin addition). Interestingly, pre-exposure of *C. glabrata* to DPI abolished the rescuing effect of DPI on caspofungin tolerance at all tested concentrations, and this was also true for rotenone at one sub-MIC caspofungin concentration (0.06 µg/ml) (Figure S5). These results suggest that chronic mitochondrial inhibition or dysfunction reduces *C. glabrata* echinocandin tolerance, whereas acute mitochondrial inhibition may enhance tolerance.

401

402 **Glucan synthase enzyme from mitochondrial mutants exhibits normal echinocandin** 403 **sensitivity *in vitro***

Echinocandins kill fungal cells by targeting the enzyme 1,3-β-D-glucan synthase (GS) and inhibiting fungal cell wall formation. Thus, the increased echinocandin susceptibility of mitochondrial mutants could potentially be due to changes in GS, which would make the enzyme less susceptible to drug action, as we had demonstrated with echinocandin resistance in *Aspergillus fumigatus* (35), or to changes in pathways acting downstream of GS inhibition. To distinguish between these possibilities, we partially purified GS complexes using our established method consisting of membrane protein extraction and partial GS purification by product entrapment (36) from two different mitochondrial mutants – the *ndi1Δ* strain and one

413 petite mutant – and a wild type control strain. The activity of the isolated GS
414 complexes and their inhibition by micafungin were assayed *in vitro* using a β -glucan
415 polymerization assay (36). We observed that GS isolated from mitochondrial mutants
416 had very similar inhibition kinetics to GS isolated from the wild type strain (Figure 7),
417 indicating that the effect of mitochondria on echinocandin sensitivity likely occurs
418 downstream of the GS inhibition step.

419

420 DISCUSSION

421 Our study presents the first examination of the transcriptional landscape of the
422 echinocandin-tolerant cell subset in the human pathogen *C. glabrata*, which is
423 presumed to serve as a precursor for stable drug resistance (8, 11). We show that the
424 transcriptional state of cells that have survived 24 hours of above-MIC echinocandin
425 exposure is distinct from the stereotypical transcriptional response to environmental
426 stresses. In particular, echinocandin-tolerant cells upregulated pathways involved in
427 chromosome structure and DNA topology and downregulated pathways involved in
428 oxidative stress response. This downregulation occurred even though *C. glabrata* cells
429 had significantly elevated ROS levels upon echinocandin exposure. Interestingly, the
430 ROS did not appear to contribute to echinocandin-induced cell death, as reducing ROS
431 using ascorbic acid did not alter cell killing. Finally, we identified a multifactorial role of
432 mitochondria in *C. glabrata* echinocandin tolerance: whereas using inhibitors of
433 respiratory chain components increased echinocandin tolerance, deletion of
434 respiratory chain components made *C. glabrata* cells hypo-tolerant to echinocandins.
435 Together, these results provide new insights into the *C. glabrata* response to
436 echinocandins and reveal the involvement of mitochondria in echinocandin tolerance.

437 A model describing the proposed role of mitochondria in echinocandin-induced cell
438 death is shown in Figure 8.

439 Several gene categories upregulated in echinocandin-tolerant cells were involved
440 in DNA conformation modification and chromosome organization. However, using
441 topoisomerase inhibitors did not alter the killing dynamics of echinocandin drugs,
442 suggesting that the pathways may not contribute to survival but instead serve other
443 roles in tolerant cells. *C. glabrata* is known for its genome plasticity: its clinical isolates
444 show remarkable genetic diversity both in terms of nucleotide sequence and
445 chromosome structure (37-39). However, it is not known under what circumstances
446 these genomic changes are acquired, as cells cultured in rich medium in the lab appear
447 to be karyotypically stable (37). Thus, cells dealing with long-term echinocandin-
448 induced stress may activate mechanisms enabling chromosomal variation to enhance
449 adaptability. In this context, it is also interesting that we did not detect an increase in
450 abundance of the “DNA damage histone” γ H2A.X during echinocandin exposure,
451 suggesting that DNA double-strand breaks (which induce γ H2A.X formation) either may
452 not be the main lesions triggering chromosomal alterations, or that they occur at lower
453 levels than can be detected by Western blotting. Nevertheless, the transcriptional
454 upregulation of DNA topology and chromosomal conformation genes suggests that
455 these cells may be in a specialized activated transcriptional state that resists drug
456 stress and has an elevated genetic potential to evolve into a genotypically stable drug-
457 resistant cell. Further studies addressing the development of resistant mutations in
458 echinocandin tolerant cells pre-exposed to this drug class will shed light on this issue.

