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Set1-mediated histone H3K4 methylation is required for azole induction of the ergosterol biosynthesis genes and antifungal drug resistance in *Candida glabrata*.

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26 **ABSTRACT**

27 *Candida glabrata* is an opportunistic pathogen that has developed the ability to
28 adapt and thrive under azole treated conditions. The common mechanisms that can
29 result in *Candida* drug resistance are due to mutations or overexpression of the drug
30 efflux pump or the target of azole drugs, Cdr1 and Erg11, respectively. However, the
31 role of epigenetic histone modifications in azole-induced gene expression and drug
32 resistance are poorly understood in *C. glabrata*. In this study, we show for the first time
33 that Set1 mediates histone H3K4 mono-, di-, and trimethylation in *C. glabrata*. In
34 addition, loss of *SET1* and histone H3K4 methylation results in increased susceptibility
35 to azole drugs in both *C. glabrata* and *S. cerevisiae*. Intriguingly, this increase in
36 susceptibility to azole drugs in strains lacking Set1-mediated histone H3K4 methylation
37 is not due to altered transcript levels of *CDR1*, *PDR1* or Cdr1's ability to efflux drugs.
38 Genome-wide transcript analysis revealed that Set1 is necessary for azole-induced
39 expression of 12 genes involved in the late biosynthesis of ergosterol including *ERG11*
40 and *ERG3*. Importantly, chromatin immunoprecipitation analysis showed that histone
41 H3K4 trimethylation was detected on chromatin of actively transcribed *ERG* genes.
42 Furthermore, H3K4 trimethylation increased upon azole-induced gene expression which
43 was also found to be dependent on the catalytic activity of Set1. Altogether, our findings
44 show that Set1-mediated histone H3K4 methylation governs the intrinsic drug resistant
45 status in *C. glabrata* via epigenetic control of azole-induced *ERG* gene expression.

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49 **IMPORTANCE**

50 *C. glabrata* is the second most commonly isolated species from *Candida* infections,
51 coming in second to *C. albicans*. Treatment of *C. glabrata* infections are difficult due to
52 their natural resistance to antifungal azole drugs and their ability to adapt and become
53 multidrug resistant. In this study, we investigated the contributing cellular factors for
54 controlling drug resistance. We have determined that an epigenetic mechanism governs
55 the expression of genes involved in the late ergosterol biosynthesis pathway, an
56 essential pathway that antifungal drugs target. This epigenetic mechanism involves
57 histone H3K4 methylation catalyzed by the Set1 methyltransferase complex
58 (COMPASS). We also show that Set1-mediated histone H3K4 methylation is needed for
59 expression of specific azole induced genes and azole drug resistance in *C. glabrata*.
60 Identifying epigenetic mechanisms contributing to drug resistance and pathogenesis
61 could provide alternative targets for treating patients with fungal infections.

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69 INTRODUCTION

70 *Candida* infections are a major health concern due to the increased frequency of
71 infections and the development of drug resistance (1, 2). Over the years, *Candida*
72 *glabrata* has become the second most common cause of candidiasis (1-3). In some
73 immunocompromised patients, such as diabetics, patients with hematologic cancer,
74 organ transplant recipients, and the elderly, it is the most predominate *Candida* infection
75 (2-6). The emergence of *C. glabrata* as a major pathogen is likely due to its intrinsic
76 drug resistance to azole antifungal drugs and ability to quickly adapt and acquire clinical
77 drug resistance during treatment (3, 7). The consequence of drug resistance leads to
78 increases in healthcare costs as well as lower success rates in treatment and an
79 increase in mortality (8-10).

80 *C. glabrata* naturally has low susceptibility to azole drugs and because of this
81 attribute, echinocandins are the preferred drug choice for treating *C. glabrata* infections
82 (11). *C. glabrata* can also acquire clinical resistance to azole drugs which is often due to
83 overexpressing the ABC-transporter drug efflux pump Cdr1 or Pdh1 (Cdr2) caused by
84 gain of function mutations in the transcription factor Pdr1 (7, 12-14). In other *Candida*
85 species, acquired clinical azole resistance can also be due to overexpression of *ERG11*
86 due to gain of function mutations in the Upc2 transcription factor or mutations in *ERG11*
87 (15-17). However, for unbeknownst reasons, *ERG11* or *UPC2* mutations are typically
88 not found in clinically drug resistant *C. glabrata* strains (7, 18-20).

89 Because pathogenic fungi can rapidly adapt to various cellular environments and
90 xenobiotic drug exposures, epigenetic mechanisms are also likely contributing to altered
91 gene expression profiles permissive for adaptation and drug resistance. Several studies

92 in *C. albicans* support this hypothesis and show that epigenetic factors such as histone
93 acetyltransferases, *CaGcn5* and *CaRtt109*, and histone deacetylases, *CaRpd3* and
94 *CaHda1* are important for either fungal pathogenesis and/or drug resistance (21-25). In
95 contrast, epigenetic factors that post-translationally modify histones have not been
96 extensively studied for their roles in drug resistance in *C. glabrata*. Nonetheless, Orta-
97 Zavalz et al., have shown that deleting histone deacetylase, *CgHST1* decreases
98 susceptibility to fluconazole which is likely attributed to an increase in transcript levels of
99 *CgPDR1* and *CgCDR1* under untreated conditions (26). In addition, a recent publication
100 by Filler et al., has indicated that *C. glabrata* strains that are deleted for *GCN5*, *RPD3*,
101 or *SPP1* have increased susceptibility to caspofungin when using high concentrations
102 (27). However, no mechanistic understanding such as gene targets or changes in
103 chromatin/histone modifications was provided for the caspofungin hypersensitive
104 phenotype.

105 Previous publications from our lab demonstrated that in *S. cerevisiae* loss of
106 *Set1*, a known histone H3K4 methyltransferase, has a hypersensitive growth defect in
107 the presence of the antifungal metabolite, brefeldin A (BFA) and clinically used azole
108 drugs (28, 29). We determined that hypersensitivity to BFA was due to a decrease in
109 ergosterol levels in *S. cerevisiae* strains lacking histone H3K4 methylation. However,
110 until this study, no mechanistic understanding has been provided why a strain lacking
111 *SET1* alters azole drug susceptibility. Furthermore, in *C. albicans*, loss of *SET1* appears
112 to alter virulence but not azole drug resistance (30). To determine if an increase in azole
113 susceptibility is conserved in a human fungal pathogen closely related to *S. cerevisiae*,

114 we investigated the role of Set1 and its mechanistic contribution to drug resistance in *C.*
115 *glabrata*.

116 In this study, we show for the first time that Set1-mediates histone H3K4 mono-,
117 di-, and trimethylation in *C. glabrata* and loss of Set1-mediated histone H3K4
118 methylation alters the azole drug susceptibility of *C. glabrata* similar to what is seen in
119 *S. cerevisiae*. This increase in susceptibility to azole drugs in *C. glabrata* strains lacking
120 Set1-mediated histone H3K4 methylation is not a consequence of altered expression
121 levels of *CDR1*, *PDR1* or their ability to efflux drugs. Interestingly, RNA-sequencing
122 (RNA-seq) revealed that Set1 is required for azole-induced expression of *ERG* genes,
123 including *ERG11* and *ERG3*. This azole-induced gene expression was dependent on
124 Set1 methyltransferase activity and associated with gene-specific increases in histone
125 H3K4 trimethylation on *ERG11* and *ERG3* chromatin. Overall, we have provided a
126 mechanistic understanding of why Set1 mediated histone H3K4 methylation governs the
127 intrinsic drug resistant status in *C. glabrata*. Identifying and understanding the
128 epigenetic mechanisms contributing to drug resistance will be important for the
129 development of alternative drug targets for treating patients with fungal infections.
130

131 **RESULTS**

132 **Loss of Set1-mediated histone H3K4 methylation in *S. cerevisiae* and *C. glabrata***

133 **alters azole drug efficacy.** Set1 is a known SET domain-containing lysine histone
134 methyltransferase that is conserved from yeast to humans and the enzymatic activity of
135 the SET domain catalyzes mono-, di-, and trimethylation on histone H3 at Lysine 4
136 (Lys4) (31, 32). Our previous work in *Saccharomyces cerevisiae* (*S. cerevisiae*) has
137 determined that loss of *SET1* in the BY4741 background strain results in increased
138 susceptibility to azole drugs suggesting that H3K4 methylation is necessary for
139 mediating wild-type azole drug resistance. To determine the role of histone H3K4
140 methylation in azole drug efficacy, we constructed histone H3K4R mutations in the
141 BY4741 background strain. Because *S. cerevisiae* has two genes encoding histone H3,
142 two yeast strains were constructed where a histone H3K4R mutation was integrated at
143 one histone H3 gene keeping the other gene wild-type (ScH3K4R-1) while the other
144 strain contained H3K4R mutations integrated at both histone H3 genes (ScH3K4R-2,
145 see supplemental table S1). To determine if loss of histone H3K4 methylation altered
146 azole drug sensitivity similar to a *set1* Δ (*Scset1* Δ) strain, a serial-dilution spot assay
147 was performed. Both *Scset1* Δ and ScH3K4R mutant strains were grown in synthetic
148 complete minimal media and spotted on SC agar plates with and without 8 μ g/mL
149 fluconazole (Fig. 1A). These data show that loss of histone H3 methylation by deleting
150 Sc*SET1* or mutating histone H3 where both histone H3 genes are mutated at K4
151 (ScH3K4R-2), resulted in similar azole drug hypersensitivity when compared to each
152 other (Fig. 1A). To confirm that histone H3K4 methylation was abolished in these
153 strains, western blot analysis was performed using methyl-specific antibodies to detect

154 histone H3K4 mono-, di-, and trimethylation (Fig. 1B). Histone H3 was used for a
155 loading control (Fig. 1B). As expected, histone methylation was abolished in *set1Δ* and
156 in H3K4R-2 mutation strains but not in the histone H3K4R-1 strain (Fig. 1B). Together
157 our data demonstrate that the presence of Histone H3K4 methylation is critical for
158 maintaining wild-type azole drug susceptibility.

159 To determine if an azole hypersensitive growth phenotype observed in *S.*
160 *cerevisiae* is also conserved in the human fungal pathogen *C. glabrata*, WT (*CgWT*)
161 and a *set1Δ* (*Cgset1Δ*) strain were spotted on SC agar plates with and without 16 μg/mL
162 fluconazole (Fig. 1C). Similar to what was observed in *S. cerevisiae*, deleting *SET1* in
163 *C. glabrata* 2001 (CBS138, ATCC2001) showed an increase in azole susceptibility
164 when compared to a *CgWT* strain (Fig. 1C). Additionally, the *Cgset1Δ* strain had a
165 significant growth delay in liquid growth cultures compared to *CgWT* when treated with
166 32 μg/mL fluconazole (Fig. 1E). Western blot analysis showed that deleting *CgSET1*
167 abolished all histone H3K4 mono-, di-, and trimethylation confirming that *CgSET1* is the
168 sole histone H3K4 methyltransferase in *C. glabrata* (Fig. 1D). Altogether, our results
169 show Set1-mediated histone H3K4 methylation in *S. cerevisiae* and *C. glabrata* is
170 conserved and is necessary for maintaining a wild-type resistance to azole drugs.

171 **Loss of *C. glabrata* Set1 complex members alters azole efficacy and histone H3K4**
172 **methylation.** In *S. cerevisiae*, Set1 forms a complex referred to as the Complex
173 Proteins Associated with Set1 or COMPASS. COMPASS forms a stable complex with 8
174 proteins which includes the catalytic subunit Set1, Swd1, Swd2, Swd3, Spp1, Bre2,
175 Sdc1, and Shg1 (33-35). Previous studies in *S. cerevisiae* have determined that Swd1,
176 Swd2, Swd3, Spp1, Bre2, and Sdc1 are necessary for Set1 to properly catalyze the

177 various states of histone H3K4 mono-, di, and trimethylation (33-38) . To determine if
178 COMPASS components are required to govern azole drug efficacy and Set1-mediated
179 histone H3K4 methylation in *C. glabrata*, we generated deletion strains lacking *SET1*,
180 *SPP1*, *BRE2* and *SWD1* and determined their MIC in RPMI media (Fig. 2A). Consistent
181 with our agar and liquid growth assays in Figure 1, the *Cgset1Δ* strain showed
182 increased susceptibility to fluconazole with an 8-fold difference in MIC compared to the
183 *CgWT* strain (Fig. 2A). A *Cgswd1Δ* strain showed a similar MIC as the *Cgset1Δ* strain
184 while the MIC of *Cgspp1Δ* and *Cgbre2Δ* deletion strains were 4-fold different than the
185 WT strain (Fig 2A). Furthermore, all *C. glabrata* COMPASS deletions strains showed an
186 increase in susceptibility to azole drugs on agar plates similar to *S. cerevisiae*
187 COMPASS deletion strains except for the *Scspp1Δ* which is likely due to differences in
188 the histone H3K4 methylation status (Fig. 2B, 2C, S1A, and (29, 33, 34, 36, 38).

