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1 **Impact of Erg11 amino acid substitutions identified in *Candida auris* clade III isolates on**
2 **triazole drug susceptibility**
3

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27 **Running title:** Analysis of *Candida auris* clade III *ERG11* mutations

28

29 **Keywords**: *Candida auris*; *ERG11*; triazole resistance; fluconazole resistance; clade III; African

30 clade; heterologous expression; mutagenesis; short- and long-tailed triazoles

31

32 **Abstract**

33 *ERG11* sequencing of 28 *Candida auris* clade III isolates revealed the presence of concomitant

34 V125A and F126L substitutions. Heterologous expression of Erg11-V125A/F126L in

35 *Saccharomyces cerevisiae* led to reduced fluconazole and voriconazole susceptibilities.

36 Generation of single substitution gene variants through site-directed mutagenesis uncovered

37 that F126L primarily contributes to the elevated triazole MICs. A similar, yet diminished pattern

38 of reduced susceptibility was observed with long-tailed triazoles posaconazole and itraconazole

39 for V125A/F126L, F126L, Y132F, and K143R alleles.

40

41 **Text**

42 *Candida auris* is an emerging fungal pathogen that has spread across the globe and

43 caused multiple healthcare center outbreaks. Strains of *C. auris* are divided into five genetically-

44 distinct, geographic clades: South Asian (I), East Asian (II), African (III), South American (IV),

45 and Iranian (V) (1). Initial spread of *C. auris* to the U.S. and other parts of the world is predicted

46 to have occurred through multiple travel-related introductions (2). Recently, several reports have

47 shown high rates of *C. auris* candidemia in hospitalized patients with severe COVID-19 (SARS-

48 CoV-2 virus) infection, particularly in severely ill patients in the ICU setting (3-5). Interestingly,

49 the pathogenicity of *C. auris* differs from other species in that it can colonize the skin, persist on

50 hospital surfaces and medical equipment, and transfer from person to person (6, 7). In addition,

51 *C. auris* exhibits elevated rates of antifungal resistance. Clinical isolates that demonstrate

52 reduced susceptibility to one or more classes of antifungals, including triazoles, polyenes

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53 (amphotericin B), and echinocandins, have been reported, with triazole resistance being the
54 most prevalent (8-10).
55 Triazole antifungals (e.g. fluconazole, voriconazole, itraconazole, posaconazole) target
56 the biosynthesis of fungal ergosterol, specifically through inhibition of lanosterol 14-alpha-
57 demethylase (Erg11p) that is encoded by the *ERG11* gene in yeast. Early reports identified
58 single Erg11 substitutions (F126L, Y132F, or K143R) in strains from multiple clades (9, 11-13).
59 These substitutions were highlighted due to their connection to triazole resistance within other
60 species of *Candida*, specifically *C. albicans* (14). In our previous study (15), we identified and
61 analyzed *C. auris ERG11* mutations in clinical isolates of clade I and IV. Using a heterologous
62 expression system, we directly linked Y132F and K143R Erg11 substitutions to fluconazole and
63 voriconazole resistance; whereas, other alterations (e.g. I466M, Y501H, and clade-specific
64 polymorphisms) were not associated with elevated minimum inhibitory concentrations (MICs).
65 Here, we investigated triazole resistance in 28 clinical isolates of *C. auris* clade III
66 obtained from South Africa (n=21), Australia (n=5), and the CDC and FDA Antimicrobial
67 Resistance (AR) Isolate Bank (n=2). *ERG11* was amplified and sequenced as described before
68 (15). In agreement with recent reports (8, 16, 17), we identified two *ERG11* mutations, T374C
69 and T376C, that lead to two amino acid substitutions, V125A and F126L, respectively, in all 28
70 isolates (**Table 1**). Antifungal susceptibility testing was performed according to CLSI
71 methodology (18, 19) and MICs were interpreted using tentative breakpoints as suggested by
72 the CDC (https://www.cdc.gov/fungal/candida-auris/c-auris-antifungal.html). These isolates
73 demonstrated reduced triazole susceptibilities, specifically to fluconazole and voriconazole
74 (**Table 1**). Of note, three clinical isolates (SA 13, 15, and 16) demonstrated fluconazole MICs in
75 the susceptible range (< 32 μg/ml), despite containing the same *ERG11* mutations as the other
76 strains (**Table 1**). This may point to additional alterations in these isolates that specifically
77 influence the fluconazole-Erg11p interaction. Further analyses on these strains are underway.

