Selection of Ethanol Tolerant Strains of *Candida albicans* by Repeated Ethanol Exposure Results in Strains with Reduced Susceptibility to Fluconazole

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Abstract

Candida albicans is a commensal yeast that has important impacts on host metabolism and immune function, and can establish life-threatening infections in immunocompromised individuals. Previously, *C. albicans* colonization has been shown to contribute to the progression and severity of alcoholic liver disease. However, relatively little is known about how *C. albicans* responds to changing environmental conditions in the GI tract of individuals with alcohol use disorder, namely repeated exposure to ethanol. In this study, we repeatedly exposed *C. albicans* to high concentrations (10% vol/vol) of ethanol—a concentration that can be observed in the upper GI tract of humans following consumption of alcohol. Following this repeated exposure protocol, ethanol small colony (Esc) variants of *C. albicans* isolated from these populations exhibited increased ethanol tolerance, altered transcriptional responses to ethanol, and cross-resistance/tolerance to the frontline antifungal fluconazole. These Esc strains exhibited chromosomal copy number variations and carried polymorphisms in genes previously associated with the acquisition of fluconazole resistance during human infection. This study identifies a selective pressure that can result in evolution of fluconazole tolerance and resistance without previous exposure to the drug.

Author Summary: Previous work has shown that *Candida albicans*, a common fungal component of the gastrointestinal (GI) microbiome, is found in high abundance in the alcoholic GI tract. However, it was unknown how repeated ethanol exposure, similar to what *C. albicans* would be exposed to in the upper GI tract of individuals with alcohol use disorder, would affect the yeast. In this work, we show that repeated ethanol exposure selects for *C. albicans* strains with altered transcriptional responses to ethanol, increased ethanol tolerance, and cross-resistance/tolerance to the common frontline antifungal fluconazole. This work shows that ethanol exposure can promote evolution of *C. albicans* to antifungal resistance without previous

52 exposure to the antifungal drug. These findings could be relevant to *C. albicans* in the upper GI
53 tract in individuals with alcohol use disorder and further studies could reveal optimized
54 antifungal regimens for *C. albicans* infections in these patients.
55

56 **Introduction**

57 *Candida albicans* is an opportunistic pathogen causing severe morbidity and mortality in
58 immunocompromised patients. *Candida spp* are the second most common cause of nosocomial
59 bloodstream infection in the US, and cause about 70% of all fungal infections [1,2]. The most
60 common species causing these fungal infections is *C. albicans*, the causative agent of oral
61 thrush, vulvovaginitis, and bloodstream infections which result in mortality rates in
62 immunocompromised patients ranging from roughly 30-56% [3–5]. Before *C. albicans* causes
63 deadly bloodstream infections, it establishes commensalism within the gastrointestinal tract of
64 humans [6,7]. Importantly, rates of antifungal resistance among *C. albicans* isolates have been
65 rising throughout recent years [8], contributing to difficulty in treating bloodstream infections.
66 Therefore, it is important to investigate factors that promote antifungal resistance whether
67 acquired during infection or the preceding commensal state.

68 *C. albicans* commensalism has been studied particularly in disease states which
69 predispose individuals to bloodstream infections. However, the environmental conditions in the
70 GI tract and how they contribute to fungal physiology have been less studied. One condition of
71 interest is the GI tract of individuals with alcohol use disorder (AUD). Recently, changes in the
72 gut microbiome of individuals with alcohol use disorder, including a loss of fungal diversity, and
73 blooms of *C. albicans* have been noted [9–11]. *C. albicans* blooms in the alcoholic GI tract
74 promote the progression and increase the severity of alcoholic liver disease. Anti-
75 *Saccharomyces cerevisiae* IgG antibodies (ASCA), which are thought to be broadly induced by
76 fungi, show an inverse correlation with 6-month and 5-year survival rates in patients with
77 alcoholic cirrhosis [9,10]. These findings suggest that fungal colonization can exacerbate

diseases associated with alcohol use and abuse. However, there are still many questions surrounding why *C. albicans* abundance increases in the alcoholic GI tract and how *C. albicans* responds to environmental changes in the alcoholic GI tract.

One of the most intuitive features of the environment in the alcoholic GI tract is the increased presence of ethanol that members of the microbiota from the mouth through the proximal small intestine would be exposed to during bouts of consumption. Following consumption of one to two standard drinks of ethanol, the percentage of alcohol in the stomach and the small intestine can reach concentrations of roughly 10% ethanol [12,13]. To test how *C. albicans* responds to conditions that mimic this environment, we repeatedly cultured C. albicans in media with increasing amounts of ethanol up to 10%. Model organisms such as *Escherichia coli* and *Saccharomyces cerevisiae* have been shown to evolve increased ethanol tolerance as a result of repeated exposure to ethanol [14,15]. We therefore hypothesized that *C. albicans* would evolve increased ethanol tolerance following repeated exposure to ethanol.

In this study, we find that populations of *C. albicans* cells repeatedly exposed to ethanol evolve increased ethanol tolerance. One ethanol tolerant strain showed increased ergosterol production during exposure to ethanol. We show that increased ergosterol content, whether induced by chemical stimulation or gain-of-function mutation, resulted in increased ethanol survival. Whole genome sequencing of strains with increased ethanol tolerance showed that these strains carried various chromosomal aneuploidies, some of which have been observed in fluconazole resistant and tolerant strains of *C. albicans*, and single nucleotide polymorphisms (SNPs). We also showed that the ethanol tolerant strains exhibited increased fluconazole resistance and tolerance. These results suggest that *C. albicans* exposed to ethanol in the alcoholic GI tract can exhibit antifungal resistance without previously being exposed to the antifungal drug.

Results

Selection of ethanol tolerant *C. albicans* isolates

The ability of *C. albicans* to evolve an altered response to ethanol exposure was investigated. To this end, *C. albicans* strain SC5314 was cultured eight times for 4 hrs each in tissue culture medium with 10% fetal bovine serum and 1X non-essential amino acids (referred to as medium A) with or without increasing concentrations of ethanol, as described in Materials and Methods (**figure 1A).** This process was repeated with five independent populations of cells.

Throughout the repeated-ethanol exposure protocol, populations from each passage were plated onto YPD agar to monitor population dynamics. An increased number of small-sized colonies was observed with greater numbers of passages and higher concentrations of ethanol (**Supplemental figure S1**). Small-sized colonies that were 50% of the size of normal sized colonies or less were rare in populations not exposed to ethanol. From these populations, small and normal sized colonies were isolated to determine their properties.

Normal-sized colonies were isolated from four different populations exposed repeatedly to ethanol, as well as from populations that were repeatedly exposed to water. Strains from normal-sized colonies were grown to post-exponential phase and added to medium A containing 10% ethanol or 10% water. Following the four-hour ethanol exposure, cells were serially diluted, and plated to determine CFU and percentage survival. The final CFU of the 10% water and 10% ethanol cultures were counted and used to determine the final percentage survival of the ethanol cultures. Values were standardized within each experiment to the survival of SC5314 in 10% ethanol (**supplemental figure S2**). Strains from normal-sized colonies from ethanol cultures showed no statistically significant increase in ethanol survival compared to SC5314 survival and were not studied further.

Ethanol small colony (Esc) variants were purified from different populations and tested as above to measure their ability to survive in ethanol. Relative survival values of SC5314, Esc6,

128 Esc7, and Esc8 are shown in **figure 1B**. Esc6, Esc7, and Esc8 were isolated from different,
129 independent populations and all showed significantly higher survival following the 4-hour ethanol
130 exposure, compared to the parent strain.

131 The next experiment asked whether Esc strains had an increased ability to survive longer
132 exposures to ethanol. SC5314, Esc6, Esc7, and Esc8 were grown overnight and then subcultured
133 in medium A with 10% ethanol. CFU were determined for cultures at 0, 2, 4, 6, 8, 10, 12, and 24
134 hours of incubation. Fractions relative to the starting CFU of the cultures are shown in **figure 1C**.
135 The results showed that Esc6, Esc7, and Esc8 outperformed WT *C. albicans* in ethanol survival
136 from 0-6 hours and Esc7 and 8 were able to maintain higher ethanol survival relative to SC5314
137 through 24 hours. Esc6 had an early advantage in the first 6 hours compared to SC5314 but
138 began to die rapidly after 12 hours. A common way to measure tolerance to antibiotics in bacterial
139 systems is to determine the minimum duration of killing x fraction of the population (MDKx) [16].
140 We estimated the MDK90 for the strains by extrapolation and found that WT *C. albicans* had an
141 MDK90 of 10 hrs, Esc6 had an MDK90 of 12 hrs, Esc7 had an MDK90 of 16 hrs, and Esc8 had
142 an MDK90 of 19 hrs. These results showed that Esc7 and Esc8 had greater ethanol tolerance
143 than SC5314 and that Esc6 had slightly higher tolerance over the first 12 hours of exposure.
144

145 **Altered gene expression in Esc strains**

146 To detect changes in gene expression that could contribute to ethanol tolerance, RNA-
147 sequencing was performed on SC5314 and Esc6, 7 and 8 during exposure to 10% ethanol
148 medium or 10% water medium. Three biological replicates of each strain were cultured in medium
149 A with 10% ethanol or 10% water and harvested. RNA-seq was performed on RNA extracted from
150 these samples as described in Materials and Methods. Initial principal coordinate analysis showed
151 that all Esc strains exhibited a distinct transcriptional profile in ethanol compared to SC5314 in
152 ethanol (**supplemental figure S3**).

153 To determine what cellular pathways were altered in these strains, GO term analyses
154 using the Candida Genome Database (CGD) were performed as previously described [17] to
155 identify enriched categories of genes. Genes that were significantly up-regulated (log2-fold
156 change >0.5 and adjusted p-value < 0.05) in Esc strains in ethanol compared to SC5314 in
157 ethanol were analyzed (1700 genes in Esc6, 1334 genes in Esc7, and 1494 genes in Esc8). The
158 top 20 processes based on corrected p-value identified by GO term process analysis are shown
159 in **supplemental figure S4**. Organonitrogen compound catabolic process was the only process
160 that was shared in the top 20 for all strains.

161 For Esc7, many processes that were involved with ergosterol biosynthesis (red arrows in
162 **figure 2A**) were enriched among the top 20 GO term processes. Analysis of individual genes
163 showed that 12 ergosterol metabolism genes were significantly up-regulated in Esc7 in ethanol
164 compared to SC5314 in ethanol (**figure 2B)**. Importantly, one of these 12 genes was *ERG11*
165 which is one of the rate-limiting steps of ergosterol biosynthesis and the target of azole antifungals
166 [18,19]. Previous work with *Saccharomyces cerevisiae* showed that ethanol impacts ergosterol
167 production and that increased ergosterol production was correlated with increased ethanol
168 tolerance [20–23], suggesting that the observed up-regulation of *ERG* genes contributes to
169 ethanol tolerance of Esc7.

170 Esc6 and Esc8 showed other enriched pathways that could be involved in ethanol
171 tolerance or response to ethanol exposure. Both Esc6 and Esc8 showed enrichment of genes
172 involved with general catabolic processes. Both strains showed enrichment of processes
173 pertaining to proteolysis and protein catabolism which could be involved with the denatured
174 protein response caused by ethanol exposure. Esc6 also showed an enrichment of genes
175 involved with autophagy, which has previously been implicated in the ethanol response of *S.*
176 cerevisiae [24]. These findings suggest that changes to proteasomal degradation/proteolysis and
177 autophagy could contribute to ethanol tolerance of *C. albicans*.