189 Western blot analysis determined that *Cgswd1Δ* strain lacked all forms of histone
190 H3K4 methylation (Fig. 2C) which is also observed in *Cgset1Δ* and *Scset1Δ* strains
191 (Fig. 2C and 1D). In contrast, deletion of *CgSPP1* and *CgBRE2* abolished all detectable
192 levels of H3K4 trimethylation and significantly reduced the levels of histone H3K4 mono-
193 and dimethylation. Taken together, our data show that when *C. glabrata* COMPASS
194 subunits *SET1* and *SWD1* are deleted, global loss of histone H3K4 methylation is
195 observed similar to what is seen when the subunits are deleted in *S. cerevisiae* (Fig 2C
196 and (33, 34, 36, 38). However, the *Cgspp1Δ* has a total loss of histone H3K4
197 trimethylation and significant loss of histone H3K4 mono- and dimethylation similar to the
198 *Cgbre2Δ* and *Scbre2Δ* strains (Fig 2C). For unknown reasons, the pattern of histone
199 H3K4 methylation is different in the *Scspp1Δ* strain which only has a reduction in

200 histone H3K4 trimethylation but not mono- or dimethylation (33-39). Altogether, these
201 results suggest that the COMPASS complex is needed to mediate proper histone H3K4
202 methylation and WT resistance to azole drugs.

203 **The methyltransferase activity of Set1 governs azole drug efficacy in *C. glabrata*.**

204 To confirm that altered azole efficacy in the *Cgset1Δ* strain was due to loss of *SET1* and
205 not a secondary mutation, a genomic fragment containing the *CgSET1* promoter,
206 5'UTR, open reading frame, and 3'UTR was amplified by PCR and cloned into the *C.*
207 *glabrata* plasmid, pGRB2.0 (40). Because a H1017K mutation in the SET domain of *S.*
208 *cerevisiae* Set1 is known to be catalytically inactive (28, 41, 42), we performed site-
209 directed mutagenesis on pGRB2.0-*CgSET1* and generated an analogous mutation in *C.*
210 *glabrata* Set1 at H1048K determined using the sequence alignment in Fig. 3A.

211 Additionally, we deleted *SET1* in *C. glabrata* 2001HTU (ATCC200989) to utilize the *ura3*
212 auxotrophic marker (43). Importantly, *Cg2001HTU* lacking *SET1* was hypersensitive to
213 azole drugs similar to when *SET1* was deleted in *Cg2001* (Fig. 1C and 3B).

214 Furthermore, transformation of pGRB2.0-*CgSET1* into the *Cg2001HTU/set1Δ* strain
215 was able to rescue azole hypersensitivity while pGRB2.0-*Cgset1H1048K* did not rescue
216 wild-type azole drug resistance as shown by serial dilution spot assays grown on SC
217 agar plates with 32 μg/mL fluconazole (Fig. 3B). MIC assays under SC-ura conditions
218 also show similar results (see Supplemental Fig. S1B). Western blot analysis indicated
219 that pGRB2.0-*CgSET1* expression in *Cg2001HTU/set1Δ* strain restored histone H3K4
220 methylation to wild-type levels while *Cgset1H1048K* did not rescue histone H3K4
221 methylation confirming that this mutation lacks catalytic activity similar to *Scset1H1017K*
222 (Fig. 3C). Importantly, quantitative real-time PCR analysis (qRT-PCR) confirmed that

223 the plasmids expressing *CgSET1* and *Cgset1H1048K* were similar to the endogenously
224 expressed *SET1* (Fig. S1C). This shows that loss of histone H3K4 methylation was not
225 due to difference in expression levels but due to the catalytic inactivation of
226 *Cgset1H1048K*. These data suggest that altered azole drug efficacy in *Cgset1Δ* strains
227 are specifically due to the loss of *SET1* and its catalytic activity.

228 **Drug efflux pump expression and function is not altered in a *C. glabrata set1Δ***
229 **strain.** In *Candida glabrata*, the major mechanisms for changes in drug resistance are
230 due to changes in expression of *CDR1*, the main drug efflux pump, or gain-of-function
231 mutations in *PDR1*, a gene that encodes the transcription factor for *CDR1* (7, 12, 19,
232 20, 44). To determine if altered drug resistance in *Cgset1Δ* cells was due to changes in
233 *CDR1* or *PDR1* expression, we analyzed the transcript levels of *CDR1* and *PDR1* via
234 qRT-PCR (Fig. 4A and B). We observed that *Cgset1Δ* cells grown with and without
235 azoles do not significantly affect transcript levels of *CDR1* or *PDR1* when compared to a
236 wild-type strain (Fig. 4A and B). Additionally, we analyzed the transcript levels of
237 transporters *SNQ2*, *YOR1*, and *PDH1*. We did not see any significant changes in *SNQ2*
238 or *YOR1*, but we did see a decrease in *PDH1* transcripts in a *set1Δ* strain upon azole
239 treatment (Fig. S2). However, previous studies have shown loss of *PDH1* alone is not
240 sufficient to lead to azole sensitivity (45). To determine if drug efflux was functional in
241 *Cgset1Δ* cells, a Nile Red fluorescence-based assay was performed. Nile Red, a
242 fluorescent lipophilic stain, has been shown to be a substrate for the ABC transporter
243 Cdr1 in *C. albicans* and *C. glabrata* (46, 47). As a control, we also generated a *Cgpdr1Δ*
244 strain, a deletion strain known to disrupt the expression of *CDR1* and subsequently
245 prevent drug or Nile Red efflux (15). The Nile Red assay showed that *Cgset1Δ* cells had

246 similar levels of Nile Red as wild-type cells but less Nile Red than *Cgpd1Δ* cells (Fig.
247 4C). To induce *CDR1* expression levels, *Cgset1Δ* and wild-type cells were treated with
248 fluconazole. Although azole treatment did reduce the amount of Nile Red in *Cgset1Δ*
249 and wild-type cells compared to untreated cells, there was no discernable differences
250 observed between *Cgset1Δ* and wild-type cells for their ability to efflux Nile Red, (Fig.
251 4C). Altogether these data suggest that cells lacking *SET1* have similar efflux
252 capabilities as wild-type cells in the presence or absence of azole treatment. This
253 suggests that the increase in azole sensitivity seen in a *Cgset1Δ* strain is not due to
254 malfunction of *Cdr1* expression or its efflux capabilities.

255 **Loss of *Set1* leads to decreased expression of genes involved in the sterol**
256 **biosynthesis pathway when treated with fluconazole.** Because drug efflux function
257 or transcript levels was not disrupted in a *Cgset1Δ* strain, we used RNA-sequencing
258 analysis to provide insight into what gene pathway might be disrupted in the *Cgset1Δ*
259 strain and explain why a loss of *SET1* alters azole drug efficacy. *CgWT* and *Cgset1Δ*
260 strains were treated with 64 µg/mL fluconazole for three hours in SC complete media
261 and RNA was extracted for RNA-sequencing. Principle component analysis (PCA) and
262 differentially expressed genes (DEG) analysis demonstrated by the volcano scatter plot
263 ($-\log_2$ false discovery rate (FDR), y-axis) versus the fold change (x-axis) of the DEGs)
264 indicate that the untreated and treated *CgWT* strain is substantially and statistically
265 different from the untreated and treated *Cgset1Δ* (Fig 5). DESeq2 analysis was used to
266 identify the differentially expressed genes (DEGs) under fluconazole treatment using an
267 FDR of 0.05. From this analysis, a total of 2389 genes were differentially expressed in
268 *Cgset1Δ* vs. *CgWT* under untreated condition (Fig. 5B). Whereas, 1508 genes were

269 differentially expressed under treated conditions, where we observed 800 (14.2%)
270 genes that were upregulated and 708 (12.6%) genes that were downregulated out of
271 5615 genes in *Cgset1Δ* compared to *CgWT* (Fig. 5C and supplemental data). After
272 applying a 1.4-fold cutoff to the data, we observed 1,644 genes differentially expressed
273 in the untreated *Cgset1Δ* vs. *CgWT* strains. In the treated strains, with a 1.4-fold cutoff,
274 we observed 543 (9.7%) genes were down in a *Cgset1Δ* vs *CgWT* and 626 (11.1%)
275 genes were up. These data show that *SET1* is important for maintaining proper gene
276 expression in *C. glabrata*.

277 Because Set1-mediated histone H3K4 methylation is known to play a key role in
278 gene activation, we focused our attention on genes downregulated in *Cgset1Δ*
279 compared to *CgWT*. For azole-treated strains. GO Term Finder of the gene sets that
280 were downregulated found significant GO terms involved in lipid, steroid and
281 sterol/ergosterol metabolism or biosynthesis (Fig. 5D). For untreated strains, GO Term
282 Finder identified significant GO terms involved in lipid metabolism but not steroid and
283 sterol/ergosterol metabolism or biosynthesis (supplemental table S6). Interestingly, our
284 data showed that 12 of the 12 genes involved in the late ergosterol biosynthesis
285 pathway are down 1.4-fold or more in a *Cgset1Δ* compared to *CgWT* under azole
286 treated conditions (Fig. 5C, S4A & B and supplemental table S7). Whereas, 5 of the 12
287 late pathway *ERG* enzyme encoding genes were down in a *Cgset1Δ* compared to
288 *CgWT* under untreated conditions using a 1.4-fold difference in gene expression as a
289 cutoff (Fig. 5D. and Supplemental table S7). Two of these differentially expressed genes
290 *ERG11*, the gene that encodes the target of azoles, and *ERG3*, the gene that encodes
291 the enzyme responsible for production of a toxic sterol when cells are treated with

292 azoles, are known to play roles in azole drug resistance in various *Candida* species (17,
293 19, 48-50). To validate results seen in RNA-sequencing analysis, *ERG11* and *ERG3*
294 transcript levels were analyzed by qRT-PCR. Our analysis showed that upon azole
295 treatment, *ERG3* and *ERG11* transcript levels are induced in a WT strain (Fig. 5C and
296 D) while loss of *SET1* prevented WT induction of both *ERG11* and *ERG3* under azole
297 conditions. Even though our untreated RNA sequencing data set did show minor
298 changes in *ERG3* and *ERG11* transcript levels, we did not detect any significant
299 changes between *Cgset1Δ* and *CgWT* cells when grown under untreated standard log-
300 phase conditions using qRT-PCR analysis (Fig. 5E and F). We also performed gene
301 expression analysis to determine if *ERG* gene transcript induction still depended on
302 Set1 in saturated cultures. We show in both exponential and saturated cultures Set1 is
303 necessary for *ERG3* and *ERG11* induction upon azole treatment in *C. glabrata* (Fig.
304 5E&F and S3A&B). Because *ERG3* transcript levels were decreased, we do not
305 anticipate azole sensitivity is due to an increase in toxic sterols but by the lack of
306 induction of *ERG11* and other *ERG* genes resulting in lower total cellular ergosterol
307 levels (51, 52).

308 **Set1-mediated histone H3K4 methylation is enriched on *ERG* gene**
309 **chromatin and is required for azole induction of *ERG* genes.** Because histone
310 H3K4 trimethylation is associated with gene induction, we wanted to determine if Set1
311 was directly catalyzing histone H3K4 methylation on chromatin at *ERG* loci. To
312 determine if histone H3K4 trimethylation was present at *ERG11* and *ERG3* chromatin,
313 chromatin immunoprecipitation (ChIP) analysis was performed using histone H3K4
314 trimethyl-specific antibodies. As expected, histone H3K4 trimethylation is highly

315 enriched at the 5'-ends of the open reading frame of *ERG11* and *ERG3* in untreated
316 conditions and further enriched upon azole treatment corresponding to increased
317 transcript levels of *ERG11* and *ERG3* in both exponential and saturated cell cultures
318 (Fig. 6A & B and S3D & E).

319 To confirm that this was due to the methyltransferase activity of Set1, we
320 performed qRT-PCR transcript analysis using the *Cg2001HTUset1Δ* strain expressing
321 pGRB2.0 only, pGRB2.0-*CgSET1*, and pGRB2.0-*Cgset1H1048K*. *Cg2001HTU*
322 expressing pGRB2.0 only was used as our WT control. As shown in Figure 6C and D,
323 pGRB2.0-*CgSET1* was able to induce *ERG11* and *ERG3* similar to WT cells under
324 azole treatment indicating that *SET1* expression could rescue the *ERG* gene expression
325 in the *Cg2001HTUset1Δ* strain. This rescue of *ERG* gene expression was dependent on
326 the catalytic activity of Set1 since expression of pGRB2.0-*Cgset1H1048K* did not
327 restore *ERG* gene expression under azole treatment. Additionally, it looked similar to
328 the *Cg2001HTUset1Δ* strain expressing pGRB2.0 indicating that the catalytic activity of
329 Set1 is required for azole gene induction. Altogether, these data show that Set1-
330 mediated histone H3K4 methylation directly targets the chromatin of *ERG* genes, and
331 this epigenetic modification is required for azole induction of *ERG* genes. Based on our
332 results, the lack of Set1 or histone H3K4 methylation on *ERG11* chromatin prevents the
333 transcriptional response for inducing *ERG* genes which consequently disrupts
334 ergosterol homeostasis, thus making the *Cgset1Δ* strains more susceptible to azole
335 drugs.

336

337

338 DISCUSSION

339 In this study, we established that loss of Set1-mediated histone H3K4
340 methylation alters azole drug susceptibility in *S. cerevisiae* and *C. glabrata*. This
341 increase in susceptibility to azole drugs in a *Cgset1Δ* strain was not because of the
342 typical changes in *CDR1* and *PDR1* expression levels or their ability to efflux drugs.
343 However, we observed that strains lacking histone H3K4 methylation failed to induce
344 *ERG* genes. This azole-induced gene expression was dependent on Set1
345 methyltransferase activity and correlated with gene-specific increases in histone H3K4
346 trimethylation on chromatin at *ERG* genes (see model, Fig. 7). Overall, we have
347 provided an epigenetic mechanism upon azole treatment that is dependent on histone
348 H3K4 methylation governing ergosterol homeostasis. Identifying and understanding this
349 Set1-*ERG* pathway and other epigenetic mechanisms contributing to altered drug
350 susceptibility will be important for the development of alternative drug targets that could
351 be used in combinatorial therapy for treating patients with drug resistant fungal
352 infections.