459 Most transcriptional studies of fungal species exposed to drugs have been
460 performed using short-term treatments and traditional bulk RNA-seq approaches with

no enrichment for surviving cells (15, 16, 40). Because echinocandins rapidly induce cell death (17), such bulk analysis performed after a few hours of treatment overwhelmingly includes dead and dying cells. In contrast, our RNA-seq approach was designed to enrich for the rare *C. glabrata* cells surviving 24 hours of above-MIC drug exposure. Thus, our approach identified a distinct transcriptional profile of echinocandin-tolerant cells compared to other studies. Because the echinocandins target cell wall assembly, previous analyses of echinocandin-induced transcriptional changes mainly focused on cell wall-related processes. For instance, Xiong et al. described two *C. albicans* transcription factors regulating cell wall maintenance and homeostasis (Efg1 and Cas5) involved in the transcriptional response to two-hour caspofungin treatment (15). Likewise, in *Aspergillus fumigatus* it has been reported that caspofungin exposure leads to communication between the cell wall-integrity and the high osmolarity-glycerol pathways through MAPK signaling (41). Other studies have also identified transcription factors that play a role in the regulation of the fungal cell wall as well as in echinocandin tolerance (40). Whereas our study also identified several upregulated cell wall maintenance genes in echinocandin-tolerant cells, including *FKS1* and *FKS2*, the cell wall integrity pathway as a whole was not present among the upregulated FUNCAT categories, suggesting that during long-term echinocandin exposure *C. glabrata* cells may switch to other strategies to survive.

In most examined human-pathogenic yeasts and molds, amphotericin B and azoles trigger intracellular ROS formation, which contributes to the antifungal action of these drugs (24, 25, 42, 43). Echinocandins are also associated with an increase in ROS production (25, 43, 44) and, at least in *C. albicans*, ROS scavenger thiourea caused a decrease in micafungin-induced killing (43). Also in *C. albicans*, genes involved in

oxidative stress responses are induced in response to caspofungin (44). Our results (both RNA-seq and qRT-PCR of individual oxidative stress responder and regulator genes) show that although echinocandins also induce robust ROS formation in *C. glabrata*, this fungus does not respond to echinocandins by upregulating oxidative stress response pathways. Indeed, the high ROS levels might be a consequence of the downregulation of expression of the ROS detoxifying enzymes. Furthermore, we could not elicit a reduction of ROS using thiourea or several other scavengers, with the exception of ascorbic acid, which strongly reduced ROS levels in echinocandin-treated cells. Despite this decrease, however, ascorbic acid did not alter the dynamics of cell killing, indicating that in *C. glabrata* the ROS do not significantly contribute to cell death but rather serve another function. Consistent with this idea, the ROS increase was not associated with detectable lipid peroxidation or DNA damage. Although these results run contrary to the traditional view that ROS are exclusively damaging and lack other physiological functions, they are consistent with observations that *C. glabrata* is exceptionally resistant to oxidative stress (45) and in fact, it has recently been described that petite mutants of *C. glabrata* show a lack of oxidative stress susceptibility when incubated with hydrogen peroxide which is combined with a constitutive upregulation of environmental stress-response and heat-shock-protein-encoding genes (46). Relative to other species, *C. glabrata* exhibit a very restrained transcriptional response to phagocyte-induced ROS with insignificant upregulation of catalase (47). Interestingly, recent evidence suggests a critical role of ROS in the communication between the mitochondria and other cellular processes to maintain homeostasis and promote adaptation to stress (48). Furthermore, some studies have also proposed a role for oxidative stress adaptation in promoting virulence and drug

509 resistance in *C. glabrata*, as several genes implicated in virulence, biofilm formation,
510 and drug transport were also induced during the oxidative adaptation response (45).
511 These studies, together with our results, suggest that in *C. glabrata* responding to
512 echinocandins ROS function as a signaling intermediate with as yet unknown specific
513 downstream targets and contribution to fitness.

514 The involvement of mitochondria in echinocandin tolerance in *C. glabrata* was
515 suggested by the RNA-seq data and the observation that mitochondrial inhibitors
516 reduced *C. glabrata* killing by echinocandins over a range of concentrations. In
517 contrast, however, deletion mutants with defective respiratory chain functions,
518 deletions of several other mitochondrial genes, as well as a series of respiration-
519 deficient petite strains of unknown etiology, were all hypo-tolerant to echinocandins,
520 showing that mitochondrial inhibition and genetically encoded mitochondrial
521 deficiency have opposite effects on echinocandin tolerance. Reduced tolerance to
522 echinocandins in some mitochondrial mutants was previously described in *C. albicans*
523 and *S. cerevisiae*. For instance, several *C. albicans* and *S. cerevisiae* mitochondrial
524 mutants are sensitive to multiple cell wall damaging agents, including calcofluor white,
525 Congo red, and echinocandins, indicating a general cell wall defect (49, 50). This defect
526 has been attributed to the role of mitochondria in phospholipid metabolism, whereby
527 mitochondrial defects compromise phospholipid production, affecting cell wall
528 biogenesis (49, 51). Similarly, *S. cerevisiae* mutants defective in producing cardiolipin, a
529 predominantly mitochondrial lipid, have general cell wall defects (52). Interestingly,
530 none of our *C. glabrata* mitochondrial mutants showed increased sensitivity to Congo
531 red or calcofluor white, indicating that the mitochondrial effect on the cell wall in *C.*
532 *glabrata* may be limited to β -glucans. Possibly relatedly, in *S. cerevisiae*, abrogation of

the cardiolipin biosynthetic pathway results in reduced β -glucan levels and can be rescued by mutation of *KRE5*, which encodes a β -1,6-glucan synthase (52). A direct link between glucan synthesis and plasma membrane lipid composition has also been demonstrated in *Aspergillus fumigatus*, where two sphingolipids, dihydrosphingosine and phytosphingosine, rendered GS insensitive to echinocandins (35). Thus, although our RNA-seq analysis did not reveal a lipid-related transcriptional signature in echinocandin-tolerant cells, the studies described above and our results obtained with mitochondrial mutants support a model wherein mitochondrial status may influence the plasma membrane lipid composition, which in turn may influence echinocandin sensitivity.