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78 Using the same approach as in (15), we cloned the Erg11 allele (V125A/F126L) from
79 isolate AR-0384 onto a low-copy plasmid (pRS416), which was then expressed in a haploid
80 strain of *S. cerevisiae* (BY4741). This heterologous system allowed us to focus solely on the
81 effects of *ERG11* mutations on triazole susceptibilities. Multiple clones were passaged on
82 selective medium (synthetic defined medium lacking uracil; SD-Ura), screened by PCR, and
83 resulting plasmid sequences verified (for primers, see (15)). *S. cerevisiae* that expressed *C.*
84 *auris* Erg11-V125A/F126L demonstrated elevated MICs to fluconazole (64 µg/ml) and
85 voriconazole (1 µg/ml). In comparison, expression of an empty vector or Erg11-wild type alleles
86 from other clades yielded MICs 4 to 8-fold more susceptible (≤ 16 µg/ml to fluconazole; ≤ 0.25
87 µg/ml to voriconazole) (**Table 2**).
88 To further dissect the specific role of V125A and F126L substitutions in triazole
89 resistance, we designed mutagenic primers to individually revert each amino acid substitution
90 (**Figure 1**). A Phusion site-directed mutagenesis kit (Thermo Scientific, MA, USA) was used to
91 introduce the desired wild-type mutations. The resulting *C. auris* Erg11-V125A and Erg11-
92 F126L plasmid constructs were expressed in *S. cerevisiae*. In addition, we performed two
93 consecutive rounds of site-directed mutagenesis to produce a strain that carried neither
94 substitution (Erg11-V125/F126) (**Figure 1D-E**). This strain represented a *de facto* clade III wild-
95 type allele. Plasmid sequences of all alleles were confirmed. Subsequent triazole susceptibility
96 assays revealed that cells expressing F126L alone exhibited elevated MICs similar in levels to
97 V125A/F126L, while V125A alone led to MICs similar to the wild-type alleles (**Table 2**). Our
98 engineered, clade III wild-type allele yielded susceptible MICs, allowing us to conclude that the
99 *ERG11* mutations, as opposed to expression levels, were mainly contributing to the observed
100 decreased susceptibility.
101 Drug binding and cloning studies have demonstrated that certain *ERG11* mutations in *S.*
102 *cerevisiae* and *C. albicans* influence susceptibility to all triazoles, while other mutations lead to
103 decreased susceptibility to only short- or long- tailed triazoles (20-22). Therefore, in addition to

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104 fluconazole and voriconazole (short-tailed triazoles), we tested all of our strains to determine
105 susceptibility to posaconazole and itraconazole (long-tailed triazoles) (**Table 2**). Changes in
106 posaconazole and itraconazole MICs were minimal, although consistent, with 2 to 4-fold
107 differences between the "resistant" alleles (V125A/F126L, Y132F, or K143R) and wild type
108 alleles (**Table 2**). These results are in alignment with the minimal differences observed in clinical
109 isolates (**Table 1**) and to those of previous studies that analyzed Y132F and K143R (or
110 equivalent changes) in *C. albicans* and *S. cerevisiae* (21, 23).

111 Crystallization of *C. albicans* Erg11 identified residue 126 (and the equivalent residue in
112 *S. cerevisiae*) as being located within the enzyme's active site and a likely player in substrate
113 binding (24, 25). Furthermore, the authors from this study predicted that alteration of this
114 residue would likely reduce affinity for all triazole drugs but would do so most extensively for
115 short-tailed azoles (24). Since all *C. auris* clade III isolates described in the literature contain
116 both V125A and F126L substitutions, it is likely that these two mutations occurred at nearly the
117 same time in the evolution of this clade. The V125A substitution may simply be a passenger
118 mutation. Alternatively, V125A may increase the stability of the Erg11 enzyme or be
119 advantageous for the yeast in another way and/or in combination with other alterations (e.g.,
120 *ERG11* copy number variants (8)). Studies have since identified *TAC1b* transcription factor
121 mutations, linked to increased expression of drug efflux pumps (e.g., *CDR1* and/or other
122 unidentified transporters), as an alternate mechanism of triazole resistance in *C. auris* (26-28).
123 Additionally, a recent report demonstrated an additive effect that concomitant *ERG11* (F444L)
124 and *TAC1b* mutations can have on triazole susceptibility (29).