353 Set1 is the catalytic subunit of a multi-subunit protein complex called COMPASS
354 that mono-, di-, and trimethylates histone H3K4. In this study, we show that *C. glabrata*
355 Set1 is the sole histone H3K4 methyltransferase under log-phase growth conditions
356 since deletion of *SET1* abolishes all forms of histone H3K4 methylation similar to what
357 is seen in *S. cerevisiae* and *C. albicans*. Deletion of the genes encoding *C. glabrata*
358 COMPASS complex subunits Swd1 and Bre2 have similar loss of histone H3K4
359 methylation as their *S. cerevisiae* counterparts (see Fig 2C and (33, 34, 36, 38)).
360 However, deleting *SPP1* in *C. glabrata* abolishes all histone H3K4 trimethylation and

361 significantly reduces the levels of histone H3K4 mono- and dimethylation which is in
362 contrast to what is found in a *Scspp1Δ* strain where only histone H3K4 trimethylation is
363 disrupted but retains WT levels of mono- and dimethylation (33, 34, 36, 38). We
364 speculate that this difference in histone H3K4 methylation pattern is due to how *CgSpp1*
365 assembles with the COMPASS complex allowing *CgSpp1* to have a greater impact on
366 the overall catalytic activity of COMPASS. Interestingly, this pattern of methylation
367 appears to correlate with sensitivity to azole drugs (compare Fig. 2B with supplemental
368 Fig. S1A) where *Scspp1Δ* grows more similar to a WT strain than *Cgspp1Δ* when grown
369 on azole containing plates.

370 Our published observation and current data show that loss of *SET1* alters ergosterol
371 homeostasis and azole susceptibility in *S. cerevisiae* and *C. glabrata* (28, 29).
372 Specifically, loss of *SET1* in *S. cerevisiae* altered expression of genes involved in
373 ergosterol biosynthesis under untreated conditions (28). In contrast, in *C. glabrata*,
374 significant changes in *ERG* gene expression were only observed under azole treated
375 conditions but not untreated conditions suggesting that histone H3K4 methylation is
376 needed for azole induced gene induction and not basal level expression (Fig. 5E and F).
377 Although regulation of ergosterol biosynthesis has been shown to be coupled to
378 expression of ABC transport genes such as *CDR1* and its transcription factor Pdr1 (53,
379 54), compensatory changes in *CDR1* and *PDR1* expression levels was not observed in
380 a *cgset1Δ* strain when treated with azole drugs (Fig. 4A and 4B). More investigation will
381 be needed to understand how *CDR1* and/or *PDR1* are epigenetically regulated in *C.*
382 *glabrata* (26, 55).

383 In *C. albicans*, Raman et al., reported that loss of *SET1* in did not alter azole
384 sensitivity but did decrease virulence in mice (30). Furthermore, *C. albicans* is naturally
385 more susceptible to azole drugs than *S. cerevisiae* and *C. glabrata*. We suspect the
386 difference observed in these organisms in azole sensitivity and gene regulation is likely
387 due to their differences in sterol uptake. For example, *C. glabrata* and *S. cerevisiae* can
388 uptake sterols under a variety of conditions where *C. albicans* does not (56).
389 Interestingly, loss of *SET1* in *S. cerevisiae* can also permit sterol uptake under aerobic
390 conditions since sterol transporter transcripts of *PDR11* and *AUS1* are increased in a
391 *Scset1Δ* strain (28). In contrast, *AUS1*, is constitutively expressed in *C. glabrata* under
392 aerobic and anaerobic conditions allowing sterol uptake. Even though *AUS1* is
393 constitutively expressed, we do observe a slight increase in *AUS1* transcript levels in a
394 *Cgset1Δ* relative to *CgWT* under untreated conditions but not treated conditions (see
395 supplemental data Fig. S3C). Alternatively, loss of *SET1* may not alter azole efficacy in
396 *C. albicans* because it does not regulate the expression of *ERG* genes or sterol
397 transporters. However, other epigenetic factors as indicated below are likely playing a
398 role in *C. albicans*. Nonetheless, additional studies will be needed to determine the
399 precise mechanistic cause of these distinct differences.

400 Overall, our data suggest that histone H3K4 methylation is an epigenetic
401 mechanism to help induce *ERG* gene expression when *C. glabrata* strains are exposed
402 to azole drugs. We propose histone H3K4 methylation and possibly other epigenetic
403 marks are contributing factors to *C. glabrata*'s natural resistance to azole drugs.
404 Interestingly, several histone deacetylases (HDACs) have been implemented in azole
405 resistance in *C. albicans* such as *CaHda1*, *CaRpd3*, and *CaHos2* (22, 23, 57-59).

406 Additionally, HDAC inhibitors have been shown to have a synergistic effect on cells
407 when combined with azoles and echinocandins (57, 58, 60, 61). Interestingly, the
408 treatment of *C. albicans* with trichostatin A (TSA) lacks the trailing effect observed in
409 MIC assays when using azole drugs and the lack of trailing effect was attributed to
410 reduced *CDR* and *ERG* gene expression (58, 62). In a similar manner, *Cgset1Δ* also
411 lacks a trailing effect in our MIC assays (personal observation) which we suspect is
412 specifically due to the lack of azole-induced *ERG* gene expression since *CDR1*
413 expression was not altered (Fig. 4A and 5). Furthermore, treatment of drug resistant
414 fungal pathogens including various isolates of *C. glabrata* with a Hos2 inhibitor
415 MGCD290 showed synergy with azole drugs which converted the MICs of azole
416 treatment from resistant to susceptible (60). Since Hos2 is known to be a key
417 component of the Set3 complex and the Set3 complex is recruited to chromatin via
418 Set1-mediated histone H3K4 methylation (63, 64), it is likely MGCD290 is mediating its
419 effect with azoles through inhibiting azole-induced *ERG* gene expression.

420 We expect that the Set1-*ERG* regulatory pathway controlling ergosterol
421 homeostasis will not only impact drug resistance but will also impact fungal
422 pathogenesis. For example, *C. albicans* strains lacking *ERG11* or *ERG3* produces
423 avirulent hyphae, decreases the adherence to epithelial cells, and reduces virulence of
424 *C. albicans* in oral mucosal infections and disseminated candidiasis (65-67). Similarly,
425 deletion of *SET1* in *C. albicans* also forms hyphae, decreases epithelial adherence, and
426 reduces virulence of *C. albicans* in disseminated candidiasis (30). Based on our current
427 data in *C. glabrata*, we speculate that loss of *SET1* in *C. albicans* reduces expression of
428 *ERG* genes and ergosterol production which in turn reduces epithelial adherence and

429 thus alters the virulence of *C. albicans*. Therefore, the loss of *SET1* could also alter the
430 virulence of *C. glabrata*. Interestingly, several genes encoding cell wall proteins and
431 adhesion factors are also down regulated in a *Cgset1Δ* strain as determined by RNA-
432 sequencing. However, future studies will be needed to determine if this Set1-*ERG*
433 regulatory pathway exists for *C. albicans* and if *SET1* is controlling virulence factors for
434 *C. glabrata*.

435 Overall, the occurrence of multidrug resistant strains is increasing across all
436 *Candida* species. In addition, with the development and identification of multidrug
437 resistant fungal species such as *C. auris*, a pathogen of urgent concern for the CDC, it
438 is imperative to find alternative treatment options. Our study along with others provide
439 compelling evidence that epigenetic modifiers are playing key roles in fungal
440 pathogenesis and drug resistance. Understanding these epigenetic events and the
441 pathways they impact are needed to develop new drug therapies so that current and
442 newly emerging multidrug resistant fungal pathogens can be effectively treated.

443 **MATERIALS AND METHODS**

444 **Plasmids and yeast strains**

445 All plasmids and yeast strains are described in Table S1 and S2. *C. glabrata* 2001
446 (CBS138, ATCC2001) and *C. glabrata* 2001HTU (ATCC200989) were purchased from
447 ATCC (43). A genomic fragment containing the *CgSET1* promoter, 5'UTR, open reading
448 frame, and 3'UTR was amplified by PCR and cloned into the pGRB2.0 plasmid. The
449 pGRB2.0 plasmid was purchased from Addgene. Standard, site-directed mutagenesis
450 was used to generate *Cgset1H1048K*. *Candida glabrata* *SET1*, *BRE2*, *SWD1*, *SPP1*,
451 and *PDR1* genes were deleted via standard homologous recombination. Briefly, drug

452 resistant selection markers were PCR amplified with Ultramer DNA Oligos (IDT) using
453 pAG32-HPHMX6 (hygromycin) or pAG25-NATMX6 (nourseothricin).

454 **Serial dilution spot and liquid growth assays**

455 For serial dilution spot assays, yeast strains were inoculated in SC media and grown to
456 saturation overnight. Yeast strains were diluted to an OD₆₀₀ of 0.1 and grown in SC
457 media to log phase shaking at 30°. The indicated strains were spotted in five-fold
458 dilutions starting at an OD₆₀₀ of 0.01 on untreated SC plates or plates containing 16,32,
459 or 64 µg/ml fluconazole (Sigma-Aldrich, St. Louis, MO). Plates were grown at 30° for 1-3
460 days. For growth assays, the indicated yeast strains were inoculated in SC media and
461 grown to saturation overnight. Yeast strains were diluted to an OD₆₀₀ of 0.1 and grown
462 in SC media to log phase shaking at 30°. The indicated strains were diluted to an OD₆₀₀
463 of 0.0001 in 100 µl SC media. Cells were left untreated or treated with 64 µg/ml
464 fluconazole and grown for 50 hrs shaking at 30°. The cell density OD₆₀₀ was determined
465 every 1 hr using the Bio-Tek Synergy 4 multimode plate reader.

466 **Cell extract and Western blot analysis**

467 Whole cell extraction and western blot analysis to detect histone modifications were
468 performed as previously described (36, 68). The histone H3K4 methylation-specific
469 antibodies were used as previously described; H3K4me1 (Upstate 07-436, 1:2,500),
470 H3K4me2 (Upstate 07-030, 1:10,000), H3K4me3 (Active motif 39159, 1:100,000) (28,
471 69). Histone H3 antibodies were used as our loading control (Abcam ab1791, 1:10,000).

472 **RNA-sequencing analysis**

473 The CBS138 *Cg2001* WT and *set1Δ* strains were inoculated in SC media and grown to
474 saturation overnight. Cells were diluted to an OD₆₀₀ of 0.1 and recovered to log phase

475 for 3 hours shaking at 30°. Prior to treatment, cells were collected for the untreated
476 sample and zero time point. Cultures were treated at an OD₆₀₀ of 0.2 with 64 µg/ml
477 fluconazole (Sigma-Aldrich, St. Louis, MO) dissolved in DMSO as previously described
478 (70). Cells were collected after 3 hours. Total RNA of three biological replicates for each
479 condition and sample were isolated by standard acid phenol purification, treated with
480 DNase (Ambion), and total RNA was purified using standard acid phenol purification.
481 The quality of the RNA was tested using an Agilent Bioanalyzer 2100 using the High
482 Sensitivity DNA Chip. The complementary DNA library was prepared by the Purdue
483 Genomics Facility using the TruSeq Stranded Kit with poly(A) selection (Illumina)
484 according to the manufacturer's instructions. The software Trimmomatic v.0.39 was
485 used to trim reads, removing adapters and low quality bases (71). STAR
486 v.2.5.4b was used to align reads to the *C. glabrata* CBS 138 reference genome,
487 version s02-m07-r23 (72). One mismatch was allowed per read. HTSeq v.0.6.1
488 was used to generate the gene count matrix on "intersection-nonempty" mode
489 (73). R version 3.5.1 and Bioconductor release 3.6 were used to perform all
490 statistical analyses on the RNA-seq data. The intersection of genes identified
491 as statistically significantly differentially expressed with a Benjamini-Hochberg
492 corrected false discovery rate of less than 5% by DESeq2 v.1.18.0 was used in
493 downstream analyses (74, 75).

494 **Quantitative real-time PCR analysis**

495 RNA was isolated from cells by standard acid phenol purification. Reverse transcription
496 was performed using the ABM all-in-one 5X RT Mastermix kit (ABM, Richmond,
497 Canada). Primer Express 3.0 software was used for designing primers and quantitative

498 real-time polymerase chain reaction (qRT-PCR) was performed as previously described
499 (28, 76, 77). A minimum of three biological replicates, including three technical
500 replicates, were performed for all samples. Data were analyzed using the $\Delta\Delta C_t$ method
501 where *RDN18* (18S ribosomal RNA) was used as an internal control. All samples were
502 normalized to an untreated, untagged WT strain.

503 **Minimal inhibitory concentration assay**

504 MIC assays were performed based on a modified version of the CLSI method for testing
505 yeast, 3rd edition (78). Briefly, yeast strains were inoculated in SC media and grown to
506 saturation overnight. The indicated strains were diluted to an OD₆₀₀ of 0.003 in SC or
507 RPMI media. Cells were mixed with fluconazole (Cayman Chemical) for a final volume
508 of 100 μ l per well in a 96 well polystyrene microplate with concentrations of fluconazole
509 ranging from 0-256 μ g/mL. Plates were incubated at 35°C and MICs were recorded at
510 24 hours. MICs were determined visually and were counted as wells where >90% of
511 growth was inhibited.