Another potential link between mitochondria and echinocandin sensitivity has been suggested by reports indicating that in *C. albicans* and in *C. glabrata* echinocandins induce cell death by apoptosis (programmed cell death) and by necrosis (53, 54). It is well known that the role of mitochondria in programmed cell death is complex, and that mitochondria have both pro- and anti-apoptotic functions (55). Thus, it may not be surprising that inhibition of certain mitochondrial activities leads to different survival phenotypes than a total lack of certain mitochondrial proteins due to gene deletions. Interestingly, our results suggest that at least one of the mitochondrial inhibitors, rotenone, which elicited the strongest rescue of cell survival, may not be acting solely via the mitochondria. Rotenone is typically considered a mitochondrial complex I inhibitor. However, *C. glabrata*, like some other fungal species, has lost the canonical NADH:ubiquinone oxidoreductase (complex I) while retaining other mitochondrial oxidative phosphorylation components, including NADH dehydrogenases (encoded by *NDI1*, *NDE1*, and *NDE2*), which largely perform the same

557 function as complex I. The observation that rotenone improved survival not just in wild
558 type cells, but also in every examined mitochondrial mutant, suggests that this
559 rotenone effect is achieved not just via mitochondrial modulation but via influencing
560 other, as yet unclear secondary pathways. To identify these pathways, transcriptomic
561 studies will likely be highly informative. In contrast, DPI (an inhibitor of NADH
562 dehydrogenases) and sodium azide (an inhibitor of complex IV) both failed to improve
563 survival in mitochondrial mutants, suggesting that their activity is indeed mediated by
564 the mitochondria. Surprisingly, the transcriptome of *C. glabrata* echinocandin tolerant
565 cells showed a downregulation of multiple genes involved in mitochondrial function,
566 e.g. *COX8*, *COX26*, *COA1*, *NDI1*, *QCR6*, *QCR8*, *RCF1* (Dataset1), which is the opposite to
567 what has been described for *A. fumigatus* cells isolated during the caspofungin
568 paradoxical effect (16). Thus, it seems the mechanism responsible for echinocandin
569 tolerance in *C. glabrata* and *A. fumigatus* may be different. This conclusion is also
570 supported by the opposite effects of rotenone in the two systems (16).

571 In sum, our results reveal a heretofore unappreciated complexity of the
572 mitochondrial involvement in *C. glabrata* response to echinocandins and
573 echinocandin-mediated killing. Whereas we show that the traditionally frequently used
574 mitochondrial inhibitors reduce fungal killing by echinocandins, which is not a desired
575 effect in the clinic, our results suggest that it may be possible to target fungal
576 mitochondria to sensitize *C. glabrata* to these drugs. Indeed, other types of
577 mitochondrial inhibitors, e.g. those that interfere with mitochondrial biogenesis by
578 blocking mitochondrial protein import (56), may be investigated in future studies as
579 potential enhancers of echinocandin fungicidal action.

580

581

582

MATERIALS AND METHODS

583 Yeast strains and media

584 The *C. glabrata* strains used in this study were ATCC2001 and mutants (gene deletions,
585 petite mutants) derived from ATCC2001 (Table S3). Cells were cultured in standard
586 yeast extract-peptone-dextrose (YPD) medium at 37°C.

587

588 RNA sequencing for echinocandin “enriched” tolerant cells

589 Overnight cultures containing 10^7 /ml *C. glabrata* cells were exposed to 0.25 µg/ml of
590 caspofungin or 0.06 µg/ml of micafungin. After a 24-hour incubation at 37°C with
591 shaking (150 rpm), cells were then collected by filtration using 0.45-µm mixed cellulose
592 ester membrane filters (Millipore) and resuspended in PBS stained with 10 µg/ml
593 propidium iodide (PI) and sorted using the BD FACSMelody (BD Biosciences).
594 Logarithmically growing “no drug” controls were collected after 2 hours of growth in
595 drug-free YPD, whereas “stationary phase” controls were collected after 24 hours of
596 growth in drug-free YPD. Each sample was collected by FACS, treated with zymolyase
597 (5U per 10 µl of cells) for 30 minutes at 37°C to digest the cell walls, and then pipetted
598 into a 96 well-plate to produce ~10 PI-negative cells per well. Thirty-six biological
599 replicates were submitted for each echinocandin treated sample (caspofungin and
600 micafungin) and twelve biological replicates were submitted for each control
601 (logarithmic and stationary). The samples were sent to Columbia University Medical
602 Center (New York, NY) for library preparation, and the “enriched” RNA sequencing of
603 ~10 live cells per well was performed as described previously (57) with some
604 modifications for the reverse transcription reaction using 40U Maxima H-

(ThermoFisher), 2X Maxima H- Buffer (ThermoFisher), 4U SupraseIN (ThermoFisher), 15% polyethylene glycol, and 2uM Template Switching Oligo (IDT) in a total volume of 7.5 µl.

