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1 Loss of C-5 sterol desaturase activity results in increased resistance to azole and  
2 echinocandin antifungals in a clinical isolate of *Candida parapsilosis*

3

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20 Running title: *ERG3*-mediated multidrug resistance in *Candida parapsilosis*

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36 **Abstract** (249 words out of 250 words max)

37       Among emerging non-*albicans* *Candida* species, *C. parapsilosis* is of particular  
38 concern as a cause of nosocomial bloodstream infections in neonatal and intensive care  
39 unit patients. While fluconazole and echinocandins are considered effective treatment  
40 of such infections, recent reports of fluconazole and echinocandin resistance in *C.*  
41 *parapsilosis* indicate a growing problem. The present study describes a novel  
42 mechanism of antifungal resistance in this organism affecting the susceptibility of azole  
43 and echinocandin antifungals in a clinical isolate obtained from a patient with prosthetic  
44 valve endocarditis. Transcriptome analysis indicated differential expression of several  
45 genes in the resistant isolate including upregulation of ergosterol biosynthesis pathway  
46 genes *ERG2*, *ERG5*, *ERG6*, *ERG11*, *ERG24*, *ERG25*, and *UPC2*. Whole-genome  
47 sequencing revealed the resistant isolate possessed an *ERG3* mutation resulting in a  
48 G111R amino acid substitution. Sterol profiles indicated a reduction in sterol  
49 desaturase activity as a result of this mutation. Replacement of both mutant alleles in  
50 the resistant isolate with the susceptible isolate's allele restored wild-type susceptibility  
51 to all azoles and echinocandins tested. Disruption of *ERG3* in the susceptible and  
52 resistant isolates resulted in a loss of sterol desaturase activity, high-level azole  
53 resistance, and an echinocandin-intermediate to -resistant phenotype. While disruption  
54 of *ERG3* in *C. albicans* resulted in azole resistance, echinocandin MICs, while elevated,  
55 remained within the susceptible range. This work demonstrates that the G111R  
56 substitution in Erg3 is wholly responsible for the altered azole and echinocandin  
57 susceptibilities observed in this *C. parapsilosis* isolate and is the first report of an *ERG3*  
58 mutation influencing susceptibility to the echinocandins.

## 59 Introduction

60 *Candida* species are among the most common causes of bloodstream infections  
61 in the United States and are associated with high morbidity and mortality. While *C.*  
62 *albicans* is the most commonly isolated causative agent of candidemia, infections due to  
63 other non-*albicans* species of *Candida* have been increasing in recent decades (1-3).  
64 Of these, *Candida parapsilosis* is of particular concern as a human fungal pathogen. It  
65 exhibits a wide growth capacity on surfaces, such as prosthetic materials and  
66 intravascular devices and grows well within parenteral nutrition. It persists in hospital  
67 environments and may be transmitted nosocomially via hand carriage. Low weight  
68 neonates and intensive care patients are among the highest at risk for infections with *C.*  
69 *parapsilosis* (4). Relative to what is known in *C. albicans*, there is little information  
70 regarding the mechanisms by which antifungal drug resistance develops in *C.*  
71 *parapsilosis*. Uniquely among *Candida* species, *C. parapsilosis* demonstrates intrinsic  
72 reduced in vitro susceptibility to the echinocandins, presumably as a result of a  
73 naturally-occurring polymorphism in *FKS1* (5). While uncommon, clinical resistance to  
74 this class of antifungals has emerged in *C. parapsilosis* (6, 7). Azole resistance, on the  
75 other hand, is more common in *C. parapsilosis*, with rates of fluconazole resistance  
76 being approximately five times that of *C. albicans* (8). Recent reports indicate that  
77 overexpression of the drug efflux pump Mdr1 as well as an increase in, or mutation of,  
78 the target of the azoles, Erg11, contributes to azole resistance in this species (9-12).

79 In this study, we investigated the mechanisms underlying antifungal drug  
80 resistance in a clinical isolate of *C. parapsilosis* by comparing it to a genetically-  
81 matched, antifungal-susceptible isolate from the same patient. Through whole

82 transcriptome and genome sequence analysis and allelic replacement, we demonstrate  
83 that a mutation in *ERG3* leads to resistance to the azole antifungals via alternate sterol  
84 production. Additionally, we discover that this mutation in *ERG3*, as well as its  
85 disruption, also causes increased resistance to the echinocandins. This is the first  
86 report of an *ERG3*-related resistance mechanism in *C. parapsilosis* and is the first  
87 evidence in any *Candida* species that such mutations also influence susceptibility to the  
88 echinocandins.

89

## 90 **Results**

### 91 *Clinical Isolates and Susceptibility Testing*

92 The *C. parapsilosis* isolates used in this study were collected over the course of multiple  
93 hospitalizations from a patient with aortic valve endocarditis, as outlined elsewhere (13).  
94 These isolates were kindly provided by Dr. Jose Vazquez. The originally reported  
95 antifungal susceptibilities (using the previous CLSI M27-A2 recommended  
96 methodologies) for both the susceptible isolate collected at initial admission (Isolate 1)  
97 as well as for the subsequently collected resistant isolate (Isolate 2) for several azoles,  
98 echinocandins, and amphotericin B are shown in Tables 3A and 3B. MIC (minimum  
99 inhibitory concentration) values for fluconazole, itraconazole, voriconazole,  
100 posaconazole, caspofungin, anidulafungin, micafungin, and amphotericin B were  
101 independently determined for this study by the Fungus Testing Laboratory at the  
102 University of Texas Health Sciences Center at San Antonio (Tables 3A and 3B). Isolate  
103 1 was susceptible to all azoles tested when read at both 24 and 48 hours, and likewise

104 to amphotericin B and all echinocandins tested (determined at 24 hours). Interestingly,  
105 when determined at 24 hours, Isolate 2 appeared susceptible to all azoles tested,  
106 whereas when determined at 48 hours, was resistant to all azoles tested. These results  
107 were consistent upon repeat testing. Isolate 2 also exhibited an increase in MIC for the  
108 echinocandins, reaching the intermediate range for micafungin and anidulafungin. No  
109 change in susceptibility to amphotericin B was observed. In separate experiments, 24  
110 and 48 hours growth curves were plotted for fluconazole MIC cultures (Figure 1). At  
111 both timepoints Isolate 2 phenocopied the *erg3Δ/erg3Δ* mutants.