512 **Nile Red Assay**

513 Fluorescence-based Nile red assays were performed as previously described (46).
514 Briefly, cells were grown overnight in SC media to saturation. Cells were back diluted to
515 an O.D.₆₀₀ of 0.1 and grown for 6 hours. Cells were collected then washed with PBS
516 twice and incubated in 1.5mL of PBS+2% glucose for 1 hour. Next, 2.87 μ L of a
517 1mg/mL stock of Nile red (Sigma) was added to each sample and incubated at 30°C
518 shaking for an additional 30 minutes. Samples were washed twice with PBS and placed
519 in triplicate in a black 96-well flat-bottomed polystyrene microplate. Fluorescence was

520 detected using a Bio-Tek Synergy 4 multimode plate reader using an excitation
521 wavelength of 553nm and an emission wavelength of 636nm.

522

523 **Chromatin Immunoprecipitation**

524 ZipChIP was performed as previously described (69). Briefly, 50 ml cultures were grown
525 to log phase (OD₆₀₀ of 0.6) in SC complete media at 30° shaking. Treated cells were
526 dosed with 64µg/mL fluconazole (Cayman Chemical) at an OD₆₀₀ of 0.2 for 3 hours.
527 Additionally, cultures were grown to saturation, back diluted to an O.D.₆₀₀ of 0.6, treated
528 with 64µg/mL fluconazole for 3 hours and collected. Cells were formaldehyde cross-
529 linked and harvested as previously described (69). Cell lysates were precleared with 5
530 µl of unbound Protein G magnetic beads for 30 min rotating at 4°. A total of 12.5 µl of
531 precleared lysate was immunoprecipitated with 10 µl of Protein G magnetic beads
532 (10004D; Life Technologies) conjugated to 1 µl of Histone H3K4me3 antibody (Millipore
533 07-473) or Histone H3 antibody (Abcam ab1791). Probe sets used in qRT-PCR are
534 described in supplemental Table S5.

535

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544

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784 **FIGURE LEGENDS**

785 **FIG 1** *Loss of Set1-mediated mono-, di-, and trimethylation at histone H3K4 in*
786 *Saccharomyces cerevisiae and Candida glabrata results in increased azole*
787 *susceptibility and delayed growth in vitro.* (A) Five-fold serial dilution spot assays of
788 the indicated *S. cerevisiae* strains were grown on SC media with and without 8
789 $\mu\text{g}/\text{mL}$ fluconazole and incubated at 30°C for 72 hours. (B & D) Whole cell extracts
790 isolated from the indicated strains were immunoblotted using histone H3K4 methyl-
791 specific mono-, di- and trimethylation antibodies of whole cell extracts isolated from
792 the indicated strains. Histone H3 was used as a loading control. (C) Five-fold serial
793 dilution spot assays of the indicated *C. glabrata* strains were grown on SC media
794 with and without 32 $\mu\text{g}/\text{mL}$ fluconazole and incubated at 30°C for 48 hours. (E)
795 Liquid growth curve assay of the indicated *C. glabrata* strains grown over 50 hours
796 with or without 32 $\mu\text{g}/\text{mL}$ fluconazole.

797 **FIG 2** *Deletion of Set1 complex members in C. glabrata results in increased azole*
798 *susceptibility and loss of histone H3K4 methylation.* (A) MIC assay of the indicated
799 strains performed in RPMI 1640 media at 35°C and results recorded after 48 hours
800 of incubation. (B) Five-fold serial dilution spot assays of the indicated *C. glabrata*
801 strains were grown on SC plates with or without 32 $\mu\text{g}/\text{ml}$ fluconazole. (C) Whole
802 cell extracts isolated from the indicated strains were immunoblotted using H3K4
803 methyl-specific mono-, di- and trimethylation antibodies. Histone H3 was used as a
804 loading control.

805 **FIG 3** *The catalytic activity of the SET domain is necessary for Set1-mediated*
806 *histone H3K4 methylation and increased azole susceptibility in C. glabrata.* (A)

807 Five-fold serial dilution spot assays of the indicated *C. glabrata* strains were grown
808 on SC plates with or without 32 µg/ml fluconazole. (B) Whole cell extracts isolated
809 from the indicated strains were immunoblotted using methyl-specific mono-, di- and
810 trimethylation antibodies. Histone H3 was used as a loading control.

811 **FIG 4** *Deletion of SET1 in C. glabrata does not alter gene expression levels or*
812 *function of the efflux drug transporter, CDR1 or transcription factor PDR1. (A and B)*
813 Expression of indicated genes was determined in *Cg*WT and *Cgset1Δ* strain cells
814 treated with and without 64 µg/ml fluconazole for 3 hr by qRT-PCR analysis. Gene
815 expression analysis was set relative to the untreated wild-type and expression was
816 normalized to *RDN18* mRNA levels. Data were analyzed from ≥ 3 biological
817 replicates with three technical replicates each. Error bars represent SD. (C) Red
818 fluorescence units were measured as output in a Nile Red assay to determine the
819 efficacy of Cdr1 in the indicated strains with and without fluconazole. A *pdr1Δ* strain
820 was used as a control. Data were analyzed from ≥ 3 biological replicates with three
821 technical replicates each. Statistics were performed using Graphpad Prism student
822 t-test version 9.2.0. *ns* represents $p < 0.05$, $**p < 0.01$, Error bars represent SD.

823 **FIG 5** *The deletion of SET1 in C. glabrata alters global and local levels of gene*
824 *expression under untreated and azole conditions. The genome-wide changes in gene*
825 *expression under azoles were performed using C. glabrata CBS138 WT and set1Δ*
826 *strains. (A) The PCA for WT and set1Δ azole treated samples relative to WT untreated*
827 *samples based on the counts per million. (B) Volcano plot showing the significance*
828 *[-log₂ (FDR), y-axis] vs. the fold change (x-axis) of the DEGs identified in the WT*
829 *untreated samples relative to set1Δ untreated samples. (C) Volcano plot showing the*

830 significance [$-\log_2$ (FDR), y-axis] vs. the fold change (x-axis) of the DEGs identified in
831 the *set1Δ* azole treated samples relative to WT azole treated samples. Genes with
832 significant differential expression (FDR < 0.05) in (B and C) are highlighted in red or
833 blue for up- and downregulated genes, respectively. Black highlighted genes are
834 considered nonsignificant. (D) Genes from the RNA-seq dataset that were statistically
835 significantly enriched (FDR < 0.05) were used for GO term determination of Set1-
836 dependent DEGs under azole conditions. Downregulated genes refer to the DEGs that
837 are dependent on Set1 for activation either directly or indirectly. Significantly enriched
838 groups of GO terms were identified as the DEGs from only *set1Δ* and WT azole treated
839 samples. (E and F) Expression of indicated genes was determined in WT and *set1Δ*
840 strain cells treated with 64 μg/ml fluconazole for 3 hr by qRT-PCR analysis. Gene
841 expression analysis was set relative to the untreated wild-type and expression was
842 normalized to *RDN18* mRNA levels. Data were analyzed from ≥ 3 biological replicates
843 with three technical replicates each. Statistics were performed using Graphpad Prism
844 student t-test version 9.2.0. **** $p < 0.0001$ and ** $p = 0.002$. Error bars represent SD..

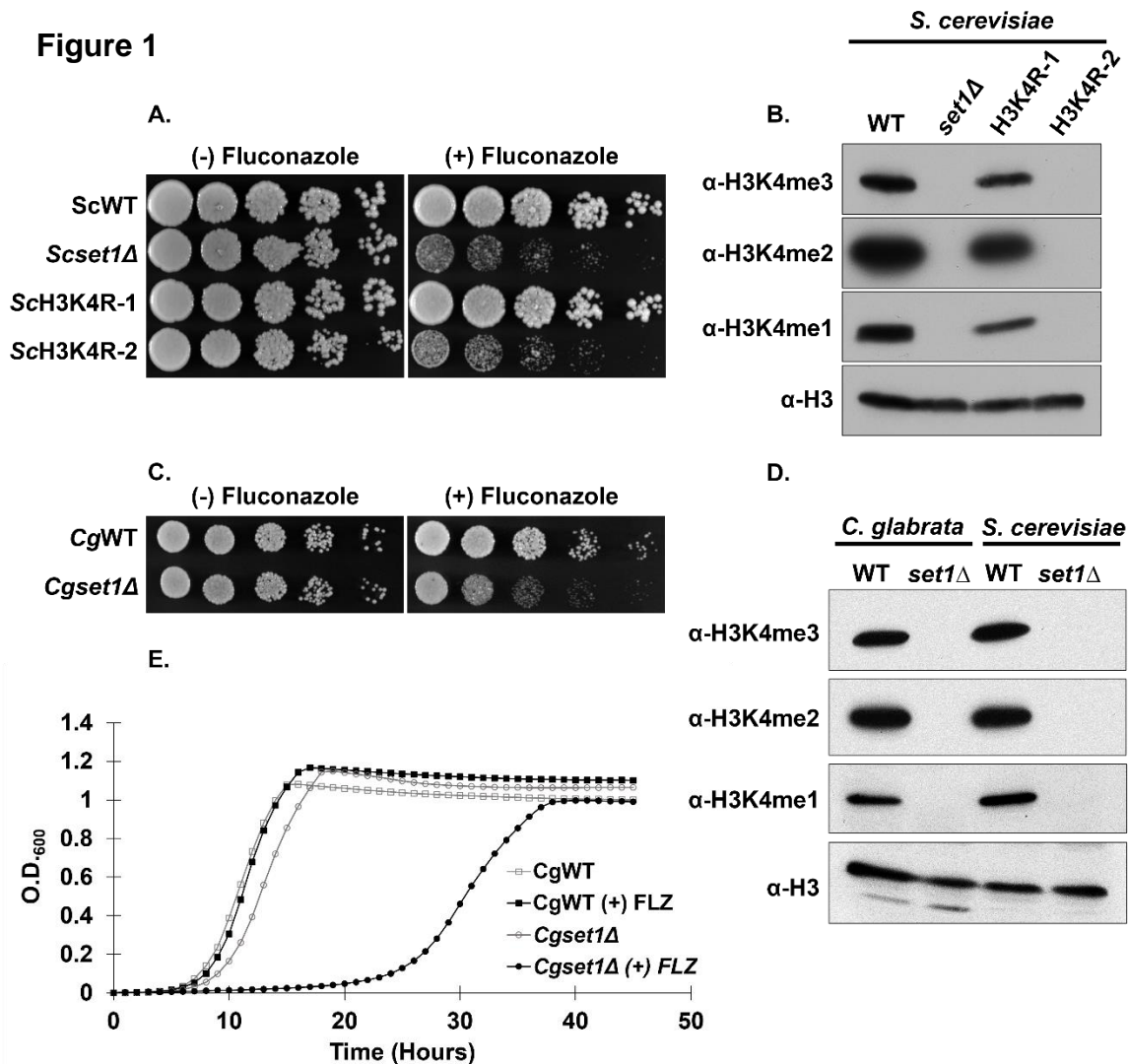
845 **FIG 6** *Histone H3K4 trimethylation is enriched on ERG gene chromatin and Set1-*
846 *mediated histone H3K4 methyltransferase activity is required for azole induction of ERG*
847 *genes. (A and B) ChIP analysis of histone H3K4 tri-methylation levels at the promoter,*
848 *5', and 3' regions of ERG11 and ERG3 in a wild-type C. glabrata strain with and without*
849 *64 μg/mL fluconazole treatment. ChIP analysis was set relative to a set1Δ strain and*
850 *normalized to histone H3 and DNA input levels. Data were analyzed from 5 biological*
851 *replicates with three technical replicates each, * $p < 0.05$. (C and D) Expression of*
852 *indicated genes was determined in the indicated mutants treated with and without 64*

853 $\mu\text{g/ml}$ fluconazole for 3 hr by qRT-PCR analysis. Gene expression analysis was set
854 relative to the untreated wild-type containing an empty vector and expression was
855 normalized to *RDN18* mRNA levels. Data were analyzed from ≥ 3 biological replicates
856 with three technical replicates each. Statistics were performed using Graphpad Prism
857 student t-test version 9.2.0. **** $p < 0.0001$ and ** $p < 0.01$. Error bars represent SD.

858 **FIG 7** *Model for the role of Set1-H3K4 methylation in epigenetic control of ERG genes*
859 (Biorender). (A) Under aerobic conditions, the Set1 complex mediates histone H3K4
860 methylation on chromatin at *ERG* genes. In the presence of azoles, azole-induced
861 transcriptional activation recruits TFs, RNA Polymerase II, and the Set1 complex to
862 increases Histone H3K4 methylation. This increase in methylation could permit
863 additional recruitment of other co-factors/epigenetic regulator (e.g., Set3 and/or SAGA
864 complex) that contain “reader” domains that recognize and bind to the H3K4 methyl
865 mark. Thus, this Set1-Erg pathway contributes to the intrinsic azole resistance in *C.*
866 *glabrata*. In the absence of Set1, histone H3K4 methylation is abolished and failure of
867 recruiting additional H3K4 methyl “readers” prevent the induction of *ERG* genes, thus
868 making the *C. glabrata* more susceptible to azole treatment.