For the RNA-seq analysis, reads were aligned to the NCBI reference, GCF_000002545.3_ASM254v2, using STAR (58). Alignments were assigned to genes using featureCounts (59). The bam files produced by featureCounts were sorted and indexed with samtools (60). The package umi_tools was used to assemble a count matrix (61). Wells were filtered by removing outliers in the distributions of total counts, genes detected, and mitochondrial expression. Highly abundant ribosomal RNAs, which would interfere with normalization, were removed prior to normalization and differential expression analysis. The package sctransform was used to normalize count data (62), and Seurat was then used perform differential expression testing with Wilcoxon rank sum test between groups (63). Raw RNA-seq data have been deposited at the Gene Expression Omnibus (accession no. GSE178797) and differential expression genes (DEGs) files are shown in Dataset 1. The list of *C. glabrata*-*S. cerevisiae* direct orthologs was downloaded from <http://www.candidagenome.org/download/homology/orthologs> and supplemented by manual curation of *C. glabrata* genes using <http://www.candidagenome.org>. Functional categories (FUNCAT) enrichment analysis was performed using FungiFun2 (<https://elbe.hki-jena.de/fungifun/>) (64). The heatmap was generated using R studio.

RNA isolation and “bulk” transcriptome analysis

Cultures containing 10^7 /ml *C. glabrata* were exposed to 0.06 µg/ml of micafungin for 24 hours and collected in triplicate in the same fashion as explained in the previous

629 section. Stationary phase cells were collected after 24 hours of growth in drug-free
 630 YPD in duplicate as no-drug control. Total RNA was extracted using the RNeasy Mini kit
 631 (QIAGEN Science) following the manufacturer's instructions. The RNA was then treated
 632 with RNase-free DNase (ThermoFisher) according to the manufacturer's
 633 recommendations and stored at -80°C until RNA sequencing was performed by
 634 Genewiz (South Plainfield, NJ). The RNAseq data were analyzed using Basepair
 635 software (Basepair, New York, NY) with a pipeline as follows: reads were aligned to the
 636 transcriptome derived from sacCer3 using STAR with default parameters, read counts
 637 for each transcript were measured using featureCounts and the differentially
 638 expressed genes were determined using DESeq2, and a cutoff of 0.05 for the adjusted
 639 P value (corrected for multiple hypotheses testing) was used for creating differentially
 640 expressed gene lists (Dataset 1). GSEA was performed on normalized gene expression
 641 counts, using gene permutations for calculating P value. The fastq RNAseq files have
 642 been deposited at the Gene Expression Omnibus (accession no. GSE178656).

643

644 **Antifungal susceptibility testing**

645 Micafungin (Astellas), caspofungin (Merck) and fluconazole (LKT Labs) susceptibility
 646 testing was performed using a broth microdilution method following CLSI standards
 647 (65) with some modifications. The media used was YPD broth and the concentrations
 648 tested ranged from 0.0035 to 2 µg/ml to echinocandins and 0.25-128 µg/ml to
 649 fluconazole. Minimum inhibitory concentrations (MICs) were visually read after 24
 650 hours of incubation at 37°C. At least three biological replicates were performed.

651

652 **Cell survival measurements after caspofungin treatment combined with inhibitors**

653 To measure the effects of topoisomerase or mitochondrial inhibitors on cell survival in
 654 the presence of caspofungin, overnight cultures containing 10^7 cells/ml were
 655 resuspended in fresh YPD and added caspofungin plus one of the inhibitors at the
 656 following concentrations. The selected concentrations for the topoisomerase inhibitors
 657 were 3 μ M, 200 μ M and 100 μ M of doxorubicin (ThermoFisher), etoposide (Millipore
 658 Sigma), and topotecan (Cayman Chemical), respectively, all dissolved in DMSO. The
 659 selected concentrations for the mitochondrial inhibitors or ROS scavengers were 0.3
 660 mM, 0.005 mM, 0.5 mM and 50 mM of rotenone, diphenyleneiodonium chloride,
 661 sodium azide, and ascorbic acid (Millipore Sigma), respectively, first two dissolved in
 662 DMSO whereas the other two dissolved in water. The selected concentration of
 663 rotenone was determined by its solubility in liquid YPD because concentrations higher
 664 than 0.3 mM resulted in precipitation. After 24 hours of drug exposure, aliquots were
 665 harvested and dilutions plated on drug-free YPD plates. Percentage of survival was
 666 calculated based on the observed number of colonies from caspofungin cultures
 667 relative to the corresponding colony counts from non-caspofungin treated cultures. At
 668 least three biological replicates were performed for every strain and condition.

