Transcriptional regulation of the synthesis and secretion of farnesol and aromatic fusel alcohols in the fungus *Candida albicans:* Examination of the Homann transcription regulator knockout collection

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Keywords

- *Candida albicans*
- Farnesol
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- Transcription factor mutant

Abstract

Candida albicans is an efficient colonizer of human gastrointestinal tracts and skin and is an opportunistic pathogen. *C. albicans* exhibits morphological plasticity and the ability to switch between yeast and filamentous morphologies is associated with virulence. One regulator of this switch is the quorum sensing molecule farnesol which is produced in *C. albicans* cells throughout growth. In addition, aromatic fusel alcohols accumulate in *C. albicans* supernatants and are implicated in regulating morphogenesis. The synthesis, secretion, and turnover of these molecules is not fully understood. To address this, we used our improved assay for farnesol and aromatic fusel alcohol quantification of whole cultures, pellets, and supernatants, to screen a transcription regulator knockout library of 164 mutants for differences in farnesol and aromatic fusel alcohol accumulation and localization. We found that all mutants produced farnesol with an average of 9.2X more farnesol localizing to the pellet in most mutants. Nineteen mutants had significant differences in accumulation and nine differed in localization. Ten mutants produced more farnesol than their SN152^{+} parent, while nine produced less. Seven mutants exhibited greater secretion of farnesol while two exhibited less. Several of these mutants have known roles in white/opaque switching and cell cycle progression such as the farnesol overproducing mutants *ahr1* and *swi4*. The aromatic fusel alcohols were similarly characterized by four over- and eleven under-producing mutants. Phenethyl alcohol and tryptophol accumulation were closely proportional to one another, but despite their structural similarity, phenethyl alcohol and tyrosol accumulation were often regulated differently.

62 **Introduction**

63 *Candida albicans* is an opportunistic pathogen that is present in most human gastrointestinal
64 tracts and is an efficient colonizer of mucosal surfaces (Neville et al. 2015). A weakened immune system
65 (HIV, chemotherapy, organ transplantation) or loss of competing flora (antibiotic treatment) allows for
66 *C. albicans* to colonize and invade host tissues leading to candidiasis (Pfaller and Diekema 2007).
67 Virulence of *C. albicans* is strongly linked to its ability to switch between yeast and filamentous
68 morphologies (Romano 1966; Kadosh 2019) as mutants that have lost the ability to switch remain
69 avirulent (Lo et al. 1997; Saville et al. 2003). Dimorphism is also important for adaptation to different
70 host environments (Noble et al. 2017; Alves et al. 2020) and evasion of host immune responses (Gow et
71 al. 2012). One regulator of dimorphic switching is the quorum sensing molecule (QSM) farnesol (Hornby
72 et al. 2001). Farnesol is produced by *C. albicans* cells continuously throughout growth (Boone et al.
73 2022). However, despite numerous publications on farnesol's role as a QSM and a virulence factor, the
74 actual mechanisms regulating its synthesis and secretion remain unclear.

75 *C. albicans* also secretes the aromatic fusel alcohols phenethyl alcohol, tyrosol, and tryptophol
76 (Ghosh et al. 2008), with most accumulation occurring in stationary phase (Boone et al. 2022). The
77 aromatic fusel alcohols are a product of the Ehrlich pathway (Hazelwood et al. 2008) in *S. cerevisiae* and
78 *C. albicans*. These molecules may also participate in yeast-mycelia morphogenesis (Chen et al. 2004;
79 Chen and Fink 2006) and biofilm formation (Alem et al. 2006), but regulation of their accumulation
80 remains unclear.