178 Further analysis of gene expression differences focused on SC5314 and Esc7. To confirm
179 the results from RNA-seq, SC5314 and Esc7 were cultured in 10% EtOH medium or 10% water
180 medium for 4 hours and RT-qPCR was used to measure gene expression. *ERG2, ERG4, ERG5,*
181 *ERG6, ERG25*, and *ERG11* were shown to have higher transcript abundance in Esc7 in
182 comparison to SC5314 in 10% ethanol **(figure 3A)**, confirming the results from RNA-Seq. Many
183 of these genes were repressed in ethanol in SC5314 and less repressed in Esc7 in ethanol. These
184 results suggested that Esc7 cells would contain increased concentrations of ergosterol compared
185 to SC5314 since increased expression of *ERG11* was shown to increase ergosterol content in *C.*
186 *albicans* [25].

187 Ethanol exposure has multiple effects that promote cell death including targeting the
188 membrane, and producing protein misfolding, mitochondrial stress, and DNA damage [26–28]. In
189 *S. cerevisiae,* increased expression of drug transporters and efflux pumps confers increased
190 ethanol tolerance [29,30] and we confirmed upregulation of *CDR1* in SC5314 and Esc7 during
191 exposure to ethanol as well as a trend towards greater expression in Esc7 compared to SC5314
192 (SC5314 vs Esc7 EtOH p-value: 0.2070, Fisher's LSD test, mean value 1.51-fold higher) (**figure**
193 **3B)**. Orthologs of genes involved in trehalose synthesis and ethanol tolerance in *S. cerevisiae,*
194 *TPS1* and *TPS2* [23], were less repressed in Esc7 compared to SC5314 in 10% EtOH (**figure**
195 **3B**).

196 Heat shock proteins (HSPs) and molecular chaperones are important for *S. cerevisiae*
197 ethanol tolerance [31–33]. In *C. albicans*, nearly all HSPs were repressed in ethanol in both Esc7
198 and SC5314 (**supplemental tables S1-S10)**. Interestingly, *HSP90* was significantly repressed in
199 Esc7 in ethanol and was more repressed than in SC5314 (**figure 6B**). HSP90 is critical to many
200 different stress responses, regulates Upc2 (a transcriptional regulator of ergosterol metabolism),
201 and regulates antifungal resistance and tolerance [34–40]. Decreased expression of *HSP90* could
202 lead to less programmed cell death during ethanol stress as has been observed with exposure to
203 hydrogen peroxide [41].

204 Another pathway of interest is the ethanol catabolic pathway. This pathway has not been
205 extensively studied in *C. albicans,* however, and some of the main players in this pathway were
206 identified based on homology. Adh1 and Adh2 are thought to convert ethanol into acetaldehyde,
207 Ald4 and Ald5 likely convert acetaldehyde into acetate, and Acs1 and Acs2 likely convert acetate
208 into acetyl-CoA [42]. Several of the genes, e.g. *ADH2*, *ADH3*, *ADH4*, and *ALD5* were
209 transcriptionally induced in ethanol in both SC5314 and Esc7 **(figure 3C**). Significant differences
210 in expression of these genes between Esc7 and SC5314 were not observed by RT-qPCR.
211 These findings highlight that many pathways are likely involved in ethanol tolerance in *C.*
212 *albicans.* Some of the pathways involved in *S. cerevisiae* ethanol tolerance may not play a role in
213 the strains described here (e.g. HSPs, ethanol catabolic pathway, etc.), although genes that do
214 not change transcriptionally may also be regulated post-transcriptionally.
215
216 ***C. albicans* strains with elevated ergosterol levels exhibit increased ethanol tolerance**
217 Twelve genes of the ergosterol pathway showed higher transcript abundance in Esc7 in
218 ethanol compared to SC5314, suggesting that elevated ergosterol content contributes to
219 increased ethanol tolerance in *C. albicans.* We therefore investigated whether the gene
220 expression changes resulted in increased ergosterol content in Esc7. SC5314 cells or Esc6, 7, 8
221 cells were cultured in medium A with 10% water or 10% ethanol for four hours and ergosterol was
222 extracted and quantitated as previously described [43,44]. Ergosterol quantity was calculated
223 relative to SC5314 in 10% H2O and is shown in **figure 4A** (8 biological replicates from four
224 different experiments). From these data, a statistically significant increase in ergosterol content
225 was seen in Esc7 in EtOH (p-value <0.0001, 2way ANOVA with Dunnett's correction, mean =
226 1.77). Esc6 and Esc8 ergosterol content were not significantly different from SC5314. These data
227 support the model that higher levels of *ERG* gene expression in Esc7 during ethanol exposure
228 promoted higher ergosterol production.

229 In *S. cerevisiae,* increased ergosterol content confers greater ethanol tolerance and in
230 synthetic bilayers, increased ergosterol leads to lower membrane disruption [20–23]. We
231 therefore tested whether elevating ergosterol content in *C. albicans* through other means would
232 confer greater ethanol tolerance. One approach utilized an SC5314 strain with a homozygous
233 *UPC2* gain of function mutation (G648D; kind gift of J. Morschhauser). $UPC2_{G648D}$ results in
234 increased ergosterol synthesis, and increased ergosterol content through upregulation of *ERG11*
235 [45]. Four hour ethanol survival of the SC5314 WT strain [45] and homozygous $UPC2_{G648D}$ mutant
236 strain was determined as described previously. Relative survival compared to SC5314 was
237 calculated. The homozygous $UPC2_{G648D}$ mutant strain exhibited a five-fold increase in ethanol
238 survival (**figure 4B**), showing that a mutation that increased ergosterol content [45] also increased
239 ethanol tolerance.
240 For a second test of the hypothesis that increased ergosterol content increases ethanol
241 tolerance, chemical stimulation of ergosterol synthesis with the anti-malarial drug artemisinin was
242 performed. Incubation of *C. albicans* with 100ug/mL of artemisinin has been shown to increase
243 ergosterol gene expression and lead to increased content of ergosterol in *C. albicans* cells [46].
244 Therefore, *C. albicans* cells were pretreated with 100ug/mL of artemisinin in DMSO or DMSO
245 alone for four hours. Artemisinin or vehicle exposure was continued during the four-hour ethanol
246 survival experiment. Survival of cells relative to SC5314 with vehicle was calculated (**figure 4C**).
247 Stimulation of ergosterol synthesis with artemisinin led to a 2-fold increased ethanol survival.
248 Therefore, cells with increased ergosterol content produced in several ways showed increased
249 ethanol tolerance. Increased ethanol tolerance in Esc7 is likely due, at least in part, to increased
250 ergosterol content in Esc7 cells.
251
252 **Esc strains show chromosome copy number variation and genomic polymorphisms**
253 Whole-genome sequencing of parental SC5314, Esc6, Esc7 and Esc8 was conducted to
254 identify possible chromosome copy number variation or sequence polymorphisms in the ethanol

255 tolerant strains. Aneuploidy is a well described mechanism of stress adaptation in *C. albicans*
256 [47–49], as well as *S. cerevisiae* ethanol tolerance [50]. Genomic DNA from the strains was
257 isolated and genome sequencing was conducted by Seq-Center (formerly MiGS) using paired-
258 end Illumina next generation sequencing. Further details on sequencing and analysis workflow
259 are described in Materials and Methods.

260 Changes in chromosome copy number were detected by uploading fastq files to the Yeast
261 Mapping Analysis Pipeline (YMAP) [51]. YMAP analyzed average read depth across all
262 chromosomes to give a read-depth based chromosome copy number estimation (**figure 5**). These
263 images also give predictions for loss of heterozygosity (LoH) regions and show which regions are
264 predicted to be composed of which alleles (A or B, AAB or ABB in trisomic regions) based on
265 sequence homology to either of these alleles. Results show that Esc strains contain various
266 aneuploidies, as well as certain LoH regions. Specifically Esc6 has a trisomy of Chr1, Esc7 has
267 a trisomy of Chr3, and a loss of the B allele of the short arm of chromosome R. Esc8 has a
268 monosomy of Chr5 (only A allele) and trisomies of Chr6, 7, and 8. Growth in ethanol may select
269 for aneuploid strains previously existing in the population or the presence of ethanol or byproducts
270 of its catabolism may stimulate chromosomal instability.

271 Additionally, we observed single nucleotide polymorphisms (SNPs) and
272 insertions/deletions in the genome sequences of Esc6, Esc7, and Esc8 (**supplemental tables**
273 **S11-S13**). There were 124 (in 64 different ORFs), 139 (in 83 different ORFs), and 135 (in 72
274 different ORFs) polymorphisms in protein coding regions in Esc6, Esc7, and Esc8 respectively.
275 Polymorphisms were comprised mostly of putative heterozygous polymorphisms in all strains
276 based on the allele depth ratio. Heterozygous and homozygous polymorphisms were classified
277 by creating a histogram of allele depth and calculating minima of a trimodal fit curve (minima =
278 Esc6:0.1845,**0.8482**; Esc7:0.1753,**0.8716**; Esc8:0.1761,**0.8628**) (**supplemental figure S5**).
279 Further analysis focused on homozygous polymorphisms classified as variant calls with an allele
280 depth ratio of greater than the highest minimum value (bolded values above). Only one gene

281 carried a homozygous polymorphism in all three Esc strains: *C6_02290C*, an uncharacterized
282 gene containing a putative zinc binding domain. Other genes of interest with homozygous
283 polymorphisms are *C4_04190C* and *C4_04200C* which are overlapping ORFs that both contain
284 a putative acetyltransferase domain and *PDC2* which was polymorphic in two strains. *PDC2*
285 encodes a homeodomain-containing transcriptional regulator which is involved in glucose
286 metabolism [52]. The role of these genes in ethanol tolerance is unknown.
287

288 **Esc strains exhibit reduced susceptibility to fluconazole**

289 Some of the chromosome aneuploidies observed in Esc7 and Esc8 have been previously
290 observed in strains with altered susceptibility to the frontline antifungal fluconazole. Aneuploidies
291 of chromosome 3, 5, 6, 7, and R have been implicated in azole resistance and tolerance [53–58].
292 Further, the ergosterol pathway, more specifically *ERG11,* is the target of azole class antifungals
293 and increased expression of *ERG11* and *CDR1* confers fluconazole resistance [34,59–61].
294 We therefore examined the susceptibility of Esc strains to fluconazole. Broth-microdilution
295 assays were carried out using the standard CLSI protocol [62]. After 24-hour incubations,
296 metabolic activity of the wells was measured with 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-
297 [(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) and MIC50 and MIC70 values were
298 calculated as described in Materials and Methods (**figure 6A**, **supplemental figure S6)**. Esc6
299 and Esc7 both exhibited fluconazole resistance phenotypes with elevated MIC50 values. Esc8
300 showed no alterations to MIC50 values but had increased MIC70 and trailing growth, suggesting
301 altered fluconazole susceptibility.
302 Disk diffusion assays were performed following standard protocols with 25ug-loaded
303 fluconazole filter disks [63,64]. Esc7 showed no zone of inhibition following a 48-hour incubation,
304 while Esc8 showed a zone of inhibition with heavy growth within the zone (**figure 6B**). These
305 results are consistent with fluconazole resistance and fluconazole tolerance respectively.
306 Interestingly, Esc6 showed a larger zone of inhibition than SC5314 in the disk-diffusion assay but

307 exhibited an elevated MIC in the broth microdilution assay. This type of discrepancy has been
308 observed rarely and could suggest a non-canonical mechanism of reduced fluconazole
309 susceptibility. In summary, all Esc strains tested exhibited altered susceptibility to fluconazole,
310 showing that *C. albicans* can evolve fluconazole resistance and tolerance through ethanol
311 exposure without previous exposure to antifungal drugs.