669

670 **Reactive oxygen species quantification**

671 The presence of intracellular reactive oxygen species (ROS), more specifically hydrogen
 672 peroxide, was assessed by the cell-permeant 2',7'-dichlorodihydrofluorescein diacetate
 673 (CFDA) (10 μ g/ml, ThermoFisher) according to the manufacturer's instructions. Cells
 674 were collected and washed with PBS, then 10 minutes before flow cytometric analysis
 675 each sample was also stained with 10 μ g/ml PI to exclude PI-positive dead cells. The
 676 superoxide indicator dihydroethidium (DHE) was used to assess superoxide radical

677 species (6.5 µg/ml in DMSO, Thermo Fisher) following the manufacturer's instructions.
678 In this case, dead cells to be excluded from the analysis were stained with Sytox Green
679 (1 µM, ThermoFisher). Percentages of ROS-positive cells were calculated relative to all
680 live (PI or Sytox Green-negative) cells after incubation with the dyes. Data were
681 analyzed using FlowJo™ software v10.6.1 (BD Biosciences).

682

683 **RNA extraction and quantitative real-time reverse-transcription (RT)-PCR**

684 Overnight cultures containing 10^7 *C. glabrata* cells/ml were resuspended in fresh YPD
685 containing echinocandins (0.25 µg/ml of caspofungin or 0.06 µg/ml of micafungin) and
686 harvested after 2, 6 or 24 hours, filtered and concentrated in the same fashion as for
687 the “enriched” RNAseq transcriptomic analysis, except that cells were resuspended in
688 RNA extraction buffer provided by the Qiagen kit instead of PBS. Non-treated controls
689 (logarithmic and stationary phase cells) and H₂O₂-treated controls (50 mM) were
690 harvested too in the same fashion, whereby the volume of overnight culture needed to
691 obtain a big enough cell pellet to isolate RNA was smaller. Total RNA was extracted
692 using the RNeasy Mini kit (QIAGEN Science) following the manufacturer's instructions.
693 The RNA was then treated with RNase-free DNase (ThermoFisher) according to the
694 manufacturer's recommendations. RNA samples were stored at -80°C. The transcript
695 levels of *SOD2*, *CTA1*, *GRX2*, *YAP1*, *MSN2*, and *MSN4* were measured by RT-PCR using
696 One Step SYBR PrimeScript RT-PCR Kit II (TaKaRa). Reactions were run on Mx3005P
697 qPCR System (Agilent Technologies) containing 10 ng RNA sample, 0.4 µM of each
698 primer (Table S4), 12.5 µl 2 × One Step SYBR RT-PCR Buffer, and 1 µl PrimeScript 1 step
699 Enzyme Mix 2 in a volume of 25 µl. Thermal cycling conditions were 42°C for 5 min for
700 the reverse-transcription and PCR cycling with initial denaturation at 95°C for 10 s,

701 followed by 40 cycles of denaturation at 95°C for 5 s and annealing and elongation at
702 60°C for 20 s; a post PCR melting curve analysis with 95°C for 5 s, 60°C for 1 min then
703 increasing to 95°C.

704 Each experiment was carried out in biological triplicates and at least two
705 technical triplicates of each biological replicate were also performed. Negative controls
706 were included in each run. The *RDN5.8* gene was used as the reference gene to
707 normalize the data (66). Comparative expression analyses were performed using the
708 $2^{-\Delta\Delta CT}$ method (67). The fold changes were determined from the normalized expression
709 of the average of treated samples relative to the normalized expression of the average
710 of stationary non-treated controls. Calculations were also done using non-treated
711 logarithmic-phase controls; all trends were similar, so these data were not shown.

712

713 ***In vitro* echinocandin tolerance assay**

714 Overnight cultures containing 10^7 *C. glabrata* cells were resuspended in fresh 1 ml YPD
715 and incubated at 37°C with shaking (150 rpm) for 24 hours in the presence of a range
716 of caspofungin concentrations (0, 0.016, 0.06, 0.25, 1, 4 µg/ml). After 24 hours, 0.1 ml
717 of the appropriate dilutions for each culture was plated onto YPD plates. Colony
718 forming units (CFU) were determined and survival percentage was obtained by
719 normalizing the CFU obtained from cultures treated with the indicated concentration
720 of drug to non-treated controls. Co-treatment with mitochondrial inhibitors or ROS
721 scavengers was performed in the same way as described above, except when there
722 was overnight pre-exposure to the mitochondrial inhibitors before caspofungin was
723 added to the media. At least three biological replicates were performed for every
724 strain and condition.

725

726 **Lipid peroxidation**

727 Suspensions of *C. glabrata* 10^7 stationary-phase cells/ml were exposed to caspofungin
728 (0.25 and 1 $\mu\text{g/ml}$) or hydrogen peroxide (5 and 10 mM) and incubated with or without
729 50 μM of diphenyl-1-pyrenylphosphine (DPPP) (ThermoFisher) at 37°C (68, 69). The
730 fluorescence intensity was measured every hour using a Tecan infinite 200 Pro plate
731 reader with a 340/380 nm excitation filter. Data are shown in arbitrary units of
732 fluorescence intensity after 2 and 6 hours of caspofungin exposure.