112

### 113 *Transcriptional Profiling Reveals Increased Expression of Ergosterol Biosynthesis*

#### 114 *Genes*

115 Global changes in gene expression between Isolates 1 and 2 were determined  
116 by RNA sequencing. A total of 378 genes (Table S1) were observed to be reproducibly  
117 upregulated by a minimum of 1.5-fold in the resistant isolate as compared to the  
118 susceptible isolate. Of note, only three of these genes (*UPC2*, *ATC1*, and  
119 *CPAR2\_400860*) have been characterized in *C. parapsilosis* at this time. Gene  
120 Ontology term analysis performed using the Candida Genome Database  
121 ([www.candidagenome.org](http://www.candidagenome.org)) revealed genes of the sterol metabolic process to be the  
122 most significantly enriched among all upregulated genes ( $p=2.96e^{-5}$ ). Of these 14  
123 genes, as shown in Table 4, the sterol regulatory transcription factor *UPC2* and the *C.*  
124 *parapsilosis* orthologs of identified CaUpc2 target genes (*ERG1*, *ERG2*, *ERG5*, *ERG6*,  
125 *ERG11*, *ERG24*, and *ERG25*) are included (14). Differential expression of key  
126 ergosterol biosynthesis genes, as well as the lack of a change in expression of the

127 multidrug transporter genes *CDR1*, *CDR2*, and *MDR1*, were confirmed with qRT-PCR  
128 (data not shown). An additional 32 genes were observed to be upregulated in the  
129 resistant isolate, but the extent of which could not be reliably quantified due to extremely  
130 low transcript counts in the susceptible isolate (Table S2). Genes which are  
131 reproducibly downregulated in the resistant isolate as compared to the susceptible  
132 isolate are listed in Table S3.

133

#### 134 *Next Generation Sequencing Identifies a SNP in ERG3 in the Resistant Isolate*

135 To further characterize this matched isolate pair, whole genome sequencing was  
136 used to identify single nucleotide polymorphisms occurring in Isolate 2 as compared to  
137 Isolate 1 (Figure 2). There was a total of 462 SNPs detected, involving 305 individual  
138 genes. Many of the genes containing SNPs are involved in cell adhesion, biofilm  
139 formation, and cytoskeletal rearrangement. A homozygous nonsynonymous mutation  
140 was detected in *ERG3*, substituting a glycine to an arginine at position 111. This  
141 polymorphism was confirmed by Sanger-based sequencing (data not shown).

142

#### 143 *Analysis of Copy Number Variation and Loss of Heterozygosity*

144 The Yeast Mapping Analysis Pipeline (Ymap; [lovelace.cs.umn.edu/Ymap](http://lovelace.cs.umn.edu/Ymap)) was  
145 used to identify chromosomal copy number variation (CNV) and loss of heterozygosity  
146 (LOH) between Isolate 1 and Isolate 2 (15). CNV and SNP analysis of Isolate 1  
147 revealed no segmental or chromosomal CNV, and scattered regions of increased  
148 heterozygosity across each chromosome (Figure 3A). Using the Isolate 1 analysis as a



149 parental reference isolate, the WGS data for Isolate 2 was then assessed. Unlike its  
150 parent isolate, Isolate 2 exhibited segmental tetraploidy in a single chromosome  
151 (Contig005809). In this region, containing 113 genes (Table S4), complete duplication  
152 across approximately 233,000 bases was observed (Figure 3B). Additionally, LOH  
153 analysis revealed that while relatively little allelic variation was present in Isolate 1,  
154 much of this variation has been lost in Isolate 2 (Figure S1).

155

156 *Loss of Erg3 Activity Confers Increased Resistance to Echinocandins in C. parapsilosis*  
157 *and to a Lesser Extent C. albicans*

158 We first deleted both alleles of *ERG3* in both the susceptible (Isolate 1) and  
159 resistant (Isolate 2) isolates. As expected, loss of *ERG3* resulted in resistance to all  
160 azoles tested (Table 3A). Surprisingly, deletion of *ERG3* also resulted in MICs that fell  
161 within the intermediate to resistant range for the echinocandins (Table 3B). In order to  
162 determine if this also occurs in the related species *C. albicans*, we tested the  
163 echinocandin susceptibilities of two independent *ERG3* deletion mutants constructed in  
164 two different backgrounds. In both cases deletion of *ERG3* resulted in negligible  
165 increases in echinocandin MICs (Table 3B).

166

167 *The G111R Substitution in Erg3 Confers Reduced Susceptibility to Azoles and*  
168 *Echinocandins*

169 In order to directly assess the role of the G111R amino acid substitution in Erg3  
170 in the reduced susceptibility to azoles and echinocandins, we introduced the wild-type

171 *ERG3* alleles from the susceptible isolate (Isolate 1) into its resistant counterpart  
172 (Isolate 2), creating a homozygous mutant, and performed susceptibility testing against  
173 azoles and echinocandins. Introduction of the wild-type *ERG3* alleles to Isolate 2  
174 restored susceptibility to all azoles tested (Tables 3A and 3B). Surprisingly,  
175 susceptibility to the echinocandins was also restored to the levels observed in Isolate 1.