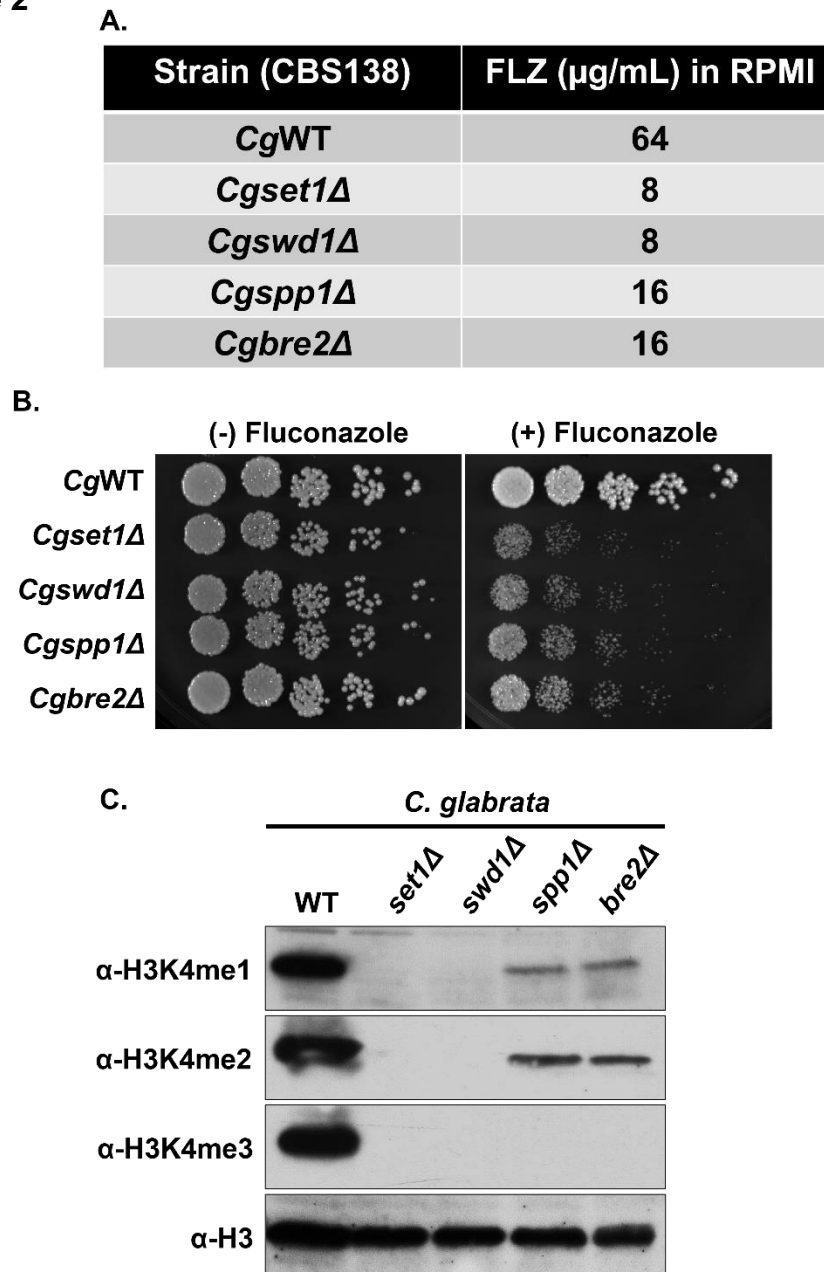
869 FIGURES

Figure 1



870 **FIG 1** Loss of Set1-mediated mono-, di-, and trimethylation at histone H3K4 in
 871 *Saccharomyces cerevisiae* and *Candida glabrata* results in increased azole
 872 susceptibility and delayed growth in vitro. (A) Five-fold serial dilution spot assays of the
 873 indicated *S. cerevisiae* strains were grown on SC media with and without 8 μg/mL
 874 fluconazole and incubated at 30°C for 72 hours. (B & D) Whole cell extracts isolated
 875 from the indicated strains were immunoblotted using histone H3K4 methyl-specific
 876 mono-, di- and trimethylation antibodies of whole cell extracts isolated from the
 877 indicated strains. Histone H3 was used as a loading control. (C) Five-fold serial dilution
 878 spot assays of the indicated *C. glabrata* strains were grown on SC media with and
 879 without 32 μg/mL fluconazole and incubated at 30°C for 48 hours. (E) Liquid growth
 880 curve assay of the indicated *C. glabrata* strains grown over 50 hours with or without 32
 881 μg/mL fluconazole.

Figure 2



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883

884 **FIG 2** Deletion of *Set1* complex members in *C. glabrata* results in increased azole

885 susceptibility and loss of histone H3K4 methylation. (A) MIC assay of the indicated

886 strains performed in RPMI 1640 media at 35°C and results recorded after 48 hours of

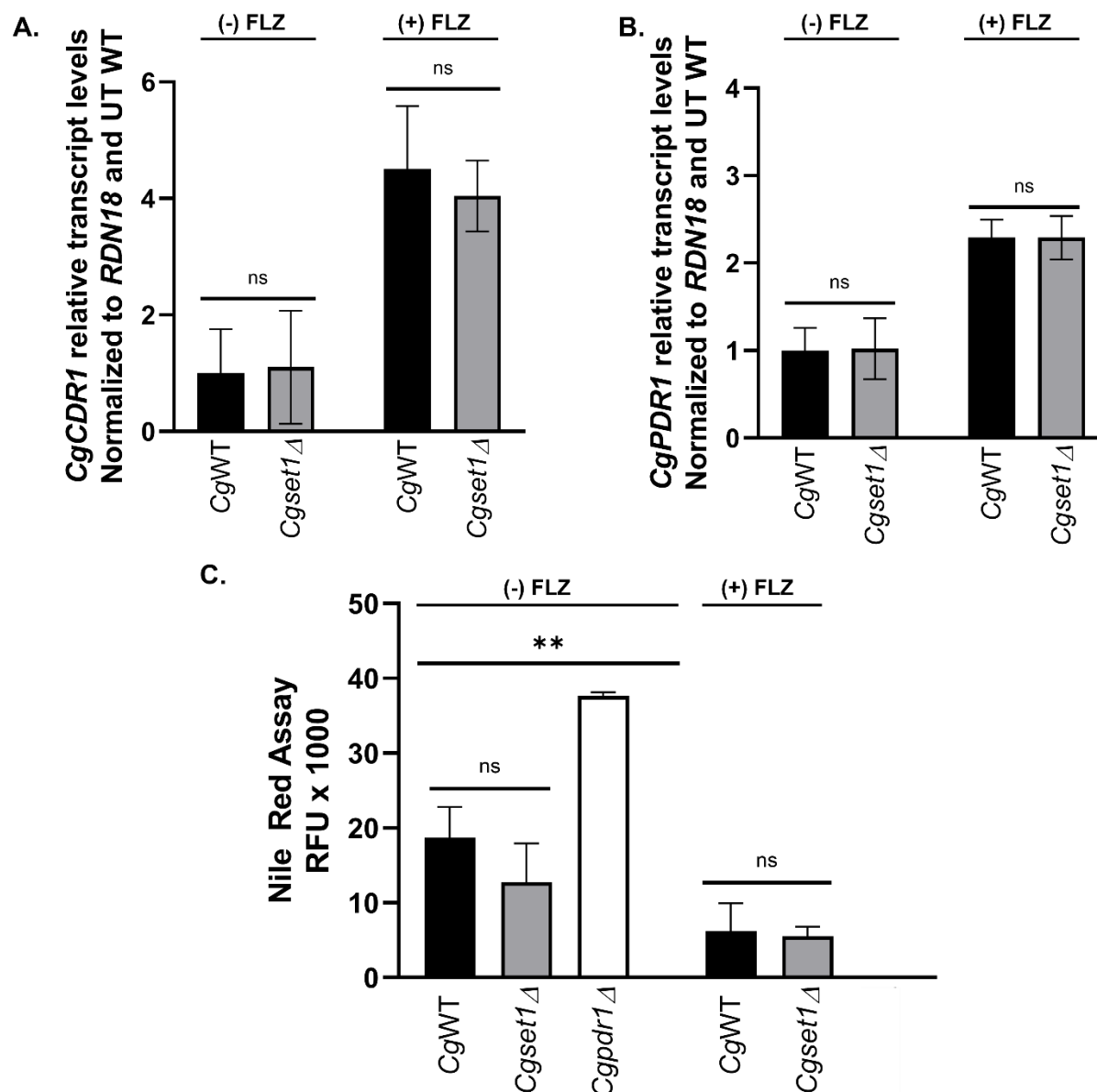
887 incubation. (B) Five-fold serial dilution spot assays of the indicated *C. glabrata* strains

888 were grown on SC plates with or without 32 $\mu\text{g/ml}$ fluconazole. (C) Whole cell extracts

889 isolated from the indicated strains were immunoblotted using H3K4 methyl-specific

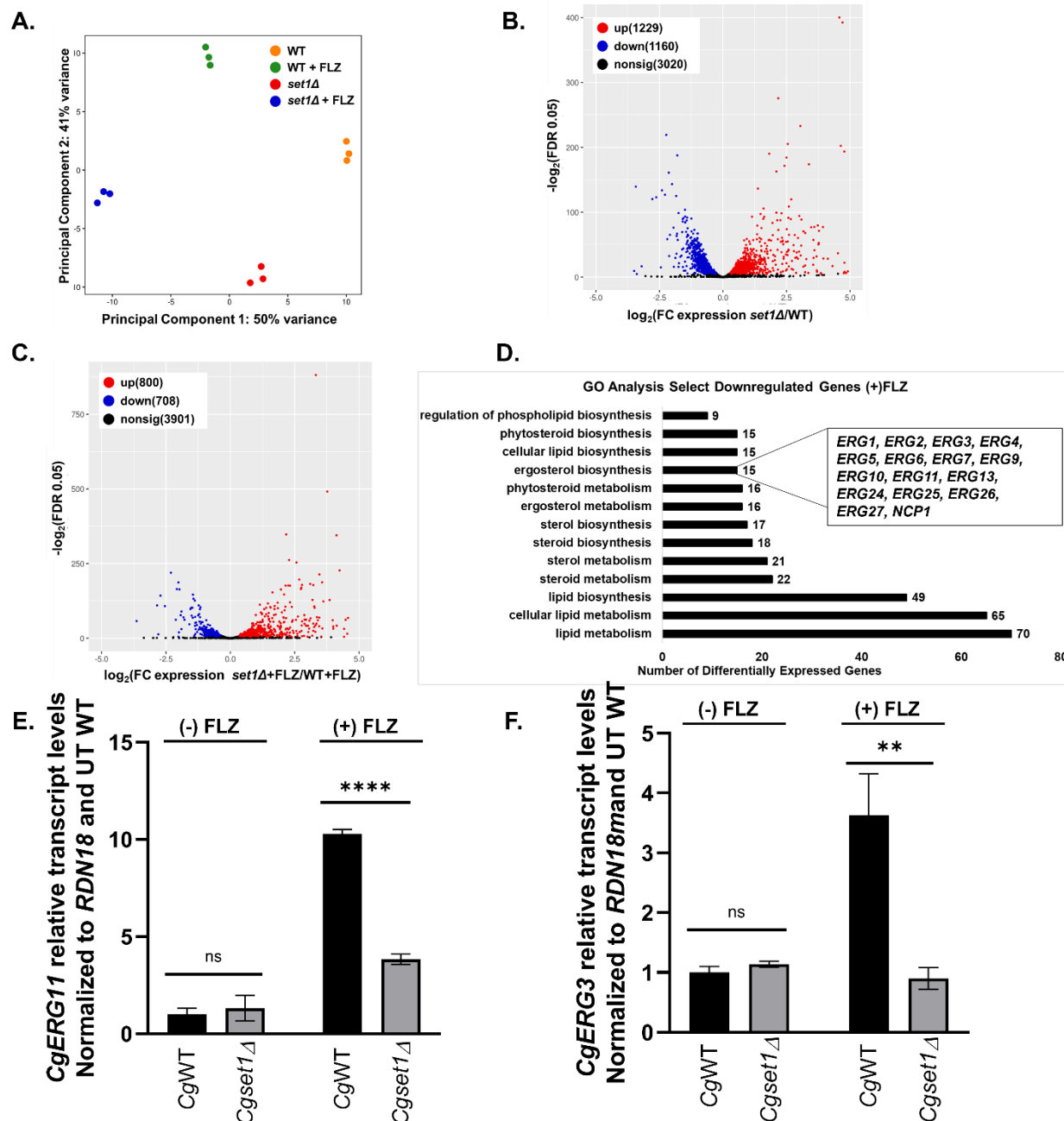
mono-, di- and trimethylation antibodies. Histone H3 was used as a loading control.