312 Due to this cross-tolerance/resistance, we compared the SNPs identified in Esc strains
313 with SNPs previously identified in strains developing fluconazole resistance. Whole genome
314 sequencing analysis of strains acquired by sequentially sampling patients during the acquisition
315 of fluconazole resistance was reported by Ford, et al [63]. In this study, sequential isolates from
316 patients with *C. albicans* oral candidiasis infections were obtained. In total 43 isolates from 11
317 patients were whole-genome sequenced. 7 of the 11 patients carried fluconazole resistant strains.
318 Genome sequences and SNPs of these strains were analyzed throughout the sequential
319 isolations and compared to SC5314 reference strains. From this study, 240 genes were observed
320 with persistent polymorphisms, defined as polymorphisms observed in fluconazole resistant
321 strains from at least 3 of the 7 patients, that were nonsynonymous, were not within a region of the
322 genome with a loss of heterozygosity event and, after arising, were found in all subsequent
323 isolates.

324 We determined how many of these 240 genes carried homozygous polymorphisms in
325 Esc6, Esc7, and Esc8. There were 8 total homozygous SNP-carrying genes in Esc6 and 5 of
326 these 8 polymorphic genes also carried persistent polymorphisms in Ford et al. Of the 6
327 homozygous polymorphic genes in Esc7, 2 of these also carried persistent polymorphisms in
328 Ford, et al. Esc8 had 5 homozygous polymorphic genes of which 3 carried persistent
329 polymorphisms in Ford, et al. This overlap in genes was greater than expected by chance (p-
330 values all < 0.0030; Chi-squared test). Genes that carry SNPs in Esc strains and Ford strains are
331 shown in **Table 1**. All polymorphisms can be found in **supplemental tables S11-S13.** These
332 results suggest an overlap in the selective pressures exerted by fluconazole and ethanol such

333 that these two pressures select for similar mutational landscapes and aneuploidies. The
334 similarities increase the likelihood that strains selected for higher ethanol tolerance will exhibit
335 cross-tolerance/resistance to fluconazole.

336 **Table 1*:* Polymorphic genes in Esc strains show overlap with genes associated with**
337 **fluconazole resistance***.*

Strain	**Observed Number of Common Genes[a]**	**Expected Number of Common Genes[b]**	**Two-tailed P-value (Chi squared)**
Esc6	5	0.3096	<0.0001
Esc7	2	0.2322	0.0029
Esc8	3	0.1935	<0.0001
	Homozygous Polymorphic Genes in Esc Strains		
Esc6	*ALS3, EAP1, GZF3, RFG1, TBF1, C6_04190C, C7_01100C, CR_02930W*		
Esc7	*PDC2, C1_14630C, C1_07690C, C4_04190C, C4_04200C, C6_04190C*		
Esc8	*FMO2, PDC2, PGA55, C6_03520C, C6_04190C*		
	Common Genes with Homozygous Polymorphisms in Esc strains and Persistent Polymorphisms in Ford, 2015		
Esc6	***C6_04190C,*** *TBF1, CR_02930W, ALS3, EAP1*		
Esc7	***C6_04190C,*** *C1_07690C*		
Esc8	***C6_04190C,*** *PGA55, FMO2*		

338

339 **Discussion**

340 In this study, we showed that repeated exposure of *C. albicans* cultures to
341 ethanol results in selection for *C. albicans* small colony variants. The small colony variants that
342 were studied exhibited increased ethanol tolerance as measured after a four-hour ethanol
343 exposure and a longer term 24-hour ethanol exposure. Interestingly, Esc strains appeared to
344 grow during the initial hours of the ethanol exposure and then lost viability at a similar rate to
345 SC5314. This observation suggests that the evolved tolerance strategies in Esc strains were
346 optimized for short ethanol exposures consistent with the four-hour exposures that were used
347 during the selection. These mechanisms did not appear to provide much advantage during

348 longer exposure times (i.e. 8-24 hours). Little is known about how *C. albicans* responds to
349 ethanol. In general, ethanol kills cells through disruption of the lipid bilayer [66,67], protein
350 denaturation [68], and intracellular membrane perturbations [69]. There is thinning of the bilayer
351 through direct interdigitation of ethanol between membrane lipids, and ultimately denaturation of
352 the membrane which will result in cell death [66,67]. Lipid metabolism alterations, aneuploidy,
353 increased production of stress responsive genes, and drug efflux pump expression have all
354 been shown to confer greater ethanol tolerance in *S. cerevisiae* [23,50,70]. We hypothesized
355 that *C. albicans* utilizes similar mechanisms to increase ethanol tolerance and improve survival
356 when exposed to relatively high concentrations of ethanol. We assessed this model with
357 transcriptomics.

358 Upon RNA-sequencing of Esc6, Esc7 and Esc8, interesting expression patterns were
359 observed. First, roughly 12 genes directly involved with ergosterol biosynthesis showed increased
360 expression in Esc7 in 10% ethanol compared to SC5314 in 10% ethanol. *ERG* genes are
361 downregulated in SC5314 in ethanol compared to growth without ethanol. Ethanol-dependent
362 *ERG* downregulation was reduced or absent in Esc7. We asked whether *UPC2* was involved in
363 the differential expression of *ERG* genes as Upc2 is a global regulator of ergosterol biosynthesis
364 [71]. However, *UPC2* expression was decreased in Esc7 in 10% ethanol compared to SC5314
365 (**supplemental table S3**), and there were no SNPs in the *UPC2* orf or promoter sequence,
366 suggesting that other novel transcriptional regulators of ergosterol synthesis are regulating *ERG*
367 gene expression in Esc7. Additionally, post-transcriptional regulatory mechanisms could lead to
368 greater activation of Upc2. For example in *S. cerevisiae* and *Candida* species, it has been
369 suggested that HSP90 acts as a pedestal for Upc2 and binds it in an inactive form [72,73]. It is
370 possible that similar mechanisms are at play in *C. albicans.* In Esc7, there is a higher ratio of
371 *UPC2*/*HSP90* transcripts in Esc7 in 10% ethanol compared to SC5314 in 10% ethanol based on
372 the RNA-Seq data (**supplemental figure S7**). Regulation of the *UPC2/HSP90* ratio could lead to
373 a greater proportion of Upc2 being in its active form, thus leading to greater ergosterol production.

374 There are likely many pathways that contribute to the increased ethanol survival of Esc
375 strains such as trehalose production, ergosterol production or reduced *HSP90* expression.
376 Ethanol affects many different pathways in cells such as protein folding, lipid bilayer integrity, and
377 mitochondrial and nuclear function [66–69,74]. In *C. albicans* specifically, ethanol also affects
378 filamentation and biofilm formation [75]. Therefore, many different pathways are under selection
379 during repeated exposure to ethanol and roughly 44% of all genes showed statistically significant
380 alterations in gene expression in the 10% ethanol comparison of Esc7 and SC5314
381 (**supplemental table S3**). These large transcriptomic changes in Esc strains in ethanol indicate
382 that many *C. albicans* pathways are likely impacted during ethanol exposure.

383 We showed that increased ergosterol content in *C. albicans* can confer increased ethanol
384 survival. This is likely due to protection from the impacts of ethanol perturbation on the plasma
385 membrane. Models of yeast plasma membranes showed that increased ergosterol content in the
386 membrane reduced ethanol interdigitation and contributed to resistance against the membrane
387 thinning effect of ethanol [67,76]. This finding shows that at least part of the overlap of selective
388 pressures between fluconazole and ethanol likely revolves around ergosterol. If a stressor
389 impacts a pathway that an antifungal drug also targets, the stressor could theoretically select for
390 drug resistant or tolerant strains. Even if there is no overlap in the phenotypic response to a
391 stimulus and an antifungal, if the resistance mechanism involves aneuploidy, cross-resistance
392 could arise due to increased dosage of genes. Previously this has been shown in *C. albicans* with
393 pre-exposure to chemotherapeutic drugs leading to decreased caspofungin susceptibility [58].
394 We show similar but novel findings—*C. albicans* can evolve fluconazole resistance and higher
395 tolerance after repeated exposure to a naturally occurring molecule, ethanol.

396 Furthermore, a peculiar fluconazole resistance phenotype was observed for Esc6, defined
397 as a susceptible disk-diffusion phenotype and a strong resistance phenotype observed by broth-
398 microdilution. There have been well documented discrepancies between broth-microdilution
399 assays and disk-diffusions [89]. However, discrepancies to the degree that we observed with Esc6

400 are uncommon. These observations suggest that Esc6 has developed a noncanonical
401 mechanism of resistance, or that Esc6 shows an environment-dependent mechanism of
402 resistance. Recently it has been shown that large discrepancies in susceptibility of vulvovaginal
403 candidiasis (VVC) isolates to fluconazole can be observed in clinical testing laboratories
404 depending on the pH of testing media. A pH of 4.5, which more accurately represents the pH of
405 the vagina, is more likely to identify a VVC strain as resistant than pH 7. Testing at pH 4.5 more
406 accurately explained treatment recalcitrance than susceptibilities obtained from testing at pH 7
407 [77]. Further studies of Esc6 will be needed to identify the basis for its fluconazole phenotypes.

408 Esc7 shows alterations previously identified in fluconazole resistant strains including Chr3
409 trisomy [53–55] and higher expression of genes in the ergosterol pathway. These genetic changes
410 have previously been implicated in fluconazole resistance [53–55]. However, Esc7 did not show
411 large changes to *CDR1*, *CDR2*, or *MDR1* expression in the conditions we examined. Previously
412 this was thought to be part of the mechanism of fluconazole resistance in strains with Chr3
413 trisomies as *CDR1, CDR2,* and *MRR1* reside on Chr3 [55,78].

414 Aneuploidies found in Esc8 included a monosomy of Chr5, and trisomies of Chr6, Chr7,
415 and ChrR. ChrR trisomy has been found in fluconazole tolerant strains and shown to confer
416 increased fluconazole tolerance [56–58]. Aneuploidies may contribute to the reduced fluconazole
417 susceptibility of Esc8.

418 Aneuploidies, while likely part of the resistance and tolerance mechanisms of these
419 strains, are not the only interesting genomic changes. Esc strains also carry SNPs in genes that
420 were altered during the acquisition of fluconazole resistance in clinical isolates [63]. Likely, some
421 of these SNPs confer a fitness advantage for Esc strains during growth in fluconazole. One of
422 these polymorphic genes of interest is *C6_04190C* which encodes an uncharacterized orf with a
423 putative zinc binding domain. *C6_04190C* was polymorphic in all Esc strains as well as the Ford,
424 et al strains. Perhaps C6_04190C plays a role in zinc uptake or zinc sequestration, which could
425 affect zinc-binding transcriptional regulators that are involved with fluconazole resistance such as

426 Mrr1, Mrr2, Tac1, and Upc2 [78–80]. Modifying the pool of biologically available zinc could affect
427 the activity of these various proteins and have effects on susceptibility to fluconazole.

428 In summary, we have shown that *C. albicans* can evolve increased ethanol tolerance
429 following repeated exposure to ethanol. This is likely due to the involvement of many different
430 pathways. Repeated ethanol exposure selects for Esc strains that have altered transcriptional
431 responses to ethanol and altered genomic structure. We also showed that increased ergosterol
432 content leads to enhanced ethanol survival. The mutational landscape of *C. albicans* Esc strains
433 resembles the landscape of genes mutated in clinical isolates upon acquisition of fluconazole
434 resistance. This finding highlights the overlap of selective pressures between the two stressors:
435 fluconazole and ethanol. Consistent with this overlap, we demonstrated that strains repeatedly
436 exposed to ethanol acquire fluconazole resistance and tolerance without previous exposure to
437 antifungal drugs.