176

### 177 *The G111R Substitution in Erg3 Leads to Alterations in Sterol Biosynthesis*

178 As the mutation in *ERG3* is not a nonsense mutation, we questioned if,  
179 alternatively, the activity of Erg3 is reduced by this mutation, leading instead to a less  
180 functional protein. Perturbations in this protein would be evident with increases in  
181 accumulated ergosta-7,22-dienol, episterol, and ergosta 7-enol. Sterol profiles were  
182 obtained using GC/MS and are shown in Table 5. Isolate 1 exhibits a reasonably  
183 normal sterol profile in which ergosterol comprised the highest percentage. On the  
184 other hand, Isolate 2 exhibits profiles in which ergosta-7-enol and ergosta-7,22-dienol  
185 represented the largest portion of the total cell fraction. This is consistent with a  
186 reduction in sterol desaturase activity. Upon replacement of the mutant *ERG3* alleles in  
187 Isolate 2 with the wild-type allele from Isolate 1, normal sterol profiles were restored.  
188 Deletion of *ERG3* in both Isolate 1 (susceptible isolate) and Isolate 2 (resistant isolate)  
189 resulted in profiles consistent with complete loss of Erg3 activity.

190

### 191 **Discussion**

192 Most of what is known about the genetic basis of antifungal resistance has been  
193 determined from *C. albicans*. Mechanisms of azole resistance in this species include  
194 overexpression of the gene encoding the target enzyme, Erg11, and nonsynonymous  
195 mutations in both *ERG11* and *ERG3*. Additionally, upregulation of drug efflux pumps  
196 represents an additional mechanism of azole resistance; ABC transporters Cdr1 and  
197 Cdr2 confer resistance to multiple azoles, while the MFS transporter Mdr1 confers  
198 resistance to fluconazole, ketoconazole, and voriconazole (16). Echinocandins, on the  
199 other hand, function by inhibiting  $\beta$ -D-glucan synthase activity, encoded by *FKS1* in *C.*  
200 *albicans* and *FKS1* and *FKS2* in *S. cerevisiae* (17). Although mutations within *FKS*  
201 genes have been linked to echinocandin resistance in several species of *Candida* (18),  
202 there are also additional mechanisms by which this resistance may occur. These  
203 include the activation of signaling pathways which regulate stress response and PKC  
204 cell wall integrity, as well as synthesis of ancillary cell wall components such as chitin  
205 and mannan (19).

206 In the present study, RNA sequencing revealed the genes that are differentially  
207 expressed between these two genetically-related clinical isolates. We did not observe  
208 overexpression of any gene encoding a characterized multidrug transporter suggesting  
209 that azole resistance was unlikely due to reduced intracellular accumulation of drug.  
210 Furthermore, the observed 1.6 to 2.6- fold increased expression of *ERG11* in the  
211 resistant isolate, as compared to the susceptible isolate, would alone not be expected to  
212 produce high-level pan-azole resistance particularly in the absence of an accompanying  
213 *ERG11* mutation. Lastly, we did not observe any change in gene expression that would

214 indicate a root cause for the observed reduction in echinocandin susceptibility, such as  
215 an increase in glucan synthase or chitin synthase expression.

216 Notably among the upregulated genes are several involved in the ergosterol  
217 biosynthesis pathway. This biosynthetic pathway results in the production of an  
218 essential fungal membrane component, ergosterol, and inhibition of the azole target  
219 sterol demethylase leads to production of toxic sterol intermediates (20). The azoles  
220 exploit this and function specifically by inhibiting the enzyme 14 $\alpha$ - lanosterol  
221 demethylase, encoded by *ERG11*, leaving the cell unable to produce ergosterol (21).  
222 The genes which were observed to be upregulated within the resistant isolate, *ERG1*,  
223 *ERG2*, *ERG5*, *ERG6*, *ERG11*, *ERG24*, *ERG25*, *ERG27*, and *DAP1*, encode various  
224 proteins involved in sterol processing within this pathway and are located both upstream  
225 and downstream of the azole target, Erg11. Additionally, the gene encoding the sterol  
226 regulatory transcription factor Upc2 was upregulated. These observations are consistent  
227 with loss of sterol desaturase activity leading to decreased membrane ergosterol and  
228 activation of Upc2. In this way, an upregulation of these critical genes represents a  
229 response to facilitate increased conversion of lanosterol to ergosterol in the event that  
230 production had declined or been disrupted. This is further supported by the results of  
231 the whole genome sequencing of the isolate pair. Among a relatively small number of  
232 SNPs detected in the resistant isolate which do not occur in the susceptible isolate, a  
233 nonsynonymous mutation was identified in *ERG3*. This gene is located downstream in  
234 the ergosterol biosynthesis pathway relative to the genes found to be upregulated by  
235 RNA sequencing. The amino acid substitution, at position 111 of the predicted protein,  
236 changes a glycine to an arginine.

237 Mutation within *ERG3* has been documented in clinical isolates as well as  
238 passage-derived isolates of *C. albicans* and linked to both amphotericin B resistance  
239 and high-level azole resistance (22, 23). When azoles inhibit the function of 14 $\alpha$ -  
240 demethylase and therefore the production of ergosterol, *ERG3* encodes an enzyme  
241 which converts the nontoxic 14 $\alpha$ -methylated sterol intermediates into the toxic sterol  
242 14 $\alpha$ -methyl-ergosta-8,24 (28)-dien-3,6-diol. A reduction in or loss of this enzyme results  
243 instead in the accumulation of nontoxic ergosta-7,22-dienol. This leads to high levels of  
244 resistance to the azole drug class.

245 The loss of ergosterol in the yeast cell membrane, the target of amphotericin B,  
246 has been reported to result in resistance to this antifungal (24-26). However, using  
247 standard CLSI methods, we did not observe a reproducible increase in amphotericin B  
248 MIC at 24 or 48 hours for the *C. parapsilosis* or *C. albicans* mutants deleted for *ERG3*  
249 or for the *C. parapsilosis* mutant expressing the G111R mutation. The majority of *ERG3*  
250 mutations reported in the literature result in premature stop codons; however, there  
251 have been cases in which the strains instead exhibit nonsynonymous mutations, as  
252 observed in this isolate pair (22, 27-29).