Figure 4



897
 898 **FIG 4** Deletion of *SET1* in *C. glabrata* does not alter gene expression levels or function
 899 of the efflux drug transporter, *CDR1* or transcription factor *PDR1*. (A and B) Expression
 900 of indicated genes was determined in *CgWT* and *Cgset1Δ* strain cells treated with and
 901 without 64 μg/ml fluconazole for 3 hr by qRT-PCR analysis. Gene expression analysis
 902 was set relative to the untreated wild-type and expression was normalized to *RDN18*
 903 mRNA levels. Data were analyzed from ≥ 3 biological replicates with three technical
 904 replicates each. Error bars represent SD. (C) Red fluorescence units were measured as
 905 output in a Nile Red assay to determine the efficacy of *Cdr1* in the indicated strains with
 906 and without fluconazole. A *pd1Δ* strain was used as a control. Data were analyzed from
 907 ≥ 3 biological replicates with three technical replicates each. Statistics were performed
 908 using Graphpad Prism student t-test version 9.2.0. *ns* represents $p < 0.05$, $**p < 0.01$,
 909 Error bars represent SD.
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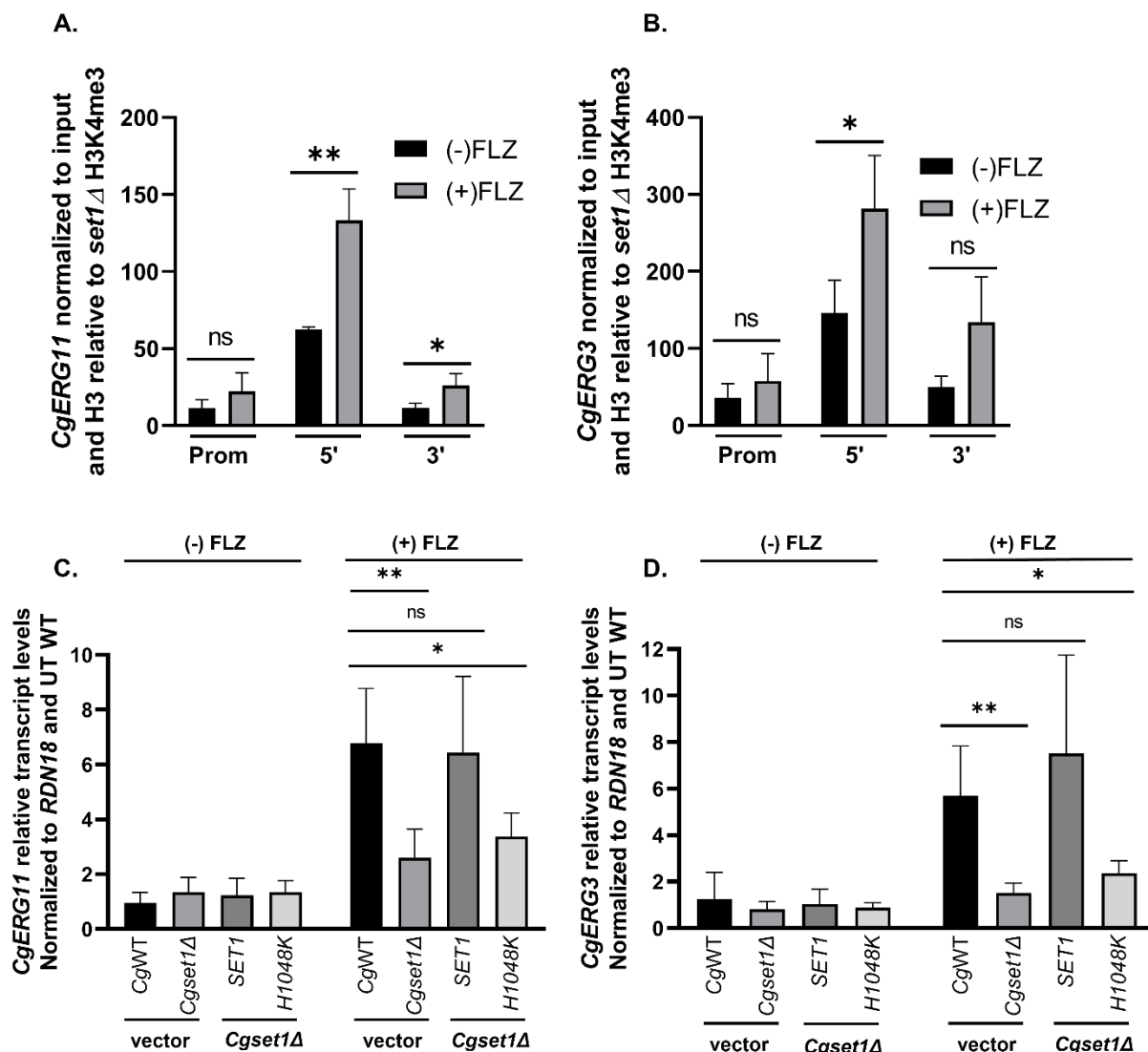
Figure 5



911
 912 **FIG 5** The deletion of *SET1* in *C. glabrata* alters global and local levels of gene
 913 expression under untreated and azole conditions. The genome-wide changes in gene
 914 expression under azoles were performed using *C. glabrata* CBS138 WT and *set1Δ*
 915 strains. (A) The PCA for WT and *set1Δ* azole treated samples relative to WT untreated
 916 samples based on the counts per million. (B) Volcano plot showing the significance
 917 [$-\log_2(\text{FDR})$, y-axis] vs. the fold change (x-axis) of the DEGs identified in the WT
 918 untreated samples relative to *set1Δ* untreated samples. (C) Volcano plot showing the
 919 significance [$-\log_2(\text{FDR})$, y-axis] vs. the fold change (x-axis) of the DEGs identified in
 920 the *set1Δ* azole treated samples relative to WT azole treated samples. Genes with
 921 significant differential expression (FDR < 0.05) in (B and C) are highlighted in red or
 922 blue for up- and downregulated genes, respectively. Black highlighted genes are

923 considered nonsignificant. (D) Genes from the RNA-seq dataset that were statistically
 924 significantly enriched (FDR < 0.05) were used for GO term determination of Set1-
 925 dependent DEGs under azole conditions. Downregulated genes refer to the DEGs that
 926 are dependent on Set1 for activation either directly or indirectly. Significantly enriched
 927 groups of GO terms were identified as the DEGs from only *set1Δ* and WT azole treated
 928 samples. (E and F) Expression of indicated genes was determined in WT and *set1Δ*
 929 strain cells treated with 64 μg/ml fluconazole for 3 hr by qRT-PCR analysis. Gene
 930 expression analysis was set relative to the untreated wild-type and expression was
 931 normalized to *RDN18* mRNA levels. Data were analyzed from ≥ 3 biological replicates
 932 with three technical replicates each. Statistics were performed using Graphpad Prism
 933 student t-test version 9.2.0. **** $p < 0.0001$ and ** $p = 0.002$. Error bars represent SD.

934 **Figure 6**
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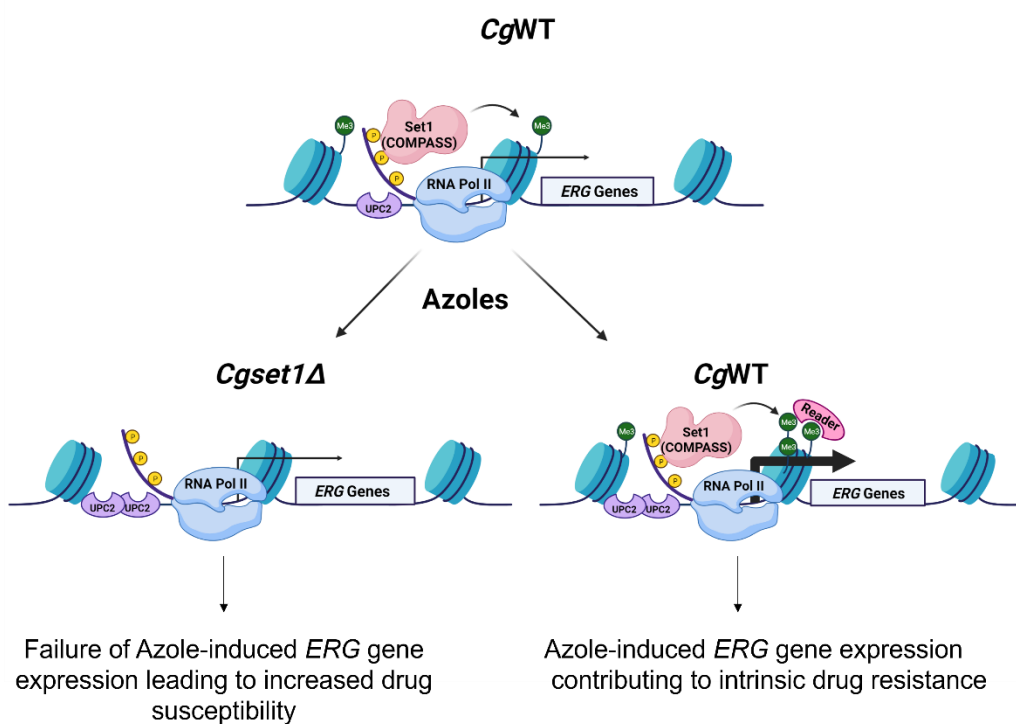


936 **FIG 6** Histone H3K4 trimethylation is enriched on ERG gene chromatin and Set1-
 937 mediated histone H3K4 methyltransferase activity is required for azole induction of ERG
 938 genes. (A and B) CHIP analysis of histone H3K4 tri-methylation levels at the promoter,

939 5', and 3' regions of *ERG11* and *ERG3* in a wild-type *C. glabrata* strain with and without
940 64 µg/mL fluconazole treatment. ChIP analysis was set relative to a *set1Δ* strain and
941 normalized to histone H3 and DNA input levels. Data were analyzed from 5 biological
942 replicates with three technical replicates each, * $p < 0.05$. (C and D) Expression of
943 indicated genes was determined in the indicated mutants treated with and without 64
944 µg/ml fluconazole for 3 hr by qRT-PCR analysis. Gene expression analysis was set
945 relative to the untreated wild-type containing an empty vector and expression was
946 normalized to *RDN18* mRNA levels. Data were analyzed from ≥ 3 biological replicates
947 with three technical replicates each. Statistics were performed using Graphpad Prism
948 student t-test version 9.2.0. **** $p < 0.0001$ and ** $p < 0.01$. Error bars represent SD.
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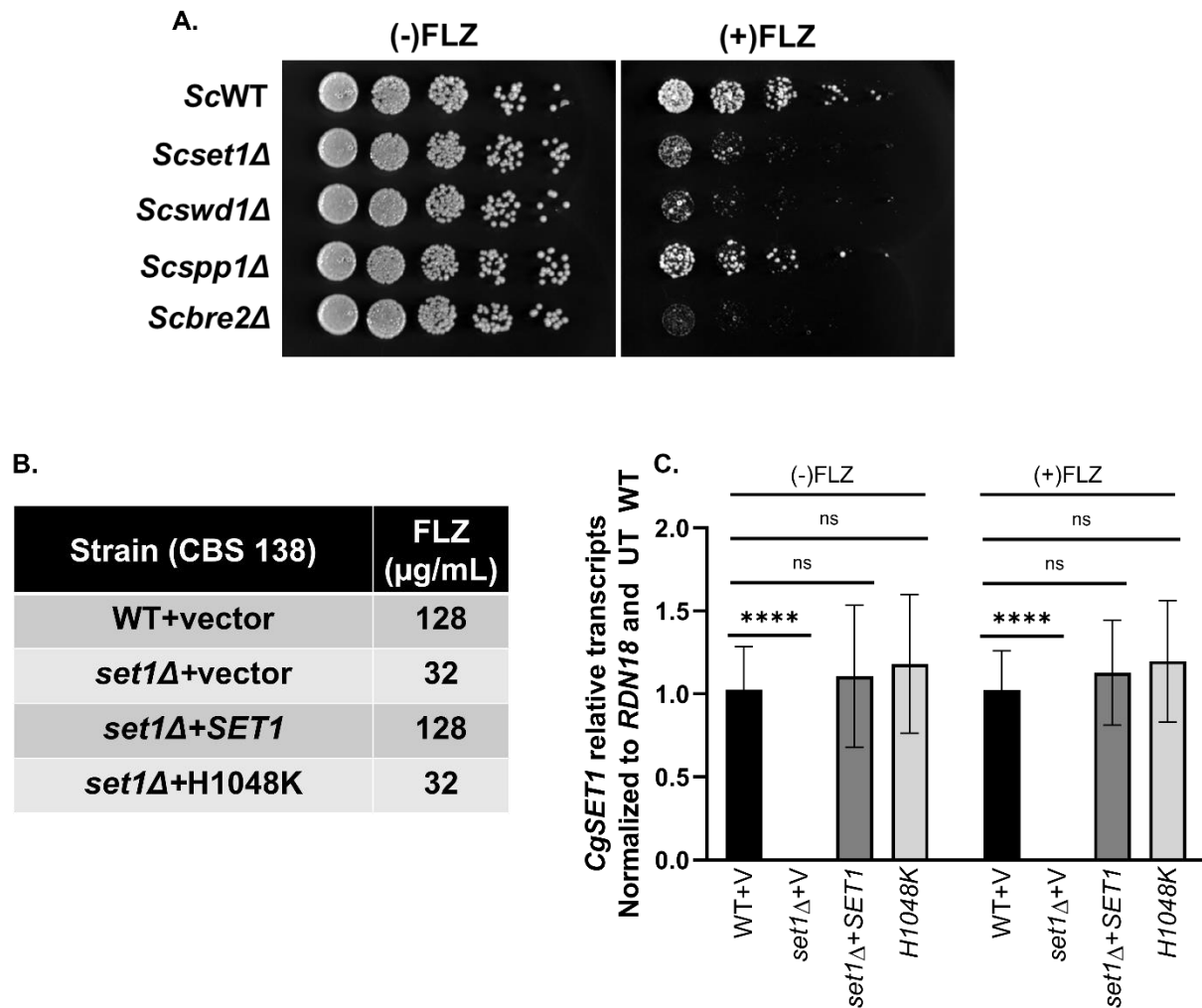
Figure 7

A.