81 As an attractive approach to identifying these genes and mechanisms, we tested the hypothesis
82 that farnesol and fusel alcohol synthesis and secretion are regulated by the transcriptional networks
83 involved in morphogenesis and virulence. The transcription regulator knockout library generated by
84 Homann and colleagues consists of deletion strains for 165 different transcriptional regulators, in most
85 cases two independently derived knockouts (Homann et al. 2009). Thus, in the present paper the strains

86 in the transcription regulator knockout library were grown in liquid culture and then assayed by the
87 improved methods of Boone et al. (2022) for total, extracellular, and intracellular farnesol and the
88 aromatic fusel alcohols. Key features of this assay include prevention of analyte loss by avoiding
89 filtration and evaporation, while incorporating simultaneous cell lysis and analyte extraction by ethyl
90 acetate with the ability to compare whole cultures with the sum of their cell pellets and supernatants. It
91 is appropriate to screen for farnesol and the aromatic fusel alcohols together because there have been
92 numerous reports that they are all QSM for the yeast-mycelial transition in *Candida albicans*.
93

94 **Materials and Methods**

95 **Strains and media**

96 The homozygous transcription regulator deletion mutants were obtained from the transcription
97 regulator knockout library provided by Dr. Alexander Johnson's lab (Homann et al. 2009) available
98 through the Fungal Genomics Stock Center (Manhattan, Kansas, USA; McCluskey et al. 2010). All assays
99 with these mutants include the paired wild-type strain located in the H12 well of the X2 and Y2
100 independently derived deletion collections. The strains were maintained at 30°C on plates with YPD
101 (1% yeast extract, 2% peptone, 2% glucose). The transcription regulator knockout mutants were
102 constructed from *C. albicans* SN152, an auxotroph for arginine, histidine, and leucine. Each of the
103 transcription regulator (TR) mutants is auxotrophic for arginine. A wild-type control strain was created
104 by reintroduction of a single allele of HIS1 and LEU2 into the parent strain (Homann et al. 2009).
105 Throughout this paper, we will refer to this control strain simply as $SN152^{+}$.
106

107 **Screening the transcription regulator deletion mutants for differences in farnesol and aromatic fusel**
108 **alcohol accumulation and secretion**

109 The transcription regulator mutant collection was transferred by a 48-pin replicator to YPD
110 plates and grown at 30°C for 48 hours. Mutants were then transferred to 3 mL YPD liquid medium and
111 grown for 16 hours at 30°C with rotary agitation at 250 RPM. This culture was used as the inoculum
112 (1:100) for 75 mL YPD in 250 mL flasks which were incubated 24 hours at 30°C, 250 RPM. 50 mL of
113 culture was used for dry weight determination and 20 mL (2 X 10 mL) was used for determination of
114 farnesol and aromatic fusel alcohol production as described by Boone et al. (2022). In this method, 10
115 mL was used to measure whole culture values while the other was centrifuged to obtain the pellet and
116 supernatant values. Due to the large number of mutants in the collection, the mutants were assayed in
117 batches of 10-20 mutants with the $SN152^+$ control assayed in triplicate as apart of each batch. Mutants
118 with farnesol or aromatic fusel alcohol accumulation of greater than 1 $\log_2$ fold above or below the
119 mean value for $SN152^+$ were chosen for follow-up analysis. In follow-up analyses, E,E-farnesol and
120 aromatic fusel alcohol accumulation were measured in at least three independent experiments using
121 both the X and Y independently-derived TR deletion mutants.
122
123 **Statistical analysis**

124 Statistical analyses were performed using Microsoft Excel (Version 16.61, Microsoft Office, Las
125 Vegas, NV, USA) and GraphPad Prism Software (Version 9.5.0, San Diego, CA, USA). All biological data
126 are represented as mean ± SD of at least 3 biological replicates in both the X and Y independent mutants
127 unless otherwise stated. Mutants with significant differences between the X and Y mutant were
128 excluded and considered a false positive. Normality and homogeneity of variance were assessed by the
129 D' Agostino-Pearson omnibus (K2) test and Brown-Forsythe test, respectively. Differences between
130 groups that were normally distributed and homoscedastic (equal variances) were assessed by One-way
131 ANOVA with Dunnett's multiple comparisons test. Differences between groups that were not normally
132 distributed or heteroscedastic were assessed by Kruskal-Wallis test with Dunn's multiple comparisons