253 The finding that the G111R amino acid substitution also explained the reduced  
254 susceptibility to the echinocandins observed in this isolate was unexpected as, until now,  
255 alterations in sterol desaturase activity have not been associated with this phenotype. It  
256 is important to note that deletion of *ERG3* had a similar effect on echinocandin  
257 susceptibility in *C. parapsilosis*, but this was not the case when *ERG3* was deleted in *C.*  
258 *albicans*. It is possible that a mutation in *ERG3* that impairs sterol desaturase activity,

259 combined with the naturally-occurring polymorphism in *FKS1* in *C. parapsilosis*,  
260 uniquely impacts echinocandin susceptibility in this *Candida* species.

261 Our finding of an *ERG3* mutation as a cause of azole resistance in this clinical  
262 isolate is supported by the recent work of Branco et al (30). In this study, a *C.*  
263 *parapsilosis* strain that was evolved to become resistant to posaconazole in the  
264 laboratory was found to have a similar (R135I) mutation in *ERG3* resulting in resistance  
265 to fluconazole, voriconazole, and posaconazole. Echinocandin susceptibilities however  
266 were not reported.

267 This matched isolate pair provided an opportunity to evaluate factors contributing  
268 to a unique case of antifungal resistance. We reasoned that the mechanism(s)  
269 underlying this resistance would be detectable among differences in gene expression  
270 and/or whole genome sequence analysis. Here we have demonstrated that a mutation  
271 in *ERG3*, which encodes a key enzyme for the production of the membrane sterol  
272 ergosterol, increases resistance to not only the azoles but also to the echinocandins in a  
273 clinical isolate. This is the first report of a mutation in *ERG3* influencing the  
274 susceptibility to the echinocandins and of a single mechanism that affects susceptibility  
275 to these two important classes of antifungals.

276

## 277 **Materials and Methods**

278 *Strains and media.* All *C. parapsilosis* isolates used in this study are listed in  
279 Table 1. Isolates were kept as frozen stock in 40% glycerol at  $-80^{\circ}\text{C}$  and subcultured  
280 on YPD (1% yeast extract, 2% peptone, and 1% dextrose) agar plates at  $30^{\circ}\text{C}$ . YPD

281 liquid medium was used for routine growth of strains, while YPM (1% yeast extract, 2%  
282 peptone, 1% maltose) liquid medium was used for induction of the *MAL2* promoter in  
283 constructed strains. Nourseothricin (200 µg/ml) was added to YPD agar plates for  
284 selection of isolates containing the *SAT1*-flipper cassette (31). One Shot *Escherichia*  
285 *coli* TOP10 chemically competent cells (Invitrogen) were used for plasmid construction.  
286 These strains were grown in Luria-Bertani (LB) broth or on LB agar plates  
287 supplemented with 100 µg/ml ampicillin (Sigma) or 50 µg/ml kanamycin (Fisher  
288 Bioreagents), when needed.

289 *Drug Susceptibility Testing.* Susceptibility testing was performed by broth  
290 microdilution according to the methods in the CLSI M27-A3 reference standard (32).  
291 Testing was performed in at least duplicate for each isolate and each antifungal agent.  
292 The starting inoculum was between  $0.5 - 2.5 \times 10^3$  cells /ml, and all testing was  
293 performed in RPMI 1640 with 0.2% glucose, buffered with 0.165M MOPS and adjusted  
294 to pH 7.0. Plates were incubated at 35°C, and MICs were read visually for the  
295 echinocandins and azoles at the lowest concentration that resulted in 50% growth  
296 inhibition compared to the drug free growth control at both 24 and 48 hours. For  
297 amphotericin B, the MIC was read as complete inhibition of growth at each of these time  
298 points.

299 The susceptibility results for fluconazole were also repeated and verified by broth  
300 microdilution using a microplate spectrophotometer with optical density readings as the  
301 endpoint. Each strain was tested at least in triplicate. Cultures were diluted to  $2.5 \times 10^3$   
302 cells/ml in sterile RPMI 1640 (Sigma, St. Louis, MO) with 2% glucose, buffered with  
303 0.165 M MOPS and adjusted to pH 7.0. Plates were incubated at 35°C for 24 and 48 h

304 with shaking. Optical density at 600 nm was read with a Biotek Synergy 2 microplate  
305 reader (Fisher Scientific, Waltham, MA); background due to medium was subtracted  
306 from all readings. The relative growth was calculated as the percentage of cell growth in  
307 drug containing medium relative to the cell growth in the absence of drug; the results  
308 were plotted as percent inhibition versus fluconazole concentration.

309 *Construction of Plasmids.* All primers used are listed in Table 2. An *ERG3*  
310 deletion construct for *C. parapsilosis* was generated by amplifying an *Apal-XhoI*-  
311 containing fragment consisting of flanking regions upstream -280 to +51 relative to the  
312 start codon of *C. parapsilosis ERG3* using primers ERG3-A and ERG3-B, as well as a  
313 *NotI-SacII*-containing fragment of downstream flanking regions +1047 to +1868 using  
314 primers ERG3-C and ERG3-D. These upstream and downstream fragments of *ERG3*  
315 were cloned upstream and downstream, respectively, of the *SAT1*-flipper cassette in  
316 plasmid pSFS2 to result in plasmid p77ERG3. Additionally, the coding region of *ERG3*  
317 was amplified from either the Isolate 1 or Isolate 2 with primers ERG3-A and ERG3-E.  
318 Each of these *Apal-XhoI*-containing fragments replaced the upstream sequence in  
319 cassette p77ERG3 to introduce the entire gene, creating plasmids p77ERG3comp and  
320 p76ERG3.

321 Similarly, an *ERG3* deletion construct for *C. albicans* was generated by  
322 amplifying an *Apal-XhoI*-containing fragment consisting of flanking regions upstream -  
323 350 to +39 relative to the start codon of *C. albicans ERG3* using primers CaERG3A-F  
324 and CaERG3B-R, as well as a *NotI-SacII*-containing fragment consisting of flanking  
325 regions +997 to +1677 downstream of the start codon using primers CaERG3C-F and  
326 CaERG3D-R. These upstream and downstream fragments were cloned upstream and



327 downstream, respectively, of the SAT1-flipper cassette in plasmid pSFS2, resulting in  
328 pCaERG3M1.