951 **FIG 7** Model for the role of Set1-H3K4 methylation in epigenetic control of ERG genes
952 (Biorender). (A) Under aerobic conditions, the Set1 complex mediates histone H3K4
953 methylation on chromatin at ERG genes. In the presence of azoles, azole-induced
954 transcriptional activation recruits TFs, RNA Polymerase II, and the Set1 complex to
955 increases Histone H3K4 methylation. This increase in methylation could permit
956 additional recruitment of other co-factors/epigenetic regulator (e.g., Set3 and/or SAGA
957 complex) that contain "reader" domains that recognize and bind to the H3K4 methyl
958 mark. Thus, this Set1-Erg pathway contributes to the intrinsic azole resistance in *C.*
959 *glabrata*. In the absence of Set1, histone H3K4 methylation is abolished and failure of
960 recruiting additional H3K4 methyl "readers" prevent the induction of ERG genes, thus
961 making the *C. glabrata* more susceptible to azole treatment.
962

963 **Figure S1**

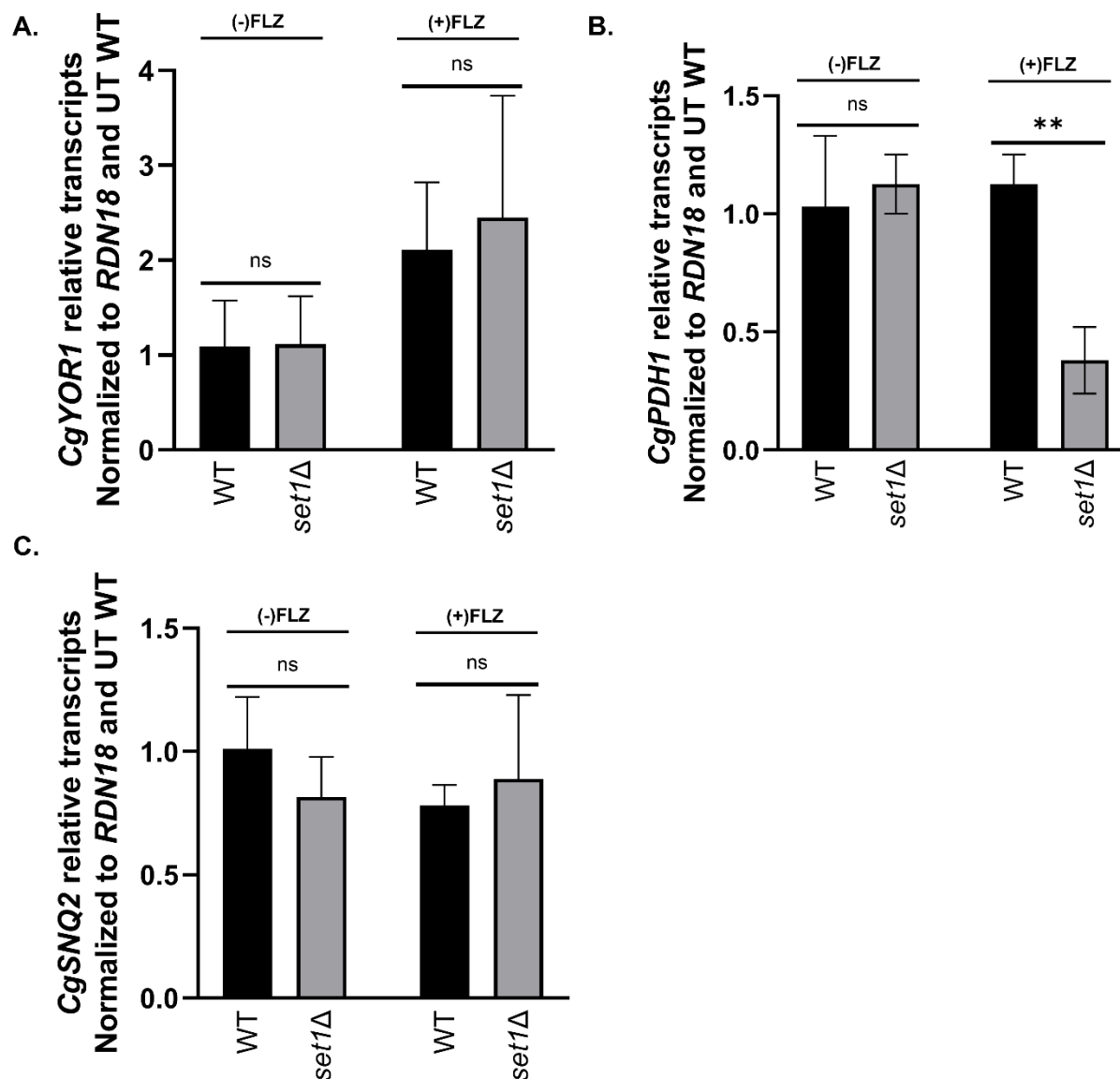


964
 965 **FIG S1.** Loss of Set1 complex members results in altered azole efficacy. (A) Five-fold
 966 serial dilution spot assays of the indicated *S. cerevisiae* strains were grown on SC
 967 plates with or without 8 μg/ml fluconazole. (B) MIC assay of the indicated strains
 968 performed in SC media at 35°C and results recorded after 24 hours of incubation. (C)
 969 Expression of *SET1* was determined in the indicated mutants treated with and without
 970 64 μg/ml fluconazole for 3 hr by qRT-PCR analysis. Gene expression analysis was set
 971 relative to the untreated wild-type and expression was normalized to *RDN18* mRNA
 972 levels. Data were analyzed from 4 biological replicates with three technical replicates
 973 each. Statistics were performed using Graphpad Prism student t-test version 9.2.0.
 974 *****p*<0.0001. Error bars represent SD.
 975

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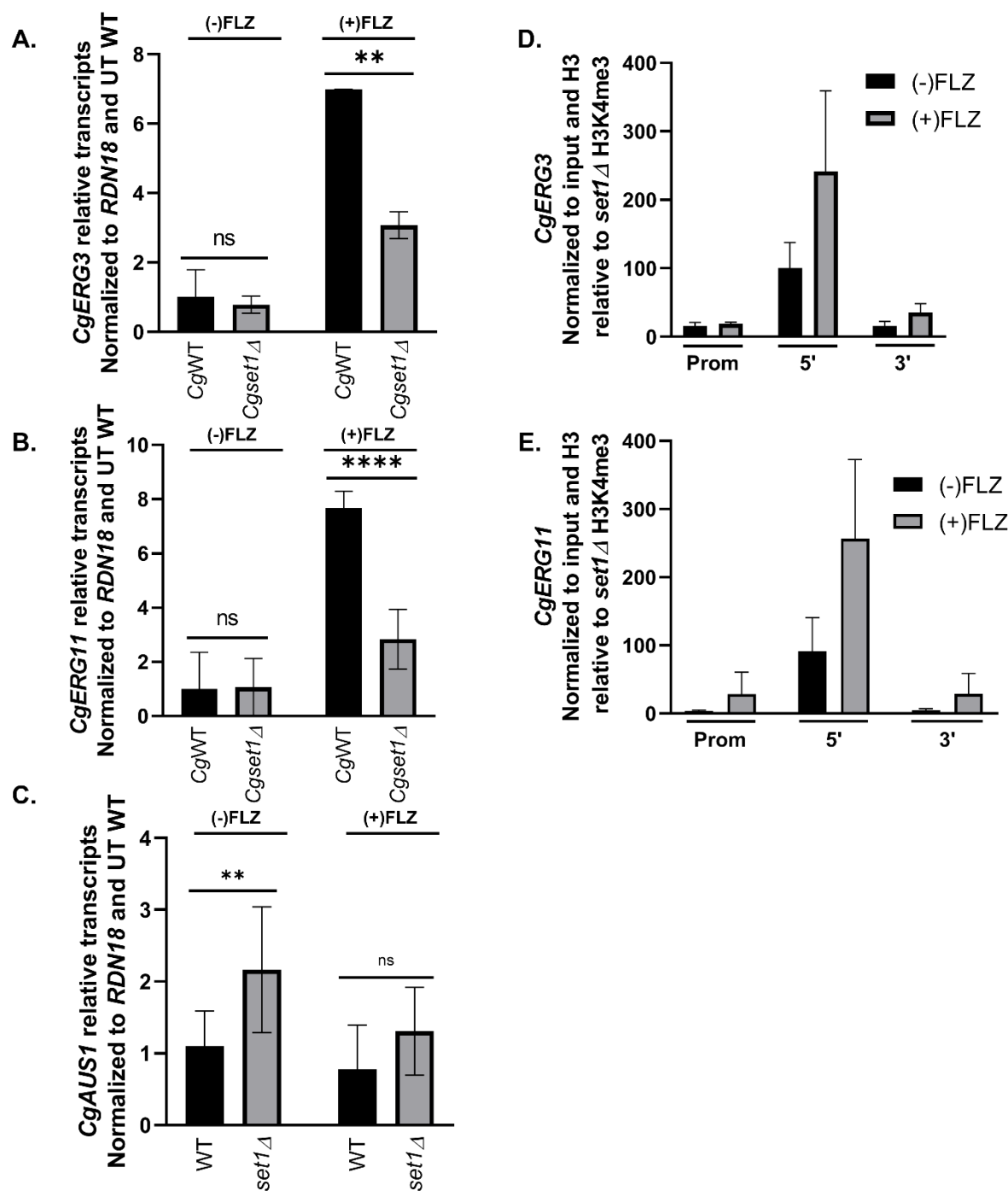
Figure S2



978

979 **Fig S2.** Transcript levels of drug transporters in a *set1Δ* strain compared to wild-type.
980 Expression of the indicated genes were determined in the indicated mutants treated
981 with and without 64 $\mu\text{g/ml}$ fluconazole for 3 hr by qRT-PCR analysis. Gene expression
982 analysis was set relative to the untreated wild-type and expression was normalized to
983 *RDN18* mRNA levels. Data were analyzed from ≥ 3 biological replicates with three
984 technical replicates each. Statistics were performed using Graphpad Prism student t-
985 test version 9.2.0. ** $p < 0.01$. Error bars represent SD.

Figure S3



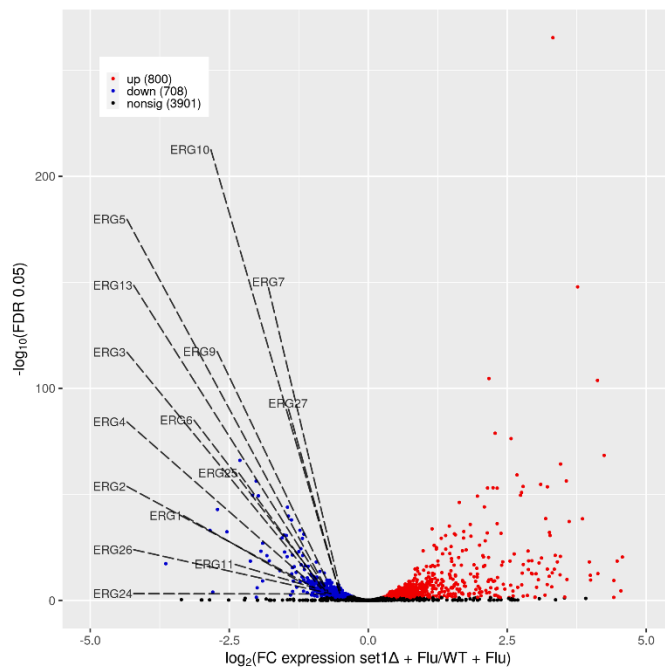
986

987 **Fig S3.** Histone H3K4 trimethylation is enriched on *ERG* gene chromatin and *Set1*-
 988 mediated histone H3K4 methyltransferase activity is required for azole induction of *ERG*
 989 genes in saturated cells. (A and B) Expression of genes was determined in the indicated
 990 strains treated with and without 64 μg/ml fluconazole in a saturated culture for 3 hr by
 991 qRT-PCR analysis. Gene expression analysis was set relative to the untreated wild-type
 992 expression was normalized to *RDN18* mRNA levels. Data were analyzed from ≥ 3
 993 biological replicates with three technical replicates each. (C) Expression of genes was

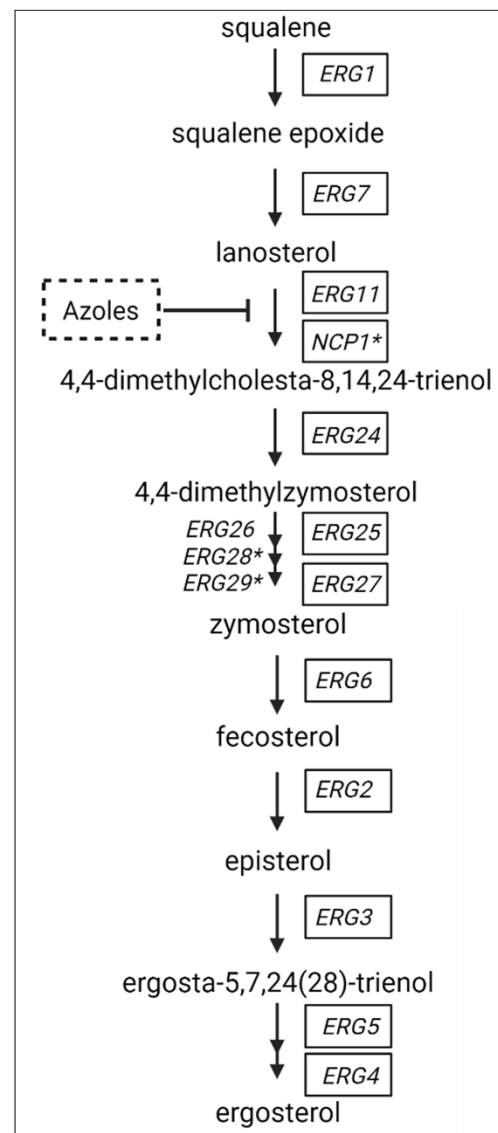
994 determined in the indicated strains treated with and without 64 $\mu\text{g}/\text{ml}$ fluconazole in an
 995 exponential culture for 3 hr by qRT-PCR analysis. Gene expression analysis was set
 996 relative to the untreated wild-type expression was normalized to *RDN18* mRNA levels.
 997 Data were analyzed from ≥ 3 biological replicates with three technical replicates each.
 998 (D and E) ChIP analysis of histone H3K4 tri-methylation levels at the promoter, 5', and
 999 3' regions of *ERG11* and *ERG3* in a wild-type *C. glabrata* strain with and without 64
 1000 $\mu\text{g}/\text{mL}$ fluconazole treatment in saturated cell cultures. ChIP analysis was set relative to
 1001 a *set1 Δ* strain and normalized to histone H3 and DNA input levels. Data were analyzed
 1002 from 3 biological replicates with three technical replicates each. Statistics were
 1003 performed using Graphpad Prism student t-test version 9.2.0. * $p < 0.05$. **** $p < 0.0001$
 1004 and ** $p < 0.01$. Error bars represent SD.