438

439 **Materials and Methods**

440

441 **Strains and Culture Conditions**

442 For the repeated ethanol exposure protocol, individual colonies of *C. albicans* strain
443 SC5314 were inoculated into 10 mL of YPD and grown overnight at 30ºC to post exponential
444 phase (16-20 hours). 5 independent cultures were split into paired cultures to be exposed to media
445 with ethanol or water. After overnight growth, cells were counted on a hemocytometer and 10^6
446 cells were inoculated from the overnight YPD cultures into 1 mL of medium A (Dulbecco's Modified
447 Eagle Medium (DMEM) with 10% fetal bovine serum and 1X non-essential amino acids) with
448 ethanol or water in 2 mL screw cap microcentrifuge tubes. The concentrations of ethanol or water
449 were 1% for the first exposure, 2% for the second exposure, 4% for the third exposure, 6% for
450 the fourth exposure, 8% for the fifth exposure, and 10% for exposures six through eight
451 (volume/volume). Cultures were incubated at 37ºC in a 5% CO_2 atmosphere for four hours to

452 mimic physiological conditions. Following ethanol or water exposure, 10 uL of the medium A
453 cultures were seeded into 10 mL YPD for recovery from the ethanol exposure and grown at 30ºC
454 until post-exponential phases were reached (1 day for passages in 6% ethanol or less or 2 days
455 for passages in 8% and 10% ethanol). 100 uL of each population was plated on YPD agar and
456 incubated for 2 days at 30ºC following each medium A exposure to monitor population dynamics
457 and normal sized and small sized colonies were counted by eye. Small colonies were defined as
458 colonies estimated to be 50% or less in size compared to normal sized colonies.

459 Ethanol small colony (Esc) strains were isolated from different populations of cells at
460 different time points in the repeated ethanol exposure protocol by picking colonies from the YPD
461 agar plates. Populations 1-4 were populations repeatedly exposed to water and 5-8 were
462 populations repeatedly exposed to ethanol. Esc6 was isolated from population 8 in the 6th passage
463 in ethanol (first exposure to 10% ethanol), Esc7 was isolated from population 6 in the 7th passage
464 in ethanol (second exposure to 10% ethanol), and Esc8 was isolated from the 5th population in
465 the 8th passage in ethanol (third exposure to 10% ethanol). SC5314-UPC2-G648D was obtained
466 from Dr. Joachim Morschhauser and has the following genotype: *UPC2-G648D::FRT/UPC2-*
467 *G648D::FRT* [45].

468

469 **Ethanol Exposure to Test for Ethanol Survival**

470 Strains were grown to post-exponential phase in YPD (16-20 hours) and then counted
471 using a hemocytometer to seed 10^6 cells in one mL of medium A with 10% H2O or 10% ethanol
472 (vol/vol) using 95% ethanol in 2 mL screw cap tubes. Cultures were incubated for 4 hours in 37ºC
473 in a 5% CO_2 atmosphere. Before incubation, 100 uL was sampled from the cultures and serially
474 diluted onto YPD-agar plates for a starting CFU determination. Following 4 hours, cultures were
475 sampled and diluted serially in 1X phosphate-buffered saline (PBS) and plated on YPD agar for
476 a final CFU determination for the water and ethanol cultures. YPD-agar plates were incubated for
477 2 days at 30ºC and counted. The final CFU from the ethanol cultures was divided by the final CFU

478 from the water cultures to determine percentage survival and then calculated as a survival relative
479 to SC5314 within each separate experiment.

480 For the extended incubation, the same conditions were used as above except cultures
481 were incubated in 37ºC incubators with ambient CO_2 concentrations. Two separate cultures for
482 each biological replicate were set up for each individual experiment. The first set of cultures was
483 sampled at 0, 2, 4, and 6 hours and the second set was sampled at 8, 10, 12, and 24 hours.
484 Survival was calculated relative to starting CFU obtained at timepoint 0 hours.

485

486 **RNA isolation and RNA-sequencing**

487 Cultures of SC5314 or Esc strains were grown to post-exponential phase and 10^8 cells for
488 each were seeded into 1 mL cultures of medium A with 10% H_2O or EtOH and incubated for 4
489 hours at 37ºC with 5% CO_2. Immediately following the incubation, cells were centrifuged at 6000
490 rpm for 1 min and supernatant was removed. Cells were then resuspended in 1 mL of RNA-later
491 (Fisher cat. AM7021) and immediately frozen at -80ºC until they were ready for extraction. Cells
492 were then thawed on ice. Cells were lysed as previously described by bead-beating [81]. RNA
493 was isolated using the Invitrogen Purelink RNA mini kit (Thermo Fisher cat: 12183-025) with the
494 on-column DNase treatment (Thermo Fisher cat: 12185-010). RNA was eluted in 40uL of
495 ultrapure H_2O and RNA yield and purity were assessed on a Nanodrop.

496 Three biological replicates of SC5314, Esc6, Esc7, or Esc8 following exposure to medium
497 A with 10% water or 10% EtOH were sent for sequencing. All samples sequenced had RNA
498 integrity scores of 9.2 or greater. cDNA libraries were made by Genewiz and RNA-seq was
499 performed by Genewiz as previously described [64] with the following alterations: gene
500 expression was considered significant only if adjusted-P-value (calculated by Benjamini-
501 Hochberg test) was less than 0.05 and log_2-fold change was greater than the absolute value of
502 0.5. RNA-sequencing was aligned to *Candida albicans* SC5314 (assembly ASM18296v3) as the
503 reference genome. Gene ontology of significant hits were assessed using Candida Genome

504 Database Go term finder as has been previously described [17] (http://candidagenome.org). For
505 volcano plots and gene expression differences, the three biological replicates from each condition
506 were averaged and significant differences were compared by relative transcript abundance
507 differences.
508
509 **Validation of RNA-sequencing by RT-qPCR**
510 RNA was extracted from additional cultures following the same water or ethanol exposure
511 protocol using the extraction protocol above. cDNA was synthesized using SSIII reverse
512 transcriptase (Thermo cat: 18080-085) following manufacturer's protocol with approximately 1 ug
513 starting RNA concentrations.. Oligo-dT primers were used for each cDNA synthesis reaction.
514 Eight biological replicates for each strain and condition were used. cDNA was diluted 1:5 in
515 ultrapure H_2O and 1 uL was used per reaction for RT-qPCR. SYBR green master mix (Fisher cat:
516 4334973) was used and qPCR reactions were run on a Roche Lightcycler 480 II using standard
517 two-step reaction procedures. Controls without cDNA were run for each primer and all primer
518 pairs either did not yield product, or yielded Cp values that were at least 10 cycles greater than
519 the sample Cp value. Melting curves were analyzed to ensure only one peak was seen and primer
520 dimerization was not observed in any of the curves analyzed. Cp values were normalized using
521 a putative housekeeping gene: *SPL1*, a tRNA splicing enzyme that showed no significant changes
522 in gene expression in all conditions and strains analyzed based on RNA-seq. The fold change in
523 gene expression relative to SC5314 in 10% H2O was then calculated and graphed by $2^{-\Delta\Delta CT}$
524 values [81]. Primers are shown in **supplemental table S14**. Primer sequences were either used
525 from previously published work or were designed using NCBI primer blast. Primer design used
526 the A allele sequence of the target gene, *C. albicans* strain SC5314 reference genome, PCR
527 product size of 90-250bp, optimized primer length of 28 nucleotides, primer melting temperatures
528 of 53-63ºC with 56ºC optimized temperature and a max primer pair difference of 5ºC. Purified
529 PCR products were sequenced to verify target specificity.

530

531 **Ergosterol Quantitation**

532 Total cellular ergosterol was quantitated as described previously [43,82,83]. 10^8 cells of
533 SC5314 or Esc strains were incubated for 4 hours at 37ºC with 5% CO_2 in medium A with 10%
534 ethanol or 10% H_2O. After the four-hour incubation, cells were pelleted and washed twice in sterile
535 PBS. Following washing, supernatant was removed, and the wet pellet weight of the cells was
536 measured. 2 mL of alcoholic 25% potassium hydroxide was added to each tube for saponification
537 and pellets were resuspended by vortexing for 1 min, transferred to borosilicate glass tubes, and
538 incubated in an 85ºC water bath for 1 hour. Following the incubation, tubes were allowed to cool
539 for 10 mins at room temperature and 2 mL of spectroscopy grade n-heptane (Sigma 1043660500)
540 and 667 uL of sterile deionized water were added to each tube and vortexed for 3 mins. The
541 organic fraction was removed from the mixture and diluted 1:2 in 100% ethanol (Sigma cat:
542 E7023-1L). Scanning absorbance values were then taken between 230nm and 290nm on a
543 Nanodrop spectrophotometer. Calculation of the amount of ergosterol and amount of dehydro-
544 ergosterol (DHE) normalized to wet pellet weight was performed as described previously
545 [43,82,83]. In summary, the calculation was **equation 1:** % (ergosterol + DHE) = [(A_{282}/290) x
546 dilution factor] / pellet weight. **Equation 2:** % DHE = [(A_{230}/518) x dilution factor]/pellet weight. %
547 ergosterol = **Equation 1 – Equation 2**. The dilution factor is the dilution factor used for diluting
548 the sterol-containing heptane solution with ethanol, 290 = E for ergosterol at 282 nm, and 518 =
549 E for DHE at 230 nm [43].

550

551

552 **Whole-Genome Sequencing**

553 Genomic DNA from SC5314 and Esc strains was extracted and sent to SeqCenter for
554 next-generation sequencing. SC5314 and Esc6 yielded consistent colony sizes so these strains
555 were struck out on YPD-agar and isolated colonies were picked, grown to post-exponential phase

556 and cells were pelleted. Esc7 and Esc8 have more unstable small colony phenotypes, therefore,
557 cells were plated on a YPD-agar plate grown for 3 days at 30ºC and small colonies were picked
558 and added to 100uL of sterile PBS. This was then used to seed 4 new plates and performed three
559 separate times. The plates with the lowest reversion rates were used for genome preps by
560 collecting small colonies from these plates and adding them to 5 mL of sterile PBS. These tubes
561 were then vortexed, and cells were pelleted. Genomes were extracted using bead-beating in
562 phenol chloroform followed by chloroform extraction and ethanol precipitation as described
563 previously [84]. DNA was purified further by the Qiagen DNeasy kit using manufacturer's protocol.
564 Whole-genome sequencing was performed by SeqCenter by paired end NGS Illumina
565 sequencing of 151 bp reads. Quality control and adaptor trimming were performed with bcl-
566 convert [85], and reads were mapped to SC5314 reference genome using bwa [86]. Genome
567 coverage from the sequencing runs ranged from 66X to 83X based on total number of reads
568 across the SC5314 reference genome length. GATK's Markduplicates was used to remove
569 duplicate reads in the alignment and GATK's Haplotype caller was used to call variants in the
570 alignment [87]. Variants with a QD < 2, or MQ < 40, or MGRankSum < -12.5, or
571 ReadPosRankSum < -8, or FS > 60.0, or SOR > 3 were filtered out. Sequence variants were
572 compiled into an excel document by SeqCenter. Due to the large number of sequence variants
573 obtained, only homozygous sequence variants of Esc strains compared to the parental SC5314
574 strain sequence were analyzed in depth. Homozygous sequence variants were determined by
575 constructing a histogram of allele depth and calculating minima of a trimodal fit curve
576 (**supplemental figure S5**). Homozygous polymorphisms were classified as variant calls with an
577 allele depth ratio greater than the high minima value. Heterozygous polymorphisms were
578 classified as those with allele depth ratios between the two minima values. Polymorphisms with
579 allele depth ratio lower than the high minima and less than a total read depth of 10 were excluded
580 from analysis. For the remaining putative homozygous polymorphisms, pre-existing heterozygous

581 polymorphisms in the B allele were excluded by examining the location of the polymorphism in a
582 multiple sequence alignment of the A and B alleles of the gene.