329 *Candida parapsilosis Transformation.* *C. parapsilosis* strains were transformed  
330 by electroporation as described previously but with some modifications (31). Cells were  
331 grown for 6 hours in 2 mL YPD liquid medium and then 4  $\mu$ L of this cell suspension was  
332 passed to 50 mL of fresh YPD liquid medium and grown overnight at 30°C in a shaking  
333 incubator. When the culture's optical density at 600 nm reached 2.0, cells were  
334 collected by centrifugation, resuspended in 1 mL 10x TE buffer, 1 mL lithium acetate,  
335 and 8 mL of deionized water, and then reincubated at 30°C for 1 hour. Freshly  
336 prepared 1M dithiothreitol was added to the cell suspension, and cells were incubated  
337 for an additional 30 minutes. Cells were then washed twice with ice-cold water and then  
338 once with ice-cold 1 mM sorbitol. Finally, the cells were resuspended in 100  $\mu$ L of fresh  
339 ice-cold 1 mM sorbitol. The gel-purified *Apal-Sacl* fragment from the appropriate  
340 plasmid was mixed with 40  $\mu$ L of competent cells and transferred into a chilled 2-mm  
341 electroporation cuvette. The reaction was carried out at 1.5 kV, using a Cellject Pro  
342 Electroporator (Thermo). Immediately following, 1 mL of YPD containing 1 M sorbitol  
343 was added and the mixture was transferred to a 1.5 mL centrifuge tube. Cells were  
344 allowed to recover at 30°C for 6 hours. Finally, 100  $\mu$ L was removed and plated to YPD  
345 agar plates containing 200  $\mu$ g/mL nourseothricin and 1 M sorbitol. Transformants were  
346 selected after at least 48-hours growth at 30°C.

347 *RNA Isolation and Sequencing.* RNA was isolated using the hot phenol method  
348 of RNA isolation described previously (33). RNA concentrations were determined using  
349 a Nanodrop spectrophotometer (Nanodrop Products), and RNA integrity was verified

350 using a Bioanalyzer 2100 (Agilent Technologies). Barcoded libraries were prepared  
351 using the Lexogen mRNA Sense kit for Ion Torrent according to manufacturer's  
352 standard protocol. Libraries were sequenced on the Ion Torrent Proton sequencer.  
353 Individual sample fragments were concatenated to form the whole sample fastq file.  
354 Files were then run through FASTQC to check data quality. Any reads with a phread  
355 score <20 were trimmed. Reads were then aligned to the *C. parapsilosis* CDC317  
356 reference transcriptome using RNA-Star long method. After alignment, transcriptome  
357 alignment counts were gathered. The read counts for each sample were normalized  
358 using transcripts per kilobase million (TPM) method. These data have been deposited  
359 in the Gene Expression Omnibus repository under the accession number GSE98986.

360 *Southern Hybridization.* Genomic DNA (gDNA) for use with Southern blotting was  
361 prepared as described previously (34), digested with appropriate restriction  
362 endonucleases, separated on a 1 % agarose gel, stained with ethidium bromide, and  
363 transferred by vacuum blotting to a nylon membrane. After UV crosslinking,  
364 membranes were probed and detected using the Amersham ECL Direct nucleic acid  
365 labeling and detection system according to the manufacturer's instructions.

366 *Whole Genome Sequencing.* Genomic DNA for library preparation was isolated  
367 using Qiagen Genomic-tip 100/G kit (Qiagen) following the manufacturer's Genomic  
368 DNA Preparation instructions. Paired-end DNA sequence libraries for Isolate 1 and for  
369 Isolate 2 were prepared and sequenced using the Ion Proton Torrent sequencer and  
370 aligned to the *C. parapsilosis* CDC317 reference genome sequence. The GATK Best  
371 Practices work flow was used, incorporating the following tools: bwa (193) v0.5.9 (aln  
372 and sample), Picard tools (<http://picard.sourceforge.net>) v1.107 (SortSam and

373 MarkDuplicates), samtools (194) v0.1.19 and GATK (195) v2.8.  
374 (RealignerTargetCreator, IndelRealigner, BaseRecalibrator, PrintReads,  
375 ReduceReads, HaplotypeCaller). A population variants file for *C. parapsilosis* was  
376 downloaded from the Broad Institute website ([https://www.broadinstitute.org/fungal-](https://www.broadinstitute.org/fungal-genome-initiative)  
377 [genome-initiative](https://www.broadinstitute.org/fungal-genome-initiative)) and used as the –knownSites file in the GATK BaseRecalibrator step.  
378 The snpEff (196) v3.5 genetic variant annotation tool was used to annotate SNP and  
379 indel variants using the c\_parapsilosis\_CDC317 snpEff database. The GATK  
380 CombineVariants and SelectVariants tools were used to select SNPs found in Isolate 2  
381 but not in Isolate 1 and SNPs that are heterozygous in Isolate 1 and homozygous in  
382 Isolate 2. The JBrowse Genome Browser (197) v1.11.6 was used to visualize  
383 sequence alignments at genomic positions in order to validate variant calls (35).  
384 Following installation of the *C. parapsilosis* CDC317 reference genome using the  
385 current chromosomal features file available at the Candida Genome Database  
386 ([www.candidagenome.org](http://www.candidagenome.org)), the whole genome sequencing (WGS) data for the parental  
387 Isolate 1 was uploaded and aligned to the reference chromosome map using Ymap  
388 ([lovelace.cs.umn.edu/Ymap](http://lovelace.cs.umn.edu/Ymap)). WGS data for Isolate 2 was then installed utilizing Isolate  
389 1 as the parental strain to allow for loss of heterozygosity (LOH) analysis (15). The  
390 NCBI accession number for these data is PRJNA361149.