125 In conclusion, the *ERG11* allele found in *C. auris* clade III isolates directly contributes to
126 reduced triazole susceptibility, in particular to fluconazole and voriconazole. Moreover, our
127 mutagenic experiments revealed that the F126L substitution was primarily responsible for the
128 elevated triazole MICs. Results of this study further improve our understanding of triazole

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129 resistance mechanisms in *C. auris* which can have a direct impact on diagnostic and treatment
130 practices.
131

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138

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246 **Table 1.** Triazole drug susceptibility and Erg11 profiles of 28 *C. auris* clinical isolates of clade III.
247

Origin	Isolate*	Specimen	Erg11	MIC† (µg/ml)			
				FLC	VRC	POS	ITC
AR Bank	**AR-0381**	N/A	WT	4	0.03	<0.03	0.03
	AR-0382	N/A	WT	4	0.03	0.06	0.06
	AR-0383	N/A	V125A/F126L	>128	1	0.03	0.125
	AR-0384	N/A	V125A/F126L	>128	1	0.06	0.25
South Africa	**SA1**	CVC tip	V125A/F126L	>128	1	0.125	0.25
	SA2	Blood	V125A/F126L	>128	0.5	0.125	0.5
	SA3	CVC tip	V125A/F126L	>128	1	0.03	0.5
	SA4	Blood	V125A/F126L	>128	1	0.03	0.5
	SA5	Tracheal aspirate	V125A/F126L	>128	2	0.03	0.5
	SA6	Blood	V125A/F126L	>128	1	0.03	0.5
	SA7	Urine	V125A/F126L	>128	1	0.125	0.5
	SA8	Urine	V125A/F126L	>128	1	0.03	0.25
	SA9	Urine	V125A/F126L	>128	1	0.03	0.5
	SA10	Blood	V125A/F126L	>128	1	0.125	0.5
	SA11	Blood	V125A/F126L	>128	1	0.03	1
	SA12	Urine	V125A/F126L	>128	1	0.03	0.25
	SA13	Urine	V125A/F126L	8	1	0.03	0.25
	SA14	Urine	V125A/F126L	>128	2	0.03	0.5
	SA15	Tracheal aspirate	V125A/F126L	8	1	0.03	0.5
	SA16	Tracheal aspirate	V125A/F126L	8	1	0.03	0.5
	SA17	Urine	V125A/F126L	>128	1	0.03	0.25
	SA18	Urine	V125A/F126L	128	0.5	0.03	0.5
	SA19	Urine	V125A/F126L	128	2	0.03	0.25
	SA22	Urine	V125A/F126L	128	0.5	0.25	0.5
	SA23	Urine	V125A/F126L	>128	8	0.25	0.5
Australia	**A3**	Sternum	V125A/F126L	>128	1	0.25	1
	A4	Sternum	V125A/F126L	>128	1	0.25	1
	A6	Axilla & Groin	V125A/F126L	>128	0.25	0.125	1
	A7	Axilla & Groin	V125A/F126L	>128	0.25	0.25	1
	A8	Catheter specimen of urine (CSU)	V125A/F126L	>128	0.5	0.25	1

248
249 *For reference, susceptibility results are presented for two Erg11 wild-type (WT) strains: AR-0381 (clade
250 II) and AR-0382 (clade I).
251 †FLC, fluconazole; VRC; voriconazole; POS, posaconazole; ITC, itraconazole.
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255
256
257 **Table 2.** Triazole susceptibilities of *S. cerevisiae* strains that express *C. auris* Erg11 plasmid
258 constructs.
259
260
261

Erg11 allele	MIC* (µg/ml) FLC	VRC	POS	ITC
Empty vector	16	0.12	0.25	0.5
wild type (clade I)	16	0.25	0.25	1
wild type (clade IV)	16	0.12	0.25	1
I466M	16	0.25	0.5	2
Y132F	128	2	0.5	4
K143R	64	1	0.5	2
V125A/F126L	64	1	0.5	2
wild type (clade III)	16	0.25	0.25	1
V125A	16	0.25	0.25	1
F126L	64	1	0.5	4

274
275 *MICs are representative of three independent experiments and obtained in both nutrient-rich YPD (Yeast
276 extract, Peptone, Dextrose) (MICs displayed) and nutrient-limited SD-Ura broth media; we observed a 2-
277 fold or less difference between media. Fluconazole and voriconazole MICs of the first six strains were
278 previously reported (15); however, these MICs were repeated in tandem with the newly engineered
279 strains and are presented here for comparison. FLC, fluconazole; VRC; voriconazole; POS,
280 posaconazole; ITC, itraconazole.
281
282

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283 **A.**

284 **Erg11-V125A/F126L**

285 ...364 acc act cca **gCt Ctc** ggg aaa ggt gtc att tac gac tgt 402...