583 Chromosome copy number variations were analyzed using the Yeast Mapping Analysis
584 Pipeline [51]. Fastq files were uploaded to YMAP and YMAP calculated the average read depth
585 across the chromosomes mapping to SC5314 as the reference genome. YMAP thus generates
586 chromosome copy number predictions based on the read depth across the genome. These
587 predictions were graphed as predicted copy number across chromosomes of the individual strains
588 relative to a ploidy of 2. Changes in ploidy were shown by thick black bars deviating from the
589 starting ploidy line at 2.

590

591 **Disk-Diffusion and Broth-Microdilution Assays**

592 For the disk-diffusion assay, cells were grown to post-exponential phase in liquid YPD
593 medium and 10^5 cells per plate were spread onto YPD-agar. Then 6mm diameter filter disks
594 impregnated with 25ug of fluconazole were placed in the center of the YPD agar. These plates
595 were incubated for 48 hours at 30ºC to assess the zone of inhibition and the fraction of growth
596 within the zone. Images of plates following incubation were taken on a Syngene gel-doc imager
597 using white light, black background, and identical iris, zoom, and focus settings for each plate.
598 Representative images of each strain were used for figures in this paper. Eight biological
599 replicates of each strain were tested in four different experiments.

600 For the broth-microdilution assay, cells were grown as stated above and then 10^4 cells/well
601 were used for each strain and experiment. Standard CLSI protocol was used for this experiment.
602 Cells were diluted in RPMI, 165 mM MOPS, 2% glucose, pH 7.0. Fluconazole was serially diluted
603 2-fold—yielding final concentrations from 64 ug/mL down to 0.125 ug/mL. In each row, a no-cell
604 well was used as a blank and sterility control, and a no-drug control was used to calculate 100%
605 metabolic activity. Plates were incubated for 24 hours at 35ºC. At 24 hours medium was carefully
606 removed from each well and 100 uL of 1 mg/mL of 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-

607 [(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) (Alfa-Aesar) in PBS with 4 uL of
608 menadione per/100 uL was added to each well. The plates were incubated for one hour at room
609 temperature and then 50 uL of solution from each well was removed and read at 492 nm to
610 determine metabolic activity of each well. The no-drug well averaged between the two duplicates
611 was used to calculate the OD492 that indicates a 50% reduction. The concentration of drug that
612 yielded a reduction in metabolic activity immediately less than 50% of the no-drug well was
613 considered the MIC50. The heat map of the metabolic activity of these wells is shown as the
614 medians of 6 biological replicates for each strain in **figure 9** or shown as individual values in
615 **supplemental figure S6**. Heat map was made using GraphPad Prism.
616

617 **Author Contributions**

618 Conceptualization, A.W.D. and C.A.K.; investigation, A.W.D.; writing—original draft preparation,
619 A.W.D.; writing—review and editing, C.A.K., A.W.D.; funding acquisition, C.A.K. All authors have
620 read and agreed to the published version of the manuscript.

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630 **Data Availability Statement:** Submission of the raw sequencing data to the NCBI Sequence
631 Read Archive is in progress.

632

633 **References**

634 1. McCarty TP, White CM, Pappas PG. Candidemia and Invasive Candidiasis. Infect Dis Clin North Am.
635 2021 Jun;35(2):389–413.

636 2. MacCallum DM. Hosting Infection: Experimental Models to Assay *Candida* Virulence. Int J Microbiol.
637 2012;2012:1–12.

638 3. Mejia-Chew C, O'Halloran JA, Olsen MA, Stwalley D, Kronen R, Lin C, et al. Effect of infectious
639 disease consultation on mortality and treatment of patients with candida bloodstream infections: a
640 retrospective, cohort study. Lancet Infect Dis. 2019 Dec;19(12):1336–44.

641 4. Fujihara N, Saito T, Kudo T, Iinuma Y, Ichiyama S, Takakura S. Clinical factors associated with
642 fluconazole resistance and short-term survival in patients with Candida bloodstream infection. Eur J
643 Clin Microbiol Infect Dis. 2004 May 1;23(5):380–8.

644 5. Kato H, Yoshimura Y, Suido Y, Shimizu H, Ide K, Sugiyama Y, et al. Mortality and risk factor analysis
645 for Candida blood stream infection: A multicenter study. J Infect Chemother. 2019 May;25(5):341–5.

646 6. Pande K, Chen C, Noble SM. Passage through the mammalian gut triggers a phenotypic switch that
647 promotes Candida albicans commensalism. Nat Genet. 2013 Sep;45(9):1088–91.

648 7. Zhai B, Ola M, Rolling T, Tosini NL, Joshowitz S, Littmann ER, et al. High-resolution mycobiota
649 analysis reveals dynamic intestinal translocation preceding invasive candidiasis. Nat Med. 2020
650 Jan;26(1):59–64.

651 8. Kotey FC, Dayie NT, Tetteh-Uarcoo PB, Donkor ES. *Candida* Bloodstream Infections: Changes in
652 Epidemiology and Increase in Drug Resistance. Infect Dis Res Treat. 2021 Jan;14:117863372110269.

653 9. Chu H, Duan Y, Lang S, Jiang L, Wang Y, Llorente C, et al. The Candida albicans exotoxin candidalysin
654 promotes alcohol-associated liver disease. J Hepatol. 2020 Mar;72(3):391–400.

655 10. Yang AM, Inamine T, Hochrath K, Chen P, Wang L, Llorente C, et al. Intestinal fungi contribute to
656 development of alcoholic liver disease. J Clin Invest. 2017 May 22;127(7):2829–41.

657 11. Hartmann P, Lang S, Zeng S, Duan Y, Zhang X, Wang Y, et al. Dynamic Changes of the Fungal
658 Microbiome in Alcohol Use Disorder. Front Physiol. 2021 Jul 19;12:699253.

659 12. Rubbens J, Brouwers J, Wolfs K, Adams E, Tack J, Augustijns P. Ethanol concentrations in the human
660 gastrointestinal tract after intake of alcoholic beverages. Eur J Pharm Sci. 2016 Apr;86:91–5.

661 13. Halsted CH, Robles EA, Mezey E. Distribution of ethanol in the human gastrointestinal tract. Am J
662 Clin Nutr. 1973 Aug;26(8):831–4.

663 14. Stanley D, Fraser S, Chambers PJ, Rogers P, Stanley GA. Generation and characterisation of stable
664 ethanol-tolerant mutants of Saccharomyces cerevisiae. J Ind Microbiol Biotechnol. 2010
665 Feb;37(2):139–49.

666 15. Turanlı-Yıldız B, Benbadis L, Alkım C, Sezgin T, Akşit A, Gökçe A, et al. In vivo evolutionary
667 engineering for ethanol-tolerance of Saccharomyces cerevisiae haploid cells triggers diploidization. J
668 Biosci Bioeng. 2017 Sep;124(3):309–18.

669 16. Balaban NQ, Helaine S, Lewis K, Ackermann M, Aldridge B, Andersson DI, et al. Definitions and
670 guidelines for research on antibiotic persistence. Nat Rev Microbiol. 2019 Jul;17(7):441–8.

671 17. Muñoz JF, Delorey T, Ford CB, Li BY, Thompson DA, Rao RP, et al. Coordinated host-pathogen
672 transcriptional dynamics revealed using sorted subpopulations and single macrophages infected
673 with Candida albicans. Nat Commun. 2019 Apr 8;10(1):1607.

674 18. Dovo EE, Zohoncon TM, Tovo SF, Soubeiga ST, Kiendrebeogo IT, Yonli AT, et al. First detection of
675 mutated ERG11 gene in vulvovaginal Candida albicans isolates at Ouagadougou/Burkina Faso. BMC
676 Infect Dis. 2022 Aug 8;22(1):678.

677 19. Du Bois AC, Xue A, Pham C, Revie NM, Meyer KJ, Yashiroda Y, et al. High-Throughput Chemical
678 Screen Identifies a 2,5-Disubstituted Pyridine as an Inhibitor of Candida albicans Erg11. Mitchell AP,
679 editor. mSphere. 2022 Jun 29;7(3):e00075-22.

680 20. Shobayashi M, Mitsueda S ichiro, Ago M, Fujii T, Iwashita K, Iefuji H. Effects of Culture Conditions on
681 Ergosterol Biosynthesis by *Saccharomyces cerevisiae*. Biosci Biotechnol Biochem. 2005
682 Jan;69(12):2381–8.

683 21. del Castillo Agudo L. Lipid content of Saccharomyces cerevisiae strains with different degrees of
684 ethanol tolerance. Appl Microbiol Biotechnol. 1992 Aug;37(5):647–51.

685 22. Kubota S, Takeo I, Kume K, Kanai M, Shitamukai A, Mizunuma M, et al. Effect of Ethanol on Cell
686 Growth of Budding Yeast: Genes That Are Important for Cell Growth in the Presence of Ethanol.
687 Biosci Biotechnol Biochem. 2004 Jan;68(4):968–72.

688 23. Ma M, Liu ZL. Mechanisms of ethanol tolerance in Saccharomyces cerevisiae. Appl Microbiol
689 Biotechnol. 2010 Jul;87(3):829–45.

690 24. Jing H, Liu H, Zhang L, Gao J, Song H, Tan X. Ethanol induces autophagy regulated by mitochondrial
691 ROS in Saccharomyces cerevisiae. J Microbiol Biotechnol. 2018 Dec 28;28(12):1982–91.

692 25. Derkacz D, Bernat P, Krasowska A. K143R Amino Acid Substitution in 14-α-Demethylase (Erg11p)
693 Changes Plasma Membrane and Cell Wall Structure of Candida albicans. Int J Mol Sci. 2022 Jan
694 31;23(3):1631.

695 26. Pais TM, Foulquié-Moreno MR, Hubmann G, Duitama J, Swinnen S, Goovaerts A, et al. Comparative
696 Polygenic Analysis of Maximal Ethanol Accumulation Capacity and Tolerance to High Ethanol Levels
697 of Cell Proliferation in Yeast. Jinks-Robertson S, editor. PLoS Genet. 2013 Jun 6;9(6):e1003548.

698 27. Ibeas JI, Jimenez J. Mitochondrial DNA loss caused by ethanol in Saccharomyces flor yeasts. Appl
699 Environ Microbiol. 1997 Jan;63(1):7–12.

700 28. Snoek T, Verstrepen KJ, Voordeckers K. How do yeast cells become tolerant to high ethanol
701 concentrations? Curr Genet. 2016 Aug;62(3):475–80.

702 29. Teixeira MC, Mira NP, Sá-Correia I. A genome-wide perspective on the response and tolerance to
703 food-relevant stresses in Saccharomyces cerevisiae. Curr Opin Biotechnol. 2011 Apr;22(2):150–6.

704 30. Song Y, Li S, Zhao Y, Zhang Y, Lv Y, Jiang Y, et al. ADH1 promotes Candida albicans pathogenicity by
705 stimulating oxidative phosphorylation. Int J Med Microbiol. 2019 Sep;309(6):151330.

706 31. Piper PW, Talreja K, Panaretou B, Moradas-Ferreira P, Byrne K, Praekelt UM, et al. Induction of
707 major heat-shock proteins of Saccharomyces cerevisiae, including plasma membrane Hsp30, by
708 ethanol levels above a critical threshold. Microbiology. 1994 Nov 1;140(11):3031–8.