733

734 **Western blotting**

735 *C. glabrata* cells exposed to echinocandin drugs (0.25 $\mu\text{g/ml}$ of caspofungin or 0.06
736 $\mu\text{g/ml}$ of micafungin), as well as positive (50 mM H_2O_2) and untreated (stationary or
737 logarithmic-phase cells) control samples, were collected after 2, 6, and 24 hours of
738 growth, except for H_2O_2 where 24-hour cultures had too few cells to proceed. Whole-
739 cell lysates were prepared by trichloroacetic acid (TCA) precipitation. Briefly, cell
740 pellets were resuspended in 20% TCA, broken by bead beating, and washed twice with
741 5% TCA. Proteins were pelleted, resuspended in sodium dodecyl sulfate-
742 polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, incubated at 95°C for 5
743 min, and centrifuged prior to loading on 16% acrylamide gels. Antibodies were
744 obtained commercially: anti-H2A (Active Motif catalog no. 39945) and anti- $\gamma\text{H2A.X}$
745 (Abcam ab15083).

746

747 **Spotting assay**

748 Cells obtained from overnight cultures grown in YPD broth at 37°C were spotted in
749 serial 10-fold dilutions on YP plates containing 2% glycerol or YPD plates containing
750 0.05 mg/ml of Congo red (Millipore Sigma) or 0.01 mg/ml of calcofluor white (Millipore
751 Sigma). Growth of each strain was evaluated after 24 hours of incubation at 37°C.
752 Petite mutants grew much slower than the other mutants, so their growth was also
753 evaluated after 48 hours.

754

755 **Microscopy mitochondrial detection**

756 Mitochondria were stained with the fluorescent dye MitoTracker Green FM
757 (ThermoFisher) according to the manufacturer's protocol but with some modifications
758 for yeast staining. The cells were harvested by centrifugation, diluted to 10^6 cells/ml in
759 10mM HEPES buffer, pH 7.4, containing 5% of glucose and stained for 30 min with 0.1
760 μ M MitoTracker Green at room temperature. Stained cells were visualized using a
761 Nikon Eclipse Ti2 inverted microscope (488 nm filter) with Hamamatsu ORCA-Flash4.0
762 camera and analyzed using NIS-Elements software.

763

764 **Construction of *C. glabrata* deletion mutants**

765 Deletion mutants were generated in-house using a CRISPR-Cas9 targeted integration
766 replacing the desired ORF by a nourseothricin (NAT) resistance cassette (Table S3). The
767 deletion constructs were generated by PCR using ~100 nucleotide-long primers
768 designed to amplify NAT and also containing homology regions flanking the locus of
769 interest. All primers used are listed in Supplementary Table 4. Gene replacement by
770 the deletion cassettes was performed using CRISPR as described previously (70).
771 Briefly, cells were made competent for electroporation using the Frozen-EZ yeast

772 transformation kit (Zymo Research) according to the manufacturer's instructions and
 773 then electroporated with Cas9-gRNA complex (Integrated DNA Technologies) and the
 774 DNA containing the deletion construct. Transformants were selected on NAT-
 775 containing plates and validated by PCR amplification and sequencing of the targeted
 776 locus using external primers (Table S4). At least two independent transformants were
 777 generated and analyzed for every deletion mutant. All primers were ordered from
 778 Integrated DNA Technologies (Coralville), and all Sanger sequencing of the above-
 779 described constructs was done by Genewiz (South Plainfield).

780

781 Measurement of inhibition of glucan synthase by micafungin

782 Three isogenic ATCC2001 background strains were selected to examine glucan
 783 synthase (GS) inhibition by echinocandins *in vitro*: wild-type, *ndi1Δ*, and caspofungin-
 784 derived petite mutant #1. The strains were grown with vigorous shaking at 37°C to
 785 early stationary phase in YPD broth, and cells were collected by centrifugation. Cell
 786 disruption, membrane protein extraction, and partial GS purification by-product
 787 entrapment were performed as described previously (36). Sensitivity to micafungin
 788 was measured at least in triplicate in a polymerization assay using a 96-well
 789 multiscree high-throughput screen filtration system (Millipore) with a final volume of
 790 100 µl. Serial dilutions of micafungin (0.01–10,000 ng/ml) were used to determine 50%
 791 inhibitory concentration (IC50) values. Inhibition profiles and IC50 values were
 792 determined using a sigmoidal response (variable-slope) curve-fitting algorithm with
 793 GraphPad Prism 8 software.

794

795 Statistical analysis

796 All shown results represent an average of three or more independent experiments
797 (biological replicates). Error bars represent the standard deviation. All data were
798 analyzed using GraphPad Prism 8 software. Statistical analyses were performed using
799 an unpaired t-test to determine significant differences between experimental groups.
800 P-values ≤ 0.05 were considered statistically significant.

801

802

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812

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1057 **Supplemental material references**

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Figure legends

Figure 1. Isolation of rare echinocandin-tolerant *C. glabrata* cells after micafungin or caspofungin exposure followed by single cell RNA sequencing. (A) *C. glabrata* cells were exposed to above-MIC concentrations of caspofungin (0.25 µg/ml) and micafungin (0.06 µg/ml) for 24 hours. Propidium iodide (PI)-negative cells, which accounted for <1% of all cells, were defined as echinocandin-tolerant. Two kinds of “no drug” controls were included: cells in either logarithmic or stationary phase. Sorted cells were analyzed using a modified single-cell RNA sequencing approach where each well contained 5-10 viable yeast cells. (B) Transcriptional profiles of caspofungin-tolerant cells and micafungin-tolerant cells were highly similar to each other but distinct from either stationary or logarithmic controls.