391 *Sequence Analysis of ERG3.* The coding sequence of *ERG3* in *C. parapsilosis*  
392 was amplified by PCR from *C. parapsilosis* genomic DNA using the primers listed in  
393 Table 2, cloned into pCR-BLUNTII-TOPO using a Zero Blunt TOPO PCR cloning kit,  
394 and transferred into *Escherichia coli* TOP10 cells with selection on LB agar plates  
395 containing 50 µg/ml kanamycin. Plasmid DNA was purified (QIAquick PCR Purification

396 Kit, Qiagen) and sequenced on an ABI model 3130XL genetic analyzer using  
397 sequencing primers, resulting in a full-length sequence from both strands of *ERG3*. The  
398 sequencing was performed using a total of six sets of clones derived from three  
399 independent PCR reactions. The coding sequence of *ERG3* in Isolate 2 described in  
400 this study has been deposited in GenBank under accession number KT277771.

401 *Sterol Analysis.* Non-saponifiable lipids were extracted using alcoholic KOH.  
402 Samples were dried in a vacuum centrifuge (Heto) and were derivatized by the addition  
403 of 100  $\mu$ l 90% BSTFA/ 10% TMS (Sigma), 200  $\mu$ l anhydrous pyridine (Sigma) and  
404 heating for 2 hours at 80°C. TMS-derivitized sterols were analyzed and identified using  
405 GC/MS (Thermo 1300 GC coupled to a Thermo ISQ mass spectrometer, Thermo  
406 Scientific) with reference to retention times and fragmentation spectra for known  
407 standards. GC/MS data files were analyzed using Xcalibur software (Thermo Scientific)  
408 to determine sterol profiles for all isolates and for integrated peak areas (20).

409

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414

415

416

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423

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- 529

530 **Table 1.** *C. parapsilosis* strains and isolates used in this study

Strain/Isolate (Name)	Strain Background	Relevant Genotype/Characteristics	Reference
<i>C. parapsilosis</i>			
35177 (Isolate 1)	N/A	Antifungal drug susceptible	13
35176 (Isolate 2)	N/A	Antifungal drug resistant	13
E77U7G3 (Isolate 1- <i>erg3Δ/Δ</i> )	Isolate 1	<i>erg3Δ::FRT/erg3Δ::FRT</i>	This study
76B2E19 (Isolate 2- <i>erg3Δ/Δ</i> )	Isolate 2	<i>erg3Δ::FRT/erg3Δ::FRT</i>	This study
76C1B4 (Isolate 2- <i>ERG3<sup>WT/WT</sup></i> )	76B2E19	<i>erg3Δ::ERG3<sup>WT</sup>-FRT/erg3Δ::ERG3<sup>WT</sup>-FRT</i>	This study
<i>C. albicans</i>			
SC5314	N/A	wild-type	-
ScERG3M4C (SC5314- <i>erg3Δ/Δ</i> )	SC5314	<i>erg3Δ::FRT/erg3Δ::FRT</i>	This study
GP1 (BR1- <i>ERG3<sup>WT/WT</sup></i> )	BWP17	<i>his1Δ/Δ::HIS1 arg4Δ/Δ::ARG4 ura3Δ/Δ::IRO1-URA3</i>	This study
E3AL1 (BR2- <i>erg3Δ/Δ</i> )	BWP17	<i>erg3Δ::ARG4/erg3Δ::HIS1 ura3Δ/Δ::IRO1-URA3</i>	This study



531 **Table 2.** Primers used in this study

Primer	Sequence <sup>a</sup>
<i>C. parapsilosis</i> Gene Disruption/Complementation	
ERG3-A	5'-GACAAACAAAATAAGGGCCCAAATTAAGG-3'
ERG3-B	5'- AGCATATAGTCTCTCGAGTAGGTAATAAT-3'
ERG3-C	5'-GATAGCCGCGGAAGATCATACAGAAGAC-3'
ERG3-D	5'-AAAATAGAGCTCCTGGGTGGGAATTAT -3'
ERG3-E	5'-AAAATACAGTTACTCGAGGGGAATTAT-3'
<i>C. parapsilosis</i> ERG3 Sequencing	
ERG3-A	5'-CCCACGTTTATTTCACTAGATCC-3'
ERG3-B	5'-GGTTGCCTTGACCAACCC-3'
ERG3-C	5'-GGGAATGGGCAATAGGGACAC-3'
ERG3-D	5'-GGTTGCCTTGACCAACCC-3'
<i>C. albicans</i> Gene Disruption	
CaERG3A-F	5'- ATCTGATTTATATATGGGCCCAAGTGTTTG-3'
CaERG3B-R	5'-AAAAGATAATAGTCTCGAGTTTCTAGTACG-3'
CaERG3C-F	5'-ATAGCCGCGGTA ACTCTTACAGAAGACC-3'
CaERG3D-R	5'-TG TGATGTGAGCTCGTTAGTATTATTTTCA-3'

532 a- Underlined sequences indicate introduced restriction sites.

533

534 **Table 3A.** Azole antifungal susceptibilities for *C. albicans* and *C. parapsilosis* isolates <sup>a</sup>