709 32. Ogawa Y, Nitta A, Uchiyama H, Imamura T, Shimoi H, Ito K. Tolerance mechanism of the ethanol-
710 tolerant mutant of sake yeast. J Biosci Bioeng. 2000 Jan;90(3):313–20.

711 33. McClellan AJ, Xia Y, Deutschbauer AM, Davis RW, Gerstein M, Frydman J. Diverse Cellular Functions
712 of the Hsp90 Molecular Chaperone Uncovered Using Systems Approaches. Cell. 2007
713 Oct;131(1):121–35.

714 34. Shapiro RS, Uppuluri P, Zaas AK, Collins C, Senn H, Perfect JR, et al. Hsp90 Orchestrates
715 Temperature-Dependent Candida albicans Morphogenesis via Ras1-PKA Signaling. Curr Biol. 2009
716 Apr;19(8):621–9.

717 35. Singh SD, Robbins N, Zaas AK, Schell WA, Perfect JR, Cowen LE. Hsp90 Governs Echinocandin
718 Resistance in the Pathogenic Yeast Candida albicans via Calcineurin. Mitchell AP, editor. PLoS
719 Pathog. 2009 Jul 31;5(7):e1000532.

720 36. Diezmann S, Michaut M, Shapiro RS, Bader GD, Cowen LE. Mapping the Hsp90 Genetic Interaction
721 Network in Candida albicans Reveals Environmental Contingency and Rewired Circuitry. Snyder M,
722 editor. PLoS Genet. 2012 Mar 15;8(3):e1002562.

723 37. Li C, Tu J, Han G, Liu N, Sheng C. Heat shock protein 90 (Hsp90)/Histone deacetylase (HDAC) dual
724 inhibitors for the treatment of azoles-resistant Candida albicans. Eur J Med Chem. 2022
725 Jan;227:113961.

726 38. Xu Y, Lu H, Zhu S, Li WQ, Jiang Y ying, Berman J, et al. Multifactorial Mechanisms of Tolerance to
727 Ketoconazole in Candida albicans. Cuomo CA, editor. Microbiol Spectr. 2021 Sep 3;9(1):e00321-21.

728 39. Van Hauwenhuyse F, Fiori A, Van Dijck P. Ascorbic Acid Inhibition of Candida albicans Hsp90-
729 Mediated Morphogenesis Occurs via the Transcriptional Regulator Upc2. Eukaryot Cell. 2014
730 Oct;13(10):1278–89.

731 40. Rogers PD. Sterol homeostasis in yeast. Nat Chem Biol. 2022 Nov;18(11):1170–1.

732 41. Dai B, Wang Y, Li D, Xu Y, Liang R, Zhao L, et al. Hsp90 Is Involved in Apoptosis of Candida albicans by
733 Regulating the Calcineurin-Caspase Apoptotic Pathway. Nielsen K, editor. PLoS ONE. 2012 Sep
734 18;7(9):e45109.

735 42. Nieminen MT, Novak-Frazer L, Rautemaa V, Rajendran R, Sorsa T, Ramage G, et al. A Novel
736 Antifungal Is Active against Candida albicans Biofilms and Inhibits Mutagenic Acetaldehyde
737 Production In Vitro. Bassilana M, editor. PLoS ONE. 2014 May 27;9(5):e97864.

738 43. Arthington-Skaggs BA, Jradi H, Desai T, Morrison CJ. Quantitation of Ergosterol Content: Novel
739 Method for Determination of Fluconazole Susceptibility of *Candida albicans*. J Clin Microbiol. 1999
740 Oct;37(10):3332–7.

741 44. Breivik ON, Owades JL. Yeast Analysis, Spectrophotometric Semimicrodetermination of Ergosterol in
742 Yeast. J Agric Food Chem. 1957 May;5(5):360–3.

743 45. Flowers SA, Barker KS, Berkow EL, Toner G, Chadwick SG, Gygax SE, et al. Gain-of-Function
744 Mutations in *UPC2* Are a Frequent Cause of *ERG11* Upregulation in Azole-Resistant Clinical Isolates
745 of Candida albicans. Eukaryot Cell. 2012 Oct;11(10):1289–99.

746 46. Zhu C, Liao B, Ye X, Zhou Y, Chen X, Liao M, et al. Artemisinin elevates ergosterol levels of Candida
747 albicans to synergise with amphotericin B against oral candidiasis. Int J Antimicrob Agents. 2021
748 Sep;58(3):106394.

749 47. Tsai HJ, Nelliat A. A Double-Edged Sword: Aneuploidy is a Prevalent Strategy in Fungal Adaptation.
750 Genes. 2019 Oct 10;10(10):787.

751 48. Berman J. Ploidy plasticity: a rapid and reversible strategy for adaptation to stress. Munro C, editor.
752 FEMS Yeast Res. 2016 May;16(3):fow020.

753 49. Mba IE, Nweze EI, Eze EA, Anyaegbunam ZKG. Genome plasticity in Candida albicans: A cutting-edge
754 strategy for evolution, adaptation, and survival. Infect Genet Evol. 2022 Apr;99:105256.

755 50. Morard M, Macías LG, Adam AC, Lairón-Peris M, Pérez-Torrado R, Toft C, et al. Aneuploidy and
756 Ethanol Tolerance in Saccharomyces cerevisiae. Front Genet. 2019 Feb 12;10:82.

757 51. Abbey DA, Funt J, Lurie-Weinberger MN, Thompson DA, Regev A, Myers CL, et al. YMAP: a pipeline
758 for visualization of copy number variation and loss of heterozygosity in eukaryotic pathogens.
759 Genome Med. 2014 Nov;6(11):100.

760 52. Kaiser B, Munder T, Saluz HP, Künkel W, Eck R. Identification of a gene encoding the pyruvate
761 decarboxylase gene regulator CaPdc2p fromCandida albicans. Yeast. 1999 May;15(7):585–91.

762 53. Perepnikhatka V, Fischer FJ, Niimi M, Baker RA, Cannon RD, Wang YK, et al. Specific Chromosome
763 Alterations in Fluconazole-Resistant Mutants of *Candida albicans*. J Bacteriol. 1999
764 Jul;181(13):4041–9.

765 54. Selmecki AM, Dulmage K, Cowen LE, Anderson JB, Berman J. Acquisition of Aneuploidy Provides
766 Increased Fitness during the Evolution of Antifungal Drug Resistance. Madhani HD, editor. PLoS
767 Genet. 2009 Oct 30;5(10):e1000705.

768 55. Kukurudz RJ, Chapel M, Wonitowy Q, Adamu Bukari AR, Sidney B, Sierhuis R, et al. Acquisition of
769 cross-azole tolerance and aneuploidy in *Candida albicans* strains evolved to posaconazole. Andrews
770 B, editor. G3 GenesGenomesGenetics. 2022 Aug 25;12(9):jkac156.

771 56. Sun L liu, Li H, Yan T hua, Fang T, Wu H, Cao Y bing, et al. Aneuploidy Mediates Rapid Adaptation to
772 a Subinhibitory Amount of Fluconazole in Candida albicans. Anderson MZ, editor. Microbiol Spectr.
773 2023 Apr 13;11(2):e03016-22.

774 57. Li X, Yang F, Li D, Zhou M, Wang X, Xu Q, et al. Trisomy of chromosome R confers resistance to
775 triazoles in Candida albicans. Med Mycol. 2015 Apr 1;53(3):302–9.

776 58. Yang F, Teoh F, Tan ASM, Cao Y, Pavelka N, Berman J. Aneuploidy Enables Cross-Adaptation to
777 Unrelated Drugs. Malik H, editor. Mol Biol Evol. 2019 Aug 1;36(8):1768–82.

778 59. Cowen LE, Sanglard D, Howard SJ, Rogers PD, Perlin DS. Mechanisms of Antifungal Drug Resistance.
779 Cold Spring Harb Perspect Med. 2015 Jul;5(7):a019752.

780 60. Lee Y, Puumala E, Robbins N, Cowen LE. Antifungal Drug Resistance: Molecular Mechanisms in
781 *Candida albicans* and Beyond. Chem Rev. 2021 Mar 24;121(6):3390–411.

782 61. Holmes AR, Lin YH, Niimi K, Lamping E, Keniya M, Niimi M, et al. ABC Transporter Cdr1p Contributes
783 More than Cdr2p Does to Fluconazole Efflux in Fluconazole-Resistant *Candida albicans* Clinical
784 Isolates. Antimicrob Agents Chemother. 2008 Nov;52(11):3851–62.

785 62. CLSI. Clinical and laboratory standards institute. Reference method for broth dilution antifungal
786 susceptibility testing of yeasts-third edition: Approved standard M27-A3. CLSI Doc M27-A3 Suppl
787 SWayne PA USA. 2008;

788 63. Ford CB, Funt JM, Abbey D, Issi L, Guiducci C, Martinez DA, et al. The evolution of drug resistance in
789 clinical isolates of Candida albicans. eLife. 2015 Feb 3;4:e00662.

790 64. Liu RH, Shang ZC, Li TX, Yang MH, Kong LY. *In Vitro* Antibiofilm Activity of Eucarobustol E against
791 Candida albicans. Antimicrob Agents Chemother. 2017 Aug;61(8):e02707-16.

792 65. Henderson CM, Block DE. Examining the Role of Membrane Lipid Composition in Determining the
793 Ethanol Tolerance of Saccharomyces cerevisiae. Müller V, editor. Appl Environ Microbiol. 2014 May
794 15;80(10):2966–72.

795 66. Vanegas JM, Contreras MF, Faller R, Longo ML. Role of Unsaturated Lipid and Ergosterol in Ethanol
796 Tolerance of Model Yeast Biomembranes. Biophys J. 2012 Feb;102(3):507–16.

797 67. Furutani N, Izawa S. Adaptability of wine yeast to ethanol-induced protein denaturation. FEMS Yeast
798 Res. 2022 Nov 25;22(1):foac059.

799 68. Yang KM, Lee NR, Woo JM, Choi W, Zimmermann M, Blank LM, et al. Ethanol reduces mitochondrial
800 membrane integrity and thereby impacts carbon metabolism of Saccharomyces cerevisiae. FEMS
801 Yeast Res. 2012 Sep;12(6):675–84.

802 69. Yang KM, Woo JM, Lee SM, Park JB. Improving ethanol tolerance of Saccharomyces cerevisiae by
803 overexpressing an ATP-binding cassette efflux pump. Chem Eng Sci. 2013 Nov;103:74–8.

804 70. Vu BG, Stamnes MA, Li Y, Rogers PD, Moye-Rowley WS. The Candida glabrata Upc2A transcription
805 factor is a global regulator of antifungal drug resistance pathways. Heitman J, editor. PLOS Genet.
806 2021 Sep 30;17(9):e1009582.

807 71. Yang H, Tong J, Lee CW, Ha S, Eom SH, Im YJ. Structural mechanism of ergosterol regulation by
808 fungal sterol transcription factor Upc2. Nat Commun. 2015 Feb 6;6(1):6129.

809 72. Tan L, Chen L, Yang H, Jin B, Kim G, Im YJ. Structural basis for activation of fungal sterol receptor
810 Upc2 and azole resistance. Nat Chem Biol. 2022 Nov;18(11):1253–62.

811 73. Cherian PP, Schenker S, Henderson GI. Ethanol-Mediated DNA Damage and PARP-1 Apoptotic
812 Responses in Cultured Fetal Cortical Neurons. Alcohol Clin Exp Res [Internet]. 2008 Aug [cited 2023
813 Aug 14]; Available from: https://onlinelibrary.wiley.com/doi/10.1111/j.1530-0277.2008.00769.x

814 74. Chauhan NM, Shinde RB, Karuppayil SM. Effect of alcohols on filamentation, growth, viability and
815 biofilm development in Candida albicans. Braz J Microbiol. 2013 Dec;44(4):1315–20.