133 tests. Both analyses were performed in GraphPad Prism. Differences were considered significant at
134 $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

135

136 **Results**

137 **Identification of transcription regulator mutants defective in accumulation of intracellular and**
138 **extracellular farnesol and aromatic fusel alcohols**

139 Homann et al. (2009) screened their collection of 165 independent transcription regulator mutants
140 for possible differences in 55 growth conditions. It was a monumental piece of work. However, for
141 practical reasons they limited themselves to phenotypes discernible via colonies on agar plates. We
142 have now extended the list of phenotypes to include accumulation of both intracellular and extracellular
143 farnesol as well as the aromatic fusel alcohols, as determined by our improved GC assay (Boone et al,
144 2022). For the initial screen, 198 cultures were screened, consisting of 164 mutants from the X plates
145 and 34 replicate controls for the $SN152^+$ parent, assayed in 12 batches including duplicate or triplicate of
146 the parent in each run, for a total of 594 GC-FID runs.

147 For the rescreen, 54 strains were selected because their farnesol and/or fusel alcohol values
148 differed from the average values for $SN152^+$ by a $\log_2$ fold change of > 1.0. Each of these values was
149 normalized on a per cell basis using the 50 mL dry weight value of the culture assayed. The initial screen
150 data set contains the same information for all 164 TR mutants with n=1. The rescreen data set examines
151 54 TR mutants, both raw data and their means ± SD with n=6, reporting normalized accumulation values
152 for phenethyl alcohol, farnesol, tyrosol, and tryptophol as detected in whole cultures, pellets, and
153 supernatants. For each mutant we also report the mean $\log_2$ fold change vs the $SN152^+$ control for each
154 of the four analytes as detected in whole cultures, pellets, and supernatants, as well as the
155 supernatant/pellet ratio for each analyte. Composite data sets for the initial screen (File S1) and
156 rescreen (File S2) are available at Figshare. We now describe several highlights from these data sets.

157 **General Design and Reproducibility**

158 Our initial screen of 164 strains, all from the X plate, was done with an n = 1 comparing whole
159 culture values versus the sum of the cell pellet and supernatant values. Strain *hfl1Δ/Δ* [orf19.3063] was
160 excluded as it exhibited a severe growth defect on YPD. The 164 TR mutants were assayed in 12 batches
161 which always included a biological duplicate or triplicate of the $SN152^+$ parent. In this initial screen, the
162 paired WT controls were run a total of 34 times and their whole culture mean values were 5.29 ± 1.12,
163 115.32 ± 31.32, 30.24 ± 5.60, and 9.52 ± 3.00 ng/µL/ 50 mL dry weight for E,E-farnesol, phenethyl
164 alcohol, tryptophol, and tyrosol, respectively. Thus, the ratio of mean values for the three fusel alcohols
165 were 12:3:1 for phenethyl alcohol, tryptophol, and tyrosol. The distribution of these 34 values for
166 $SN152^+$ is shown in Fig. 1 where they can be seen in comparison with the means and distribution of the
167 164 TR mutants. For reference, these values are denoted by the red X in Figs. 2-5. Fig. 1 confirms that
168 the vast majority of the 164 TR mutants were unchanged from the $SN152^+$ replicates. As expected
169 (Boone et al. 2022), the whole culture (W) and pellet (P) + supernatant (S) values were always very close
170 to one another with the average WPS relative error values (W-(P+S)/W) being: phenethyl alcohol 0.12 ±
171 0.09, E,E-farnesol 0.07 ± 0.06, tyrosol 0.21 ± 0.15, and tryptophol 0.19 ± 0.28.