Isolate	Fluconazole				Voriconazole				Itraconazole				Posaconazole			
	24h		48h		24h		48h		24h		48h		24h		48h	
<i>C. parapsilosis</i>																
Isolate 1 <sup>b</sup>			1				0.03									
Isolate 2 <sup>b</sup>			<b>&gt;64</b>				<b>&gt;16</b>									
Isolate 1	0.25	0.25	0.25	0.5	≤0.03	≤0.03	0.06	≤0.03	≤0.03	≤0.03	≤0.03	0.06	≤0.03	≤0.03	≤0.03	≤0.03
Isolate 2	≤0.125	≤0.125	<b>&gt;64</b>	<b>&gt;64</b>	≤0.03	≤0.03	<b>&gt;16</b>	<b>&gt;16</b>	≤0.03	≤0.03	<b>&gt;16</b>	<b>&gt;16</b>	≤0.03	≤0.03	<b>&gt;16</b>	<b>&gt;16</b>
Isolate 1- $\Delta erg3$	≤0.125	≤0.125	<b>&gt;64</b>	<b>&gt;64</b>	≤0.03	≤0.03	<b>&gt;16</b>	<b>&gt;16</b>	≤0.03	≤0.03	<b>&gt;16</b>	<b>&gt;16</b>	≤0.03	≤0.03	<b>&gt;16</b>	<b>&gt;16</b>
Isolate 2- $\Delta erg3$	≤0.125	≤0.125	<b>&gt;64</b>	<b>&gt;64</b>	≤0.03	≤0.03	<b>&gt;16</b>	<b>&gt;16</b>	≤0.03	≤0.03	<b>&gt;16</b>	<b>&gt;16</b>	≤0.03	≤0.03	<b>&gt;16</b>	<b>&gt;16</b>
Isolate 2- <i>ERG3</i> <sup>WT/WT</sup>	0.25	0.25	≤0.125	0.5	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	0.06	0.125	≤0.03	≤0.03	0.06	≤0.03
<i>C. albicans</i>																
SC5314	≤0.125	≤0.125	≤0.125	≤0.125	≤0.03	≤0.03	≤0.03	≤0.03	0.06	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03
SC5314- $\Delta erg3$	<b>&gt;64</b>	<b>&gt;64</b>	<b>&gt;64</b>	<b>&gt;64</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>
BWP17	≤0.125	≤0.125	≤0.125	≤0.125	≤0.03	≤0.03	0.06	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	0.125	≤0.03	0.06	≤0.03
BWP17- $\Delta erg3$	<b>&gt;64</b>	<b>&gt;64</b>	<b>&gt;64</b>	<b>&gt;64</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>	<b>&gt;16</b>

535 a- All values are in µg/ml; values in bold are resistant.

536 b- MIC values as reported previously determined using the previous CLSI M27-A2 recommended methodologies (13).

537

538 **Table 3B.** Echinocandin and amphotericin B susceptibilities for *C. albicans* and *C.*  
539 *parapsilosis* isolates <sup>a</sup>

Isolate	Anidulafungin		Caspofungin			Micafungin		Amphotericin B		
	24h	48h	24h	48h	24h	48h	24h	48h		
<i>C. parapsilosis</i>										
Isolate 1 <sup>b</sup>	-	-	1	-	-	2	-	-	<b>8</b>	0.25
Isolate 2 <sup>b</sup>	-	-	2	-	-	<b>&gt;16</b>	-	-	<b>&gt;16</b>	0.5
Isolate 1	2	2	-	1	1	-	2	2	-	0.5 0.5
Isolate 2	<b>4</b>	<b>4</b>	-	2	1	-	<b>4</b>	<b>4</b>	-	0.5 0.5
Isolate 1- $\Delta erg3$	<b>4</b>	<b>4</b>	-	2	2	-	<b>8</b>	<b>8</b>	-	0.5 1
Isolate 2- $\Delta erg3$	<b>4</b>	<b>4</b>	-	2	2	-	<b>4</b>	<b>4</b>	-	0.5 0.5
Isolate 2- <i>ERG3</i> <sup>WT/WT</sup>	2	2	-	1	0.5	-	2	2	-	0.25 0.5
<i>C. albicans</i>										
SC5314	≤0.015	≤0.015	-	0.25	0.25	-	0.03	0.03	-	0.25 0.5
SC5314- $\Delta erg3$	0.06	0.06	-	0.25	<b>0.5</b>	-	0.06	0.06	-	0.5 0.5
BWP17	≤0.015	≤0.015	-	0.25	0.25	-	0.03	0.03	-	0.25 0.5
BWP17- $\Delta erg3$	0.06	0.06	-	0.25	0.25	-	0.06	0.06	-	0.5 0.5

540 a- All values are in µg/ml; values in bold are non-susceptible according to CLSI clinical  
541 breakpoints.

542 b- MIC values as reported previously determined using the previous CLSI M27-A2  
543 recommended methodologies (13).

544

545 **Table 4.** Genes involved in sterol metabolic processes overrepresented among the  
546 upregulated genes in Isolate 2 relative to Isolate 1.

Gene ID	<i>C. parapsilosis</i> Gene Name	<i>S. cerevisiae</i> Gene Name	<i>C. albicans</i> Gene Name	Fold	Fold
				Change	Change
				Sample A	Sample B
CPAR2_601530	-	<i>GRE2</i>	-	4.8	5.0
CPAR2_201490	-	-	-	7.2	1.7
CPAR2_405010	-	<b><i>ERG6</i></b>	<b><i>ERG6</i></b>	2.3	3.8
CPAR2_202280	-	<i>DAP1</i>	<i>DAP1</i>	3.3	1.6
CPAR2_405900	-	<b><i>ERG24</i></b>	<b><i>ERG24</i></b>	2.0	2.7
CPAR2_103490	-	<i>ATF2</i>	-	2.3	2.2
CPAR2_801560	-	<i>ERG27</i>	<i>ERG27</i>	2.2	2.2
CPAR2_303740	<b><i>ERG11</i></b>	<b><i>ERG11</i></b>	<b><i>ERG11</i></b>	1.6	2.6
CPAR2_801410	-	<b><i>ERG25</i></b>	<b><i>ERG25</i></b>	1.8	2.2
CPAR2_105000	-	<i>YEH1</i>	-	2.0	2.0
CPAR2_210480	-	<b><i>ERG1</i></b>	<b><i>ERG1</i></b>	1.7	2.2
CPAR2_207280	<b><i>UPC2</i></b>	<b><i>UPC2</i></b>	<b><i>UPC2</i></b>	1.8	1.8
CPAR2_703970	-	<b><i>ERG5</i></b>	<b><i>ERG5</i></b>	1.6	1.6
CPAR2_109890	-	<b><i>ERG2</i></b>	<b><i>ERG2</i></b>	1.6	1.5