1005 **Figure S4**

A.



B.



1006 **Fig S4.** Genes encoding enzymes of the late ergosterol pathway are down in a *set1Δ*
 1007 strain upon azole treatment in *C. glabrata*. (A) Volcano plot showing the significance
 1008 $[-\log_2(\text{FDR})]$, y-axis] vs. the fold change (x-axis) of the DEGs identified in the *set1Δ*
 1009 azole treated samples relative to WT azole treated samples. Genes with significant
 1010 differential expression (FDR < 0.05) are highlighted in red or blue for up- and
 1011 downregulated genes, respectively. Down-regulated *ERG* genes are labelled in the plot
 1012 which include 12 of the *ERG* genes in the late pathway and two *ERG* genes in the early
 1013 pathway. Black highlighted genes are considered nonsignificant. (B) Depiction of the
 1014 late ergosterol pathway in *C. glabrata*. Azoles inhibit lanosterol 14- α -demethylase, the
 1015 enzyme encoded by *ERG11*. *NCP1** and *ERG28** interact with ergosterol synthesizing
 1016 enzymes. *ERG29** is a protein of unknown function involved in ergosterol biosynthesis.
 1017 Loss of *SET1* results in lower transcript levels of 12 of the 12 ergosterol synthesizing
 1018 enzymes of the late pathway compared to a wild-type strain upon azole treatment.
 1019 Genes with decreased transcript levels due the loss of *SET1* are surrounded by a solid
 1020 square.

1021 **Supplemental Tables**

Table S1.	Yeast Strains		
Strains	Genotype	Reference	Strain Name
<i>S. cerevisiae</i> BY4741	<i>MATα his3Δ leu2Δ0 LYS2 met15Δ0 ura3Δ0</i>	Open Biosystems	ScWT
<i>Scswd1Δ</i>	<i>MATα his3Δ leu2Δ0 LYS2 met15Δ0 ura3Δ0 swd1Δ::KanMX</i>	Open Biosystems	<i>Scswd1Δ</i>
<i>Scspp1Δ</i>	<i>MATα his3Δ leu2Δ0 LYS2 met15Δ0 ura3Δ0 spp1Δ::KanMX</i>	Open Biosystems	<i>Scspp1Δ</i>
<i>Scbre2Δ</i>	<i>MATα his3Δ leu2Δ0 LYS2 met15Δ0 ura3Δ0 bre2Δ::KanMX</i>	Open Biosystems	<i>Scbre2Δ</i>
SDBY1420	<i>MATα his3Δ leu2Δ0 LYS2 met15Δ0 ura3Δ0 set1Δ::HphMX</i>	Zhang et al. Yeast 2017	<i>Scset1Δ</i>
SDBY1600	<i>MATα his3Δ leu2Δ0 LYS2 met15Δ0 ura3Δ0 HHT1::K4R</i>	This study	<i>ScH3K4R1</i>
SDBY1601	<i>MATα his3Δ leu2Δ0 LYS2 met15Δ0ura3Δ0 HHT1::K4R HHT2::K4R</i>	This study	<i>ScH3K4R2</i>
ATCC 2001 CgWT	<i>C. glabrata</i> wild type strain	www.atcc.org	CgWT
SDBY1602	<i>set1Δ::HphMX</i>	This study	<i>Cgset1Δ</i>
SDBY1603	<i>swd1Δ::HphMX</i>	This study	<i>Cgswd1Δ</i>
SDBY1604	<i>spp1Δ::HphMX</i>	This study	<i>Cgspp1Δ</i>
SDBY1605	<i>bre2Δ::HphMX</i>	This study	<i>Cgbre2Δ</i>
SDBY1606	<i>pdr1Δ::NatMX</i>	This study	<i>Cgpdr1Δ</i>
ATCC 200989 CgWT	<i>his3Δ trp1Δ ura3Δ</i>	www.atcc.org	CgWT

SDBY1607 *his3Δ trp1Δ ura3Δ set1Δ::HphMX* This study *Cgset1Δ*

1022

Table S2. Plasmids

Plasmid	Inserted Gene	Promoter	Vector	Source
pGRB2.0	None		pGRB2.0	Zordan et al.
pGRB2.0	<i>SET1</i>	<i>SET1</i>	pGRB2.0	Zordan et al.
pGRB2.0	<i>SET1/H1048K</i>	<i>SET1</i>	pGRB2.0	Zordan et al.

1023

Table S3. Primers for qRT-PCR

Primer Name	Sequence
<i>CgRDN18-001F</i>	ACGGAGCCAGCGAGTCTAAC
<i>CgRDN18-002R</i>	CGACGGAGTTTCACAAGATTACC
<i>CgERG3-001F</i>	TGGGAGCACCACGGTCTAAG
<i>CgERG3-002R</i>	CAGTCGGTGAAGAAGATGAAAGTG
<i>CgERG11-001F</i>	GGGTCCAAAGGGTCACGAA
<i>CgERG11-002R</i>	GCAGCTTCAGCGGAAACATC
<i>CgCDR1-001F</i>	GTCTATGGAAGGTGCCGTCAA
<i>CgCDR1-002R</i>	TGAACCAGGTCTACCTAGCACAAC
<i>CgPDR1-001F</i>	TCGGCGAGGGTAAATTCAAC
<i>CgPDR1-002R</i>	CAACTGCGTTTGATTCCCTTAAGC
<i>CgSET1-001F</i>	CCAACCAAAGCCGATACTCATC
<i>CgSET1-002R</i>	GCGTTGACTACCGCGAGATT

1024

Table S4. Probe sets for ChIP Analysis

Probe Name	Sequence 5'-3'
<i>ERG11 Promoter</i>	/56-FAM/CCTTGTTCC/ZEN/AACTACAATCGAGTGAGCT/3IABkFQ/ CGAATACGAGGCCATTTGTAAAC CTGTGCTCCCATCTCACTATAAC
<i>ERG11 5'</i>	/56-FAM/TCGTACTTC/ZEN/CAAGCTCTGCCATTGG/3IABkFQ/ GAGTACGTGAAGCTTGGTCTT TGGCAAGGCGACCATAATAG
<i>ERG11 3'</i>	/56-FAM/CGGCATGAC/ZEN/TTAAGCTGGTTGTTTCG/3IABkFQ/ ACGGGATATACGCTGATTCATT AGCAGCAAAGCCCTCTAAA
<i>ERG3 Promoter</i>	/56-FAM/AGCGAGAGC/ZEN/TGCTAGAGCTGAGAA/3IABkFQ/ GAGACTATACGAGTGTGCTCTTTG TCTTCTTCCAGGCCTCATCT
<i>ERG3 5'</i>	/56-FAM/TCGACGACT/ZEN/CGTTGGTCAATGCTT/3IABkFQ/ CGACGATGTGTATGCCAAGA ATGCAGCAGCGTAGAGTTAG
<i>ERG3 3'</i>	/56-FAM/CCAAGAGGT/ZEN/GGAAGGTGACGACAC/3IABkFQ/ AGAAACCGCCGCTTACAT CCGGTGTTCCTGTCTAGTT

1025

Table S5.		qRT-PCR Values				
Figure 4A: qRT-PCR						
Gene	Strain	Condition	Mean RQ	Standard Deviation	N	P-Value
<i>CgCDR1</i>	<i>CgWT</i>	Untreated	1	0.750623	7	n.s.
<i>CgCDR1</i>	<i>CgWT</i>	(+) fluconazole	4.51	1.071572	7	n.s.
<i>CgCDR1</i>	<i>Cgset1Δ</i>	Untreated	1.1	0.972961	7	n.s.
<i>CgCDR1</i>	<i>Cgset1Δ</i>	(+) fluconazole	4.04	0.608125	7	n.s.
Figure 4B: qRT-PCR						
Gene	Strain	Condition	Mean RQ	Standard Deviation		P-Value
<i>CgPDR1</i>	<i>CgWT</i>	Untreated	1	0.257724	3	n.s.
<i>CgPDR1</i>	<i>CgWT</i>	(+) fluconazole	2.29	0.204429	3	n.s.
<i>CgPDR1</i>	<i>Cgset1Δ</i>	Untreated	1.02	0.3498374	3	n.s.
<i>CgPDR1</i>	<i>Cgset1Δ</i>	(+) fluconazole	2.29	0.249992	3	n.s.
Figure 5E: qRT-PCR						
Gene	Strain	Condition	Mean RQ	Standard Deviation		P-Value
<i>CgERG11</i>	<i>CgWT</i>	Untreated	1	0.32	3	n.s.
<i>CgERG11</i>	<i>CgWT</i>	(+) fluconazole	10.29	0.23	3	n.s.
<i>CgERG11</i>	<i>Cgset1Δ</i>	Untreated	1.32	0.65	3	n.s.
<i>CgERG11</i>	<i>Cgset1Δ</i>	(+) fluconazole	3.83	0.26	3	<0.0001
Figure 5F: qRT-PCR						
Gene	Strain	Condition	Mean RQ	Standard Deviation		P-Value
<i>CgERG3</i>	<i>CgWT</i>	Untreated	1	0.1	3	n.s.
<i>CgERG3</i>	<i>CgWT</i>	(+) fluconazole	3.63	0.69	3	n.s.
<i>CgERG3</i>	<i>Cgset1Δ</i>	Untreated	1.14	0.05	3	n.s.
<i>CgERG3</i>	<i>Cgset1Δ</i>	(+) fluconazole	0.9	0.18	3	<0.01
Figure 6C: qRT-PCR						
Gene	Strain	Condition	Mean RQ	Standard Deviation		P-Value
<i>CgERG11</i>	<i>CgWT+V</i>	Untreated	0.951	0.384	4	n.s.
<i>CgERG11</i>	<i>CgWT+V</i>	(+) fluconazole	6.791	1.988	4	n.s.
<i>CgERG11</i>	<i>Cgset1Δ+V</i>	Untreated	1.342	0.549	4	n.s.
<i>CgERG11</i>	<i>Cgset1Δ+V</i>	(+) fluconazole	2.603	1.043	4	<0.01
<i>CgERG11</i>	<i>Cgset1Δ/SET1</i>	Untreated	1.227	0.631	4	n.s.
<i>CgERG11</i>	<i>Cgset1Δ/SET1</i>	(+) fluconazole	6.438	2.783	4	n.s.
<i>CgERG11</i>	<i>Cgset1Δ/H1048K</i>	Untreated	1.346	0.429	4	n.s.
<i>CgERG11</i>	<i>Cgset1Δ/H1048K</i>	(+) fluconazole	3.370	0.870	4	<0.05
Figure 6D: qRT-PCR						
Gene	Strain	Condition	Mean RQ	Standard Deviation		P-Value
<i>CgERG3</i>	<i>CgWT+V</i>	Untreated	1.266	1.138	4	n.s.
<i>CgERG3</i>	<i>CgWT+V</i>	(+) fluconazole	5.686	2.144	4	n.s.
<i>CgERG3</i>	<i>Cgset1Δ+V</i>	Untreated	0.814	0.345	4	n.s.
<i>CgERG3</i>	<i>Cgset1Δ+V</i>	(+) fluconazole	1.512	0.429	4	n.s.
<i>CgERG3</i>	<i>Cgset1Δ/SET1</i>	Untreated	1.034	0.64	4	n.s.
<i>CgERG3</i>	<i>Cgset1Δ/SET1</i>	(+) fluconazole	7.518	4.232	4	<0.001

<i>CgERG3</i>	<i>Cgset1Δ/H1048K</i>	Untreated	0.888	0.206	4	n.s.
<i>CgERG3</i>	<i>Cgset1Δ/H1048K</i>	(+) fluconazole	2.357	0.54	4	<0.05

Figure 6A: ChIP qRT-PCR

Gene	Strain	Condition	Mean RQ	Standard Deviation		P-Value
<i>CgERG11</i>	<i>CgWT</i> promoter	Untreated	11.24	5.568	3	n.s.
<i>CgERG11</i>	<i>CgWT</i> promoter	(+) fluconazole	22.138	12.012	3	n.s.
<i>CgERG11</i>	<i>CgWT</i> 5'	Untreated	62.531	1.452	3	n.s.
<i>CgERG11</i>	<i>CgWT</i> 5'	(+) fluconazole	133.274	20.388	3	<0.001
<i>CgERG11</i>	<i>CgWT</i> 3'	Untreated	11.412	3.193	3	n.s.
<i>CgERG11</i>	<i>CgWT</i> 3'	(+) fluconazole	26.019	7.85	3	<0.05

Figure 6B: ChIP qRT-PCR

Gene	Strain	Condition	Mean RQ	Standard Deviation		P-Value
<i>CgERG3</i>	<i>CgWT</i>	Untreated	35.768	18.274	3	n.s.
<i>CgERG3</i>	<i>CgWT</i>	(+) fluconazole	57.996	35.319	3	n.s.
<i>CgERG3</i>	<i>CgWT</i> 5'	Untreated	145.877	42.565	3	n.s.
<i>CgERG3</i>	<i>CgWT</i> 5'	(+) fluconazole	281.392	69.117	3	<0.05
<i>CgERG3</i>	<i>CgWT</i> 3'	Untreated	50.262	13.737	3	n.s.
<i>CgERG3</i>	<i>CgWT</i> 3'	(+) fluconazole	133.95	59.062	3	n.s.

1026

1027