Figure 2. Echinocandin-tolerant *C. glabrata* cells upregulate genes involved in DNA topology and chromosome structure and downregulate genes involved in oxidative stress responses. (A) Heatmap showing the log2 ratios of “bulk” vs. tolerant cell-enriched (“enriched”) RNAseq samples. (B) Heatmap showing the log2 ratios of “enriched” RNAseq samples relative to “no drug” log phase or stationary phase controls. (C) Genes upregulated relative to both “no drug” controls were enriched for functional categories of ATP binding, DNA conformation modification and topology, and chromosome organization, whereas downregulated genes were enriched for functional categories of oxidative stress response, NAD/NADH binding, and oxygen and radical detoxification.

Figure 3. Mitochondrial inhibitors but not topoisomerase inhibitors increase echinocandin tolerance in *C. glabrata*. Chemical inhibitors were used to modulate the most significantly up- or downregulated pathways and examine the resulting effects on echinocandin tolerance. (A) Topoisomerase inhibitors did not alter echinocandin tolerance in *C. glabrata*, whereas (B) mitochondrial inhibitors increased the fraction of caspofungin-surviving cells. Statistical significance was calculated using an unpaired t-test and is indicated with asterisks (* p-value ≤0.05, ** p-value ≤0.01, *** p-value ≤0.001) or with n.s. (not significant).

Figure 4. Caspofungin treatment leads to increased reactive oxygen species (ROS) levels but decreased expression of genes involved in oxidative stress responses. (A) Two different ROS-sensitive dyes, CFDA or DHE, were used to detect hydrogen peroxide or superoxide species, respectively. Dead cells were gated out using PI or Sytox Green (SG). 50 mM H₂O₂ as a positive control. Caspofungin induced a strong increase in both CFDA and DHE staining, and this increase was abolished in an *fks1* mutant, which lacks wild type glucan synthase (echinocandin target enzyme). NT indicates caspofungin non-treated cells. (B) Oxidative stress response (OSR) genes as well as transcription factors involved in the regulation of the OSR pathway were downregulated by caspofungin and micafungin treatment but were either induced or unaltered by the H₂O₂ control. (C) Heatmap comparing log2 ratios of treatment/control obtained using different molecular approaches for the six selected genes involved in oxidative stress responses. The statistical significance was calculated using an unpaired

t-test and is indicated with asterisks (* p-value ≤ 0.05 , ** p-value ≤ 0.01 , *** p-value ≤ 0.001 , **** p-value ≤ 0.0001) or with ns (not significant).

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Figure 5. Mitochondrial inhibitors but not an ROS scavenger improve survival of *C. glabrata* cells in caspofungin. (A) Mitochondrial inhibitors rotenone, DPI and sodium azide increased the fraction of caspofungin-surviving cells over a wide range of caspofungin concentrations. (B) The mitochondrial inhibitors also reduced ROS production as detected by CFDA and DHE. (C) Ascorbic acid, an ROS scavenger, strongly decreased ROS levels induced by caspofungin. (D) Ascorbic acid did not alter cell survival in the presence of caspofungin. (E) Despite the elevation of ROS, no increase in lipid peroxidation, as measured by the DPPP fluorometric assay, was detected in the presence of caspofungin. (F) No increase in DNA damage as measured by γ H2A.X abundance was detected in the presence of caspofungin or micafungin. The statistical significance was calculated using an unpaired t-test and is indicated with asterisks (* p-value ≤ 0.05 , ** p-value ≤ 0.01 , *** p-value ≤ 0.001 , **** p-value ≤ 0.0001) or with ns (not significant).

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Figure 6. Mutants lacking various mitochondrial respiratory chain components and mitochondrial-defective petite mutants showed a caspofungin hyper-susceptible phenotype, which was rescued by rotenone. (A) Deletion mutant of mitochondrial respiratory chain component *NDI1* (NADH dehydrogenase, the yeast equivalent of complex I) and *COX4* (cytochrome-c oxidase of complex IV) showed a mild hyper-susceptibility to caspofungin that was rescued by rotenone (B). (C) Deletion mutants of three components of the mitochondrial complex V ATP synthase (*ATP1*, *ATP2* and *ATP10*) also showed a hyper-susceptible phenotype to a wide range of caspofungin concentrations below the MIC-value that was rescued by rotenone (D). (E) Respiration-deficient petite mutants generated during caspofungin exposure showed the strongest hyper-susceptible phenotypes that were also rescued by rotenone (F).

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Figure 7. 1,3- β -D-glucan synthase (GS) isolated from mitochondrial mutants shows unaltered micafungin inhibition kinetics *in vitro*. Echinocandin inhibition profiles and biochemical sensitivity (IC₅₀) for product-entrapped GS enzyme complexes was assessed by the incorporation of [³H]glucose into radiolabeled product. Micafungin titration curves and EC₅₀ values are shown for two different mitochondrial mutants – the *ndi1Δ* strain and one petite mutant – and the wild type control strain.