547

548 Fold change values represents genes upregulated in Isolate 2 as compared to Isolate 1  
549 a minimum of 1.5- fold in samples from independent experiments A and B. Genes  
550 shown in bold have orthologs regulated by *CaUPC2*

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553 **Table 5.** Sterol composition for each isolate and each *ERG3* mutant (shown as percent  
554 of total sterols)

Sterol			Isolate 1	Isolate 2
	Isolate 1	Isolate 2	<i>erg3Δ/ erg3Δ</i>	<i>ERG3<sup>WT</sup>/ERG3<sup>WT</sup></i>
Ergosta-5,7,24(28)- tetranol	4.0	0.5	0.4	0.5
Unknown m/z 470	--	--	7.0	--
Zymosterol	4.2	7.5	--	1.9
Ergosterol	65.4	9.1	--	51.8
Ergosta-7,22-dienol	9.3	71.7	76.8	22.3
Fecosterol (E8, 24(28))	1.4	0.6	0.9	0.7
Ergosta-8-enol	--	0.8	1.2	0.6
Ergosta 5,7 dienol	6.3	0.4	0.3	8.6
Episterol (E7, 24(28))	3.2	3.3	4.2	3.2
Ergosta-7-enol	1.9	4.2	8.4	6.6
Lanosterol/obtusifoliol	2.8	0.5	0.3	2.7
4,4,-diemthyl cholesta- 8,24-dienol	1.7	1.4	0.6	1.1

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558 **Figure 1.** Fluconazole MIC growth curves. Isolates 1 and 2 and their related strains  
559 were grown for (A) 24 hours and (B) 48 hours in the presence of the indicated  
560 concentrations of fluconazole.

561 **Figure 2.** Schematic representation of RNA and whole genome sequencing data for  
562 Isolate 2 as compared to Isolate 1. Colored circle segments represent contig  
563 boundaries. The outer track represents RNA sequence data, such that the green lines  
564 indicate those genes which are overexpressed in Isolate 2 by at least 1.5-fold and red  
565 lines indicate those genes which are underexpressed at 0.5-fold or less, relative to  
566 Isolate 1. The inner track represents sequence variants which are found in Isolate 2 but  
567 not in Isolate 1 for the whole genome sequence data. Overexpressed genes of the  
568 ergosterol biosynthetic process are labeled with *S. cerevisiae* ortholog gene names.

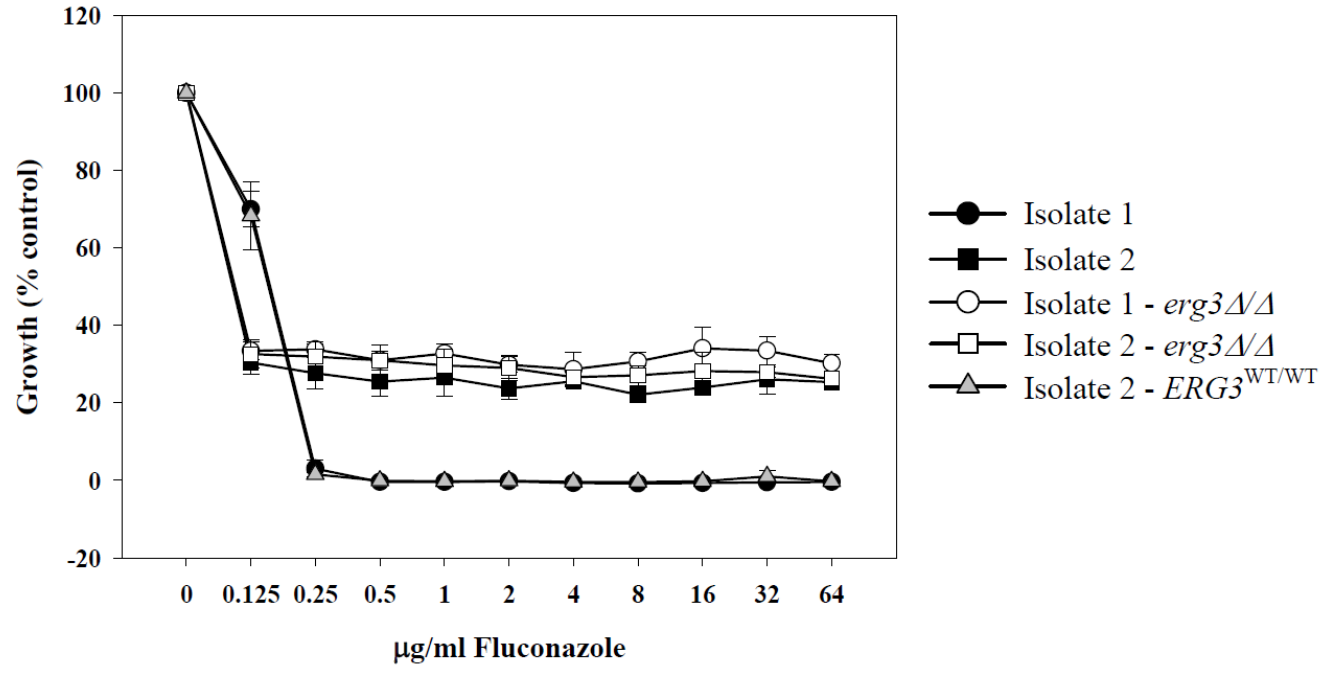
569 **Figure 3.** Horizontal tracks represents the *C. parapsilosis* contig as labeled. The black  
570 line running horizontally through each contig represents the the predicted local copy  
571 number, with the center set equal to two, and divergences up or down indicating  
572 increased or decreased copy number at that loci, respectively. A region of segmental  
573 tetraploidy is denoted with an asterisk. Gray vertical lines (A) represent local allelic  
574 variation with the darker gray indicating higher concentrations of heterozygosity. Red  
575 vertical lines (B) represent loss of heterozygosity relative to the parent isolate (Isolate  
576 1), with darker red indicating a greater degree of change in heterozygosity.

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A.



B.