286 ...122 T T P **A** **L** G K G V I Y D C 134...

287

288 **B.**

289 **Erg11-V125A only**

290 5′ act cca **gCt Ttc** ggg aaa ggt gtc att 3′

291 ... T P **A** **F** G K G V I ...

292

293 **C.**

294 **Erg11-F126L only**

295 5′ act cca **gTt Ctc** ggg aaa ggt gtc att t 3′

296 ... T P **V** **L** G K G V I ...

297

298 **D.**

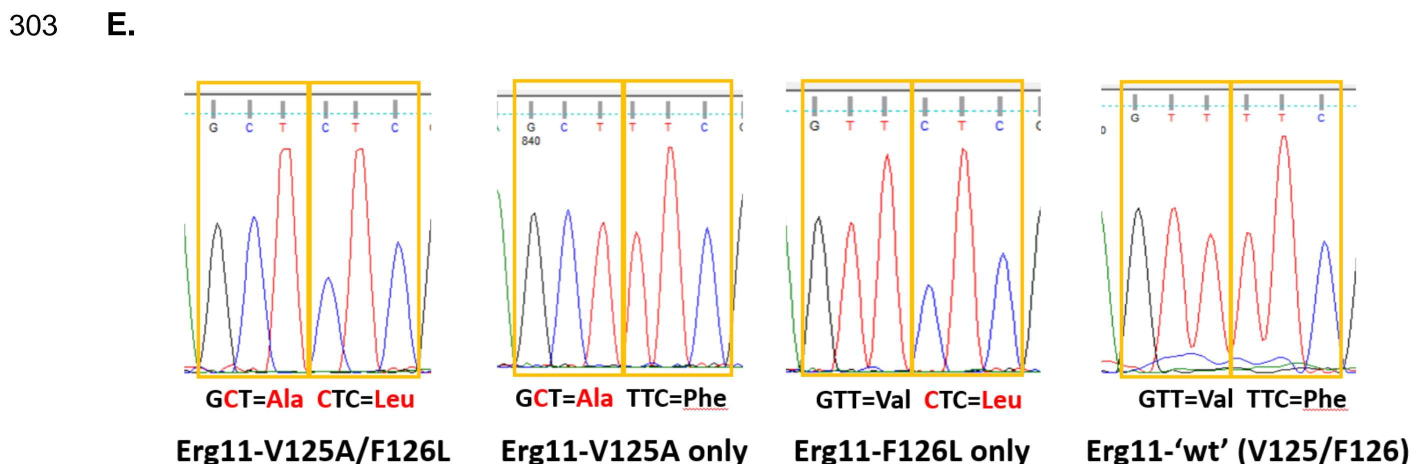
299 **Erg11-'wild type'**

300 5′ act cca **gTt Ttc** ggg aaa ggt gtc att 3′

301 ... T P **V** **F** G K G V I ...

302

303 **E.**



304

305 **Figure 1. Molecular dissection of *C. auris* Erg11 V125A and F126L amino acid**

306 **substitutions.**

307 **A**, Region of clade III *ERG11* DNA that displays nucleotide mutations (T374C/T376C) in red and

308 resulting protein alterations (V125A/F126L) in yellow highlight. **B**, Forward mutagenic primer

309 used to revert Leucine (L) back to Phenylalanine (F). After mutagenesis, this construct

310 contained only V125A (pCauErg11-V125A). **C**, Forward mutagenic primer used to revert

311 Alanine (A) back to wild type Valine (V). After mutagenesis, this construct contained only F126L

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312 (pCauErg11-F126L). **D**, Forward mutagenic primer used to revert Leucine (L) back to
313 Phenylalanine (F) using the pCauErg11-F126L plasmid as a template. After mutagenesis, this
314 construct contained both wild type nucleotides and amino acids (pCauErg11-'wt'). **E**, Plasmid
315 sequencing chromatograms of relevant codons corresponding to the 125th and 126th amino
316 acids following mutagenesis and propagation in *E. coli*.
317
318