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Figure 8. Graphical representation of the proposed model for the role of mitochondria in echinocandin tolerance. (A) Echinocandin exposure in *Candida glabrata* cells inhibits GS activity, leading to cell death via mitochondria-dependent as well as mitochondria-independent mechanisms. The mitochondrial involvement also results in increased ROS production. (B) When echinocandin exposure is combined with mitochondrial inhibitors, mitochondria-dependent mechanisms of cell death are reduced, along with ROS production. (C) Chronic mitochondrial dysfunction leads to hypo-tolerance to echinocandins characterized by decreased survival at below-MIC concentrations. In this case, rotenone still partially rescues survival, likely due to non-mitochondrial mechanisms. Created with BioRender.com

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Figure S1. Functional categories enriched among differentially upregulated and downregulated genes. (A) Functional categories in “bulk” RNAseq samples relative to stationary “no drug” control. (B) Functional categories in tolerant cell-enriched (“enriched”) RNAseq samples relative to stationary “no drug” controls. (C) Functional categories in tolerant cell-enriched (“enriched”) RNAseq samples relative to log phase “no drug” controls. Functional categories found in at least two datasets are marked in violet.

Figure S2. Mitochondrial defective mutants showed wild type sensitivity to cell wall damaging agents. Mitochondrial deletion mutants were photographed after 24 hours. Petite mutants of unknown etiology, which were very slow growing, were photographed after 48 hours. YPG = YP + 2% glycerol. Congo red (CR) was used at 0.05 mg/ml and calcofluor white (CW) was used at 0.01 mg/ml.

Figure S3. Characterization of *C. glabrata* mitochondrial mutants. (A) MitoTracker Green detection by fluorescence microscopy showed mitochondrial content in petite *C. glabrata* strains. (B) Ethidium bromide-derived petite mutants were hypo-tolerant at sub-MIC caspofungin concentration and showed variable survival at above-MIC caspofungin concentrations. (C) Deletion mutants lacking mitochondrial proteins not directly involved in the respiratory chain showed a caspofungin hypo-tolerant phenotype. The examined deletion mutants were a major ADP/ATP carrier of the mitochondrial inner membrane (*PET9*), a translocase of the inner mitochondrial membrane (*TIM18*), and a catalytic subunit of i-AAA protease complex responsible for degradation of misfolded mitochondrial gene products encoding (*YME1*).

Figure S4. Diphenyleneiodonium chloride (DPI) and sodium azide did not improved survival in deletion mutants lacking mitochondrial respiratory chain components. Deletion mutants of mitochondrial respiratory chain components *ND11* (NADH dehydrogenase of equivalent of complex I) and *COX4* (cytochrome-c oxidase of complex IV) (A), and complex V ATP synthase (*ATP1*, *ATP2* and *ATP10*) (B) were not rescued by DPI in the caspofungin tolerance assay. (C) Sodium azide did not rescue survival of the *cox4Δ* mutant.

Figure S5. Exposure of *C. glabrata* cells to the mitochondrial inhibitors (A) rotenone and (B) diphenyleneiodonium chloride (DPI) prior to caspofungin treatment reduced cell survival. Rotenone was still able to rescue growth at several caspofungin concentrations above the MIC.

Table S1. Sorting cells that did not stain with propidium iodide resulted in the strongest enrichment for cells capable of producing colonies. Fluorescence-activated cell sorting (FACS) was used with different combinations of live/dead dyes and fluorescent markers in cells cultured in the presence of either 0.25 µg/ml caspofungin or 0.06 µg/ml micafungin for 24 hours, followed by plating of the sorted fractions on drug-free medium and counting the colony forming units (CFU). PI (propidium iodide), CFDA-AM (5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester), SG (Sytox Green), RFP (red fluorescence protein), GFP (green fluorescence protein).

1210 **Table S2. MIC susceptibility testing of selected concentration of inhibitors in**
 1211 **combination to caspofungin and micafungin showed identical MIC-values than**
 1212 **echinocandins by themselves.** The inhibitor concentration selected was based on the
 1213 MIC-value obtained in the susceptibility testing together with its solubility in YPD liquid
 1214 media. DPI (diphenyleneiodonium chloride). * Rotenone is non-soluble >0.3 mM.
 1215 **Other authors have used 2.5-10 mM of ascorbic acid combined with other drug
 1216 compounds in *Candida* species (1-3).

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 1218 **Table S3.** List of *Candida glabrata* strains used in this study and their fluconazole
 1219 susceptibilities. ECH (echinocandins).

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 1221 **Table S4.** Sequences of primers used in this study for RTqPCR transcriptional
 1222 experiments and generation of CRISPR knock-out mutants. * Primers described in a
 1223 different study (4).

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 1226 **Dataset 1.** “Bulk” and “enriched” RNAseq data.

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Functional gene categories in “enriched” RNAseq samples relative to both stationary and log phase cells

	WT	<i>ndi1</i> Δ	<i>petite</i>
IC50	8.881	8.432	10.85