816 75. Tierney KJ, Block DE, Longo ML. Elasticity and Phase Behavior of DPPC Membrane Modulated by
817 Cholesterol, Ergosterol, and Ethanol. Biophys J. 2005 Oct;89(4):2481–93.

818 76. Sobel JD. Resistance to Fluconazole of Candida albicans in Vaginal Isolates: a 10-Year Study in a
819 Clinical Referral Center. Antimicrob Agents Chemother. 2023 May 17;67(5):e00181-23.

820 77. Morschhäuser J, Barker KS, Liu TT, Blaß-Warmuth J, Homayouni R, Rogers PD. The Transcription
821 Factor Mrr1p Controls Expression of the MDR1 Efflux Pump and Mediates Multidrug Resistance in
822 Candida albicans. Johnson AD, editor. PLoS Pathog. 2007 Nov 2;3(11):e164.

823 78. Nishimoto AT, Zhang Q, Hazlett B, Morschhäuser J, Rogers PD. Contribution of Clinically Derived
824 Mutations in the Gene Encoding the Zinc Cluster Transcription Factor Mrr2 to Fluconazole
825 Antifungal Resistance and *CDR1* Expression in *Candida albicans*. Antimicrob Agents Chemother.
826 2019 May;63(5):e00078-19.

827 79. MacPherson S, Larochelle M, Turcotte B. A Fungal Family of Transcriptional Regulators: the Zinc
828 Cluster Proteins. Microbiol Mol Biol Rev. 2006 Sep;70(3):583–604.

829 80. Junier A, Weeks A, Alcaraz Y, Kumamoto CA. Bypass of Dfi1 Regulation of Candida albicans Invasive
830 Filamentation by Iron Limitation. Mitchell AP, editor. mSphere. 2022 Feb 23;7(1):e00779-21.

831 81. Zhang JD, Ruschhaupt M, Biczok R. ddCt method for qRT–PCR data analysis. Citeseer.
832 2013;48(4):346–56.

833 82. Alizadeh F, Department of Microbiology, Yasooj Branch, Islamic Azad University, Yasooj, Iran.,
834 Khodavandi A, Department of Biology, Gachsaran Branch, Islamic Azad University, Gachsaran, Iran,
835 Zalakian S, Department of Microbiology, Yasooj Branch, Islamic Azad University, Yasooj, Iran.
836 Quantitation of ergosterol content and gene expression profile of ERG11 gene in fluconazole-
837 resistant Candida albicans. Curr Med Mycol. 2017 Mar 1;3(1):13–9.

838 83. Santos JRA, Gouveia LF, Taylor ELS, Resende-Stoianoff MA, Pianetti GA, César IC, et al. Dynamic
839 Interaction between Fluconazole and Amphotericin B against Cryptococcus gattii. Antimicrob Agents
840 Chemother. 2012 May;56(5):2553–8.

841 84. White SJ, Rosenbach A, Lephart P, Nguyen D, Benjamin A, Tzipori S, et al. Self-Regulation of Candida
842 albicans Population Size during GI Colonization. Cormack BP, editor. PLoS Pathog. 2007 Dec
843 7;3(12):e184.

844 85. Illumina. BCL Convert: a proprietary Illumina software for the conversion of BCL files to basecalls.
845 2021;

846 86. Li H. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. ArXiv Prepr
847 ArXiv13033997. 2013;

848 87. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The Genome Analysis
849 Toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. Genome Res.
850 2010 Sep;20(9):1297–303.

851 88. Cassel JB. Wolfram| Alpha: a computational knowledge “Search” engine. Google It Total Inf Aware.
852 2016;267–99.

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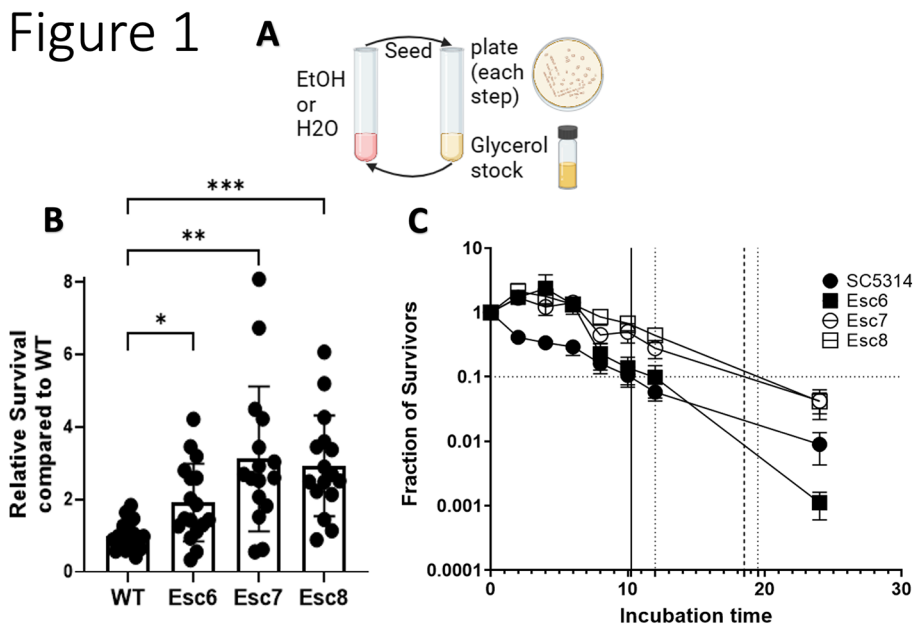
856 **Figures and Legends**



857

858 **Figure 1 Isolation of Ethanol Tolerant Strains. A)** The repeated ethanol exposure protocol is
859 illustrated. 10^6 cells of *C. albicans* strain SC5314 from 5 different biological replicate populations
860 were inoculated into medium A with increasing concentrations of ethanol (1-10% vol/vol) or
861 medium A diluted with water. Populations were exposed to 8 passages in ethanol or paired
862 mock-exposed populations exposed to 8 passages in media with water. After each 4 hour
863 ethanol or water exposure step, cells were seeded into YPD for recovery, and cultures were
864 plated onto YPD-agar for examination of the populations. Recovery cultures were grown to post-
865 exponential phase, and a glycerol stock was made. Cells from the recovery culture were then
866 used for the next step. **B)** Relative survival of ethanol small colony (Esc) strains following a 4-
867 hour ethanol exposure relative to WT ethanol survival was measured as described in Materials
868 and Methods. Symbols show results from biological replicate cultures, bars show mean with
869 standard deviation. Outliers were removed through ROUT, Q=1%, and statistical significance
870 calculated by Brown-Forsythe and Welch ANOVA test. Each group represents 16-18 biological
871 replicate cultures. (*, $p < 0.0170$; **, $p < 0.0037$; ***, $p < 0.0004$) **C)** Fraction of survivors was

872 measured for 8-16 biological replicate cultures for each point. Timepoints taken were 0, 2, 4, 6,
873 8, 10, 12, or 24 hours of incubation time in 10% ethanol. Error bars show standard error for
874 each timepoint. Horizontal line represents 90% death. Minimum duration of killing 90% (MDK90)
875 values are plotted as vertical lines with solid black line representing WT and dashed lines
876 representing Esc strains.

877

Figure 2

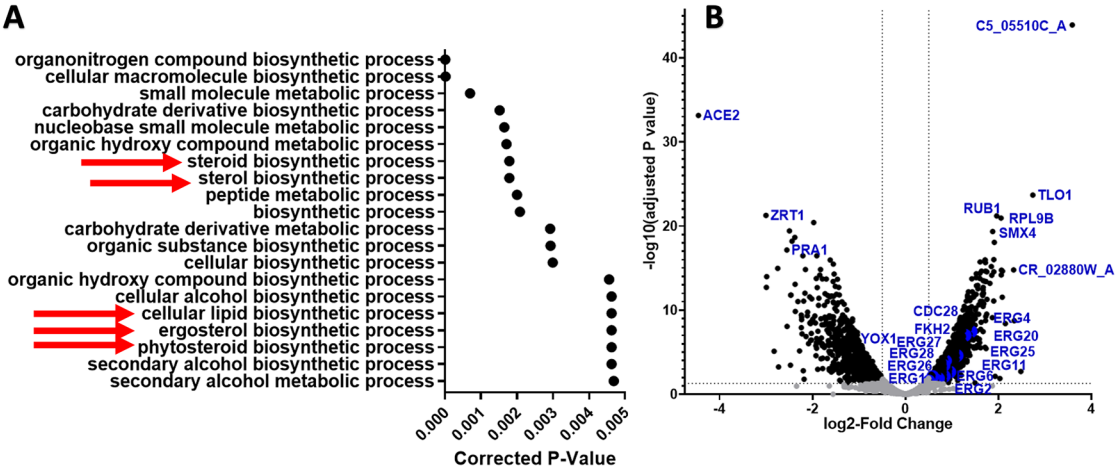


Figure 2: Differential gene expression in Esc7 versus SC5314 in 10% Ethanol. **A)** Transcripts expressed more highly in Esc7 in 10% ethanol compared to SC5314 were analyzed using GO term search on Candida Genome Database. Enriched processes are listed. The X axis shows corrected p-value for the top 20 hits (from most significant at top and descending statistical significance moving to the bottom) (triangles). Red arrows indicate processes related to lipid or ergosterol synthesis. **B)** Volcano plot representing differentially expressed genes in Esc7 in ethanol compared to SC5314 in ethanol. Log2-Fold change in transcript abundance is shown on the x-axis and –log10 adjusted p-value is shown on the y-axis. RNA-seq was performed with 3 biological replicates for each condition. Genes of interest are labeled in blue and blue dots show ergosterol synthesis genes. Significant increases or decreases in transcript abundance in Esc7 shown in black dots, nonsignificant shown in gray dots.

Figure 3

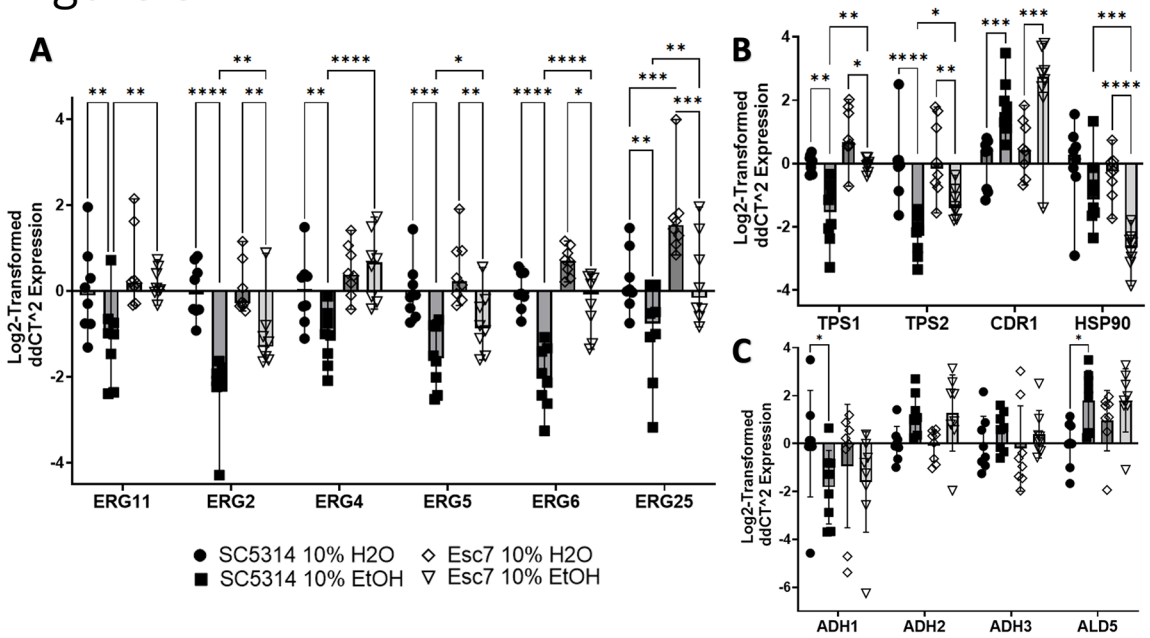


Figure 3: Differential gene expression in Esc7 versus SC5314 with and without ethanol. 8 biological replicate cultures were grown for 4 hours in medium A with 10% ethanol or water. Gene expression was measured by RT-qPCR. Expression was normalized to expression of *SPL1* (tRNA splicing gene that had unchanged expression in all conditions based on RNA-seq). Genes are indicated on the x-axis and Log_2-fold change in expression is shown relative to SC5314 in 10% water on the y-axis. **A)** Genes of the ergosterol pathway. **B)** Genes/pathways implicated in *S. cerevisiae* ethanol tolerance. **C)** Genes in the ethanol catabolic pathway. Each symbol shows results from an individual culture, error bars show standard deviation, and statistical significance for each of the RT-qPCR figures was tested by Fisher's LSD test. Only significant differences within one variable comparisons are shown. (*, p< 0.455, **, p< 0.0063, ***, p< 0.0004, ****, p< 0.0001)

Figure 4

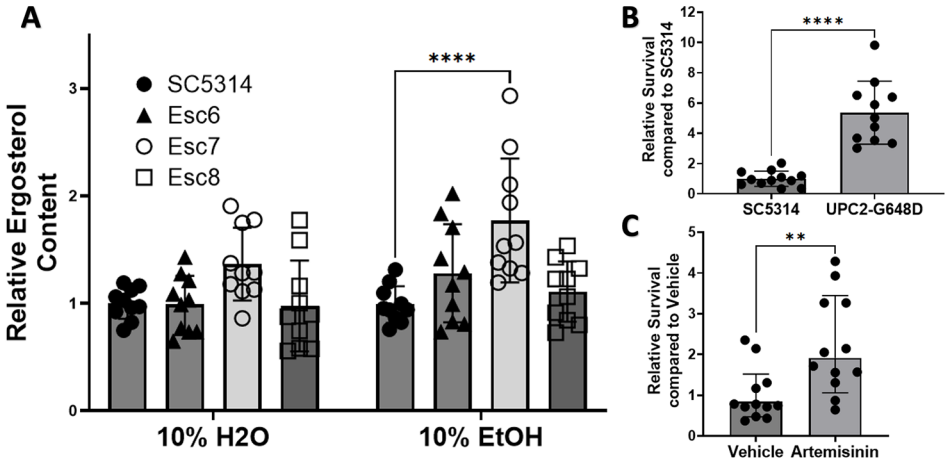
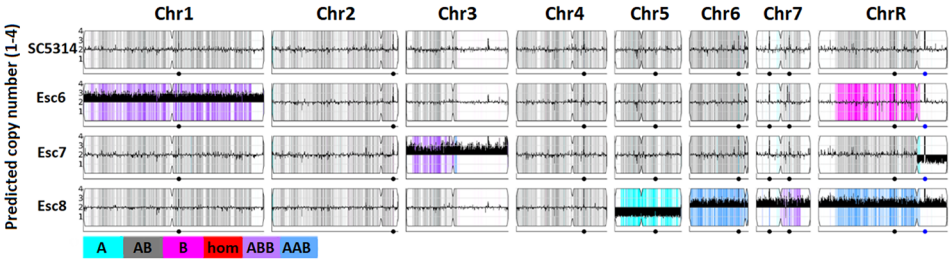


Figure 4: Cells with increased ergosterol content show increased ethanol tolerance. A) Cells were grown for 4 hours in medium A with 10% ethanol or water and harvested. Ergosterol was measured as a percentage ergosterol by mass normalized to wet-pellet weight calculated as previously described ([43,82,83] and Materials and Methods). Relative ergosterol content was calculated by standardizing ergosterol % within each experiment to SC5314 in 10% H2O. 4 separate experiments with 10 total biological replicate cultures for each condition are shown. Symbols show individual replicate values relative to SC5314 within the condition and bars show means with standard deviation (****, $p < 0.0001$). Statistical significance calculated with 2way ANOVA with Dunnett's multiple comparison, only significant comparisons are shown. **B)** Ethanol survival was measured after a 4-hr exposure of SC5314 or SC5314 with a *UPC2* gain of function mutation (G648D) [(45)]. Relative survival was standardized to SC5314 survival within each experiment. 11-12 biological replicates total from 3 different experiments are shown. One high outlier was removed from UPC2-G648D by ROUT (Q=1%). Symbols show individual replicate cultures and bars show mean with standard deviation (****, $p < 0.0001$). Statistical significance calculated by unpaired t-test. **C)** Ethanol survival from SC5314 cells pre-treated for 4 hrs with DMSO or DMSO with 100ug/mL of Artemisinin was measured. Each condition has 12 biological replicates. Ethanol exposure and standardization are the same as in B. Symbols show

922 standardized ethanol survival of individual replicates and bars show median survival with error
923 bars representing 95% confidence intervals. Statistical significance was tested using a Mann-
924 Whitney test (**, $p<0.0053$).

925

Figure 5

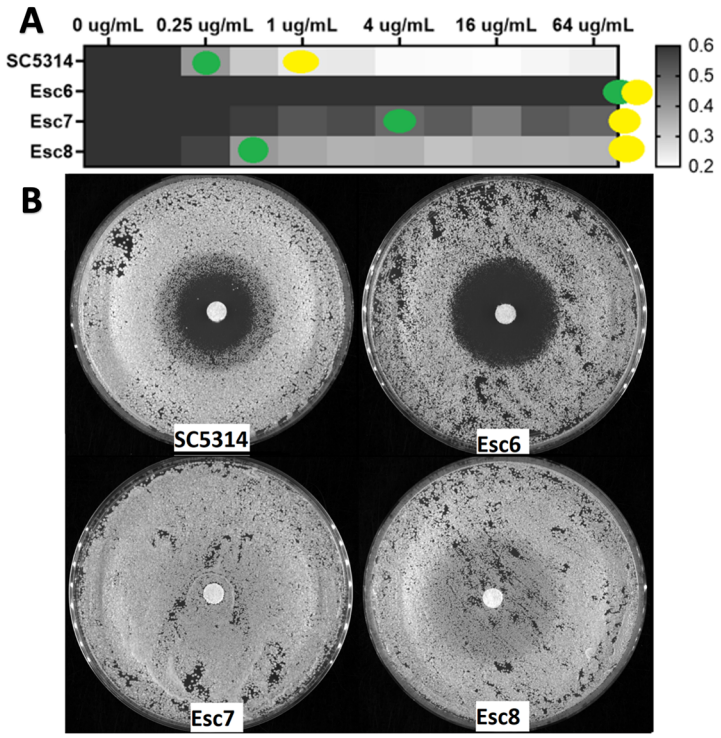


926

927 **Figure 5: Chromosome copy numbers in Esc strains.** Whole genome sequencing of
928 SC5314, Esc6, 7, and 8 was conducted as described in Materials and Methods. Copy number
929 estimates were determined based on read depth and estimated copy number is plotted along
930 chromosomes using YMAP[51]. C. albicans is diploid so euploid chromosomes show an
931 average copy number of 2 on the Y-axis as shown for SC5314. Thick black bars denote
932 aneuploidies, shown by predicted copy number greater than or less than 2. Images from YMAP
933 [(51)].

934

Figure 6



935

936 **Figure 6: Esc strains exhibit altered fluconazole susceptibility**. **A)** Strains were grown in
937 liquid cultures containing increasing concentrations of fluconazole as described in Materials and
938 Methods. Growth was detected after a 24 hour incubation using the XTT assay for metabolic
939 activity. Metabolic activity relative to the 0ug/mL well is shown as a heat map. Yellow dots show
940 the well that corresponds to MIC70 and green dots show MIC50. Median metabolic activity
941 values from 6 biological replicates performed in 6 separate experiments are shown. **B)** Strains
942 were grown to post exponential phase in YPD liquid and then used to seed the disk diffusion
943 plates. Disk diffusion assays were performed on YPD agar with 10^5 cells/plate with 25ug
944 fluconazole disks incubated for 48 hours at 30ºC. Images were taken using a Syngene Imager.
945 Representative images from an experiment repeated 4 times are shown.

946

947 **Supplemental Information**

948 **Supplemental Figure S1: Quantification of small colony variants throughout passaging of**
949 **four separate populations of cells that were exposed repeatedly to increasing**
950 **concentrations of ethanol in medium A or repeatedly exposed to water in medium A.**

951 **Supplemental Figure S2: Enc and Wnc survival in 10% ethanol**.

952 **Supplemental Figure S3: Principal component analyses for SC5314 vs Esc6, Esc7, or**
953 **Esc8 in 10% ethanol from RNA sequencing**.

954 **Supplemental Figure S4: Gene Ontology Terms of the Top 20 Processes of Significantly**
955 **Enriched Transcripts in Esc strains exposed to ethanol compared to SC5314 exposed to**
956 **ethanol**..

957 **Supplemental Figure S5: Histogram Fit Curve of Esc6 Allele Depth Ratio.**

958 **Supplemental Figure S6: MIC50 values from broth-microdilution experiments of SC5314**
959 **and Esc6, Esc7, and Esc8**.

960 **Supplemental Figure S7: UPC2 to HSP90 Ratio of SC5314 vs Esc7 in different conditions**.

961 **Supplemental Table S1 Transcripts showing significant changes in abundance between**
962 **SC5314 with ethanol vs water medium by RNASeq**
963 **Supplemental Table S2 Transcripts showing significant changes in abundance between**
964 **Esc7 with ethanol vs water medium by RNASeq**
965 **Supplemental Table S3 Transcripts showing significant changes in abundance between**
966 **Esc7 with ethanol vs SC5314 with ethanol medium by RNASeq**
967 **Supplemental Table S4 Transcripts showing significant changes in abundance between**
968 **Esc7 with water vs SC5314 with water medium by RNASeq**

969 **Supplemental Table S5 Transcripts showing significant changes in abundance between**

970 **Esc6 with ethanol vs water medium by RNASeq**

971 **Supplemental Table S6 Transcripts showing significant changes in abundance between**

972 **Esc8 with ethanol vs water medium by RNASeq**

973 **Supplemental Table S7 Transcripts showing significant changes in abundance between**

974 **Esc6 with ethanol vs SC5314 with ethanol medium by RNASeq**

975 **Supplemental Table S8 Transcripts showing significant changes in abundance between**

976 **Esc8 with ethanol vs SC5314 with ethanol medium by RNASeq**

977 **Supplemental Table S9 Transcripts showing significant changes in abundance between**

978 **Esc6 with water vs SC5314 with water medium by RNASeq**

979 **Supplemental Table S10 Transcripts showing significant changes in abundance between**

980 **Esc8 with water vs SC5314 with water medium by RNASeq**

981 **Supplemental Table S11 Esc6 Genomic Polymorphisms**

982 **Supplemental Table S12 Esc7 Genomic Polymorphisms**

983 **Supplemental Table S13 Esc8 Genomic Polymorphisms**

984 **Supplemental Table S14: Primers used in this study.**

985