172 We then identified all mutants which were > 1 $\log_2$ above or below the mean values, as
173 indicated by the 4 dotted lines in Fig. 2, and designated them as mutants of interest to be rescreened
174 with greater accuracy. There were 31 mutants with higher or lower farnesol accumulation and 40
175 mutants with higher or lower aromatic fusel alcohols; they made a cohort of 49 strains because 22
176 strains had overlapping differences. To these we added 5 strains (*upc2Δ/Δ, tac1Δ/Δ, dal81Δ/Δ,*
177 *aro80Δ/Δ, and csr1Δ/Δ*) because prior reports from the literature indicated they were likely to have
178 altered farnesol or aromatic fusel alcohol accumulations. These 54 mutant strains were then rescreened
179 five more times, two from the X plate and three times from the Y plate (total n =6) in 10 batches
180 including the $SN152^+$ parent in biological duplicate or triplicate, requiring 292 flasks and 876 GC

181 measurements. Ten mutants (*cta7Δ/Δ, cwt1Δ/Δ, rim101Δ/Δ, crz1Δ/Δ, zcf1Δ/Δ, ofi1Δ/Δ, leu3Δ/Δ,*
182 *tea1Δ/Δ, ume7Δ/Δ, rme1Δ/Δ*) were designated as false positives because their rescreened values from
183 the X and Y plates differed significantly or their rescreen analyte values differed significantly from the
184 initial screen. Thus, the TR mutants which were identified as being statistically different included ten
185 with elevated farnesol and nine with reduced farnesol (Fig. 6A-B, Table 1), four with elevated aromatic
186 fusel alcohols and 11 with reduced aromatic fusel alcohols (Table 2, Fig. 7A-B), and seven with elevated
187 farnesol supernatant/pellet ratios and two with lowered farnesol supernatant/pellet ratios (Fig. 6C).
188 These constituted only 35 mutants of interest because three mutants (*swi4Δ/Δ, rap1Δ/Δ, and cas5Δ/Δ)*
189 were on all three lists, and another ten mutants were on two.

190 **Mutants altered in farnesol accumulation**

191 Of the 19 mutants with statistically significant differences in farnesol accumulation, only
192 *nrg1Δ/Δ* had been identified in prior literature (Kebaara et al. 2008). Seven mutants (*swi4Δ/Δ, ahr1Δ/Δ,*
193 *nrg1Δ/Δ, rap1Δ/Δ, pho4Δ/Δ, tye7Δ/Δ, aaf1Δ/Δ*) had increased farnesol accumulation either in the whole
194 culture or supernatant fraction with a $\log_2$ fold change of 1 or greater compared to the $SN152^+$ parent
195 strain (Table 1). The two highest accumulators (*swi4Δ/Δ* and *ahr1Δ/Δ)* have known roles in cell cycle
196 progression (Hussein et al. 2011; Sellam et al. 2019). Two farnesol over accumulating mutants (*tye7Δ/Δ*
197 and rtg1*Δ/Δ*) have roles in carbohydrate metabolism (Askew et al. 2011; Moreno-Velásquez et al. 2020).
198 Four farnesol over accumulating mutants (*ahr1Δ/Δ, nrg1Δ/Δ, ash1Δ/Δ, ssn6Δ/Δ*) have a role in
199 dimorphism (Fu et al. 1998; Murad et al. 2001; Inglis and Johnson 2002; García-Sánchez et al. 2005).
200 Three farnesol over accumulating mutants (*ahr1Δ/Δ, ssn6Δ/Δ, wor2Δ/Δ)* have roles in white/opaque
201 switching (Zordan et al. 2007; Wang et al. 2011; Hernday et al. 2016). This is of particular interest as
202 another regulator of white/opaque switch, Czf1, has a known role in coordinating the response to
203 farnesol (Langford et al. 2013), and farnesol production is turned off in both anaerobically grown cells
204 (Dumitru et al. 2004) and opaque cells (Dumitru et al. 2007).

205 Eight mutants (*orf19.6874Δ/Δ, msn4Δ/Δ, cph2Δ/Δ, fcr1Δ/Δ, sko1Δ/Δ, gzf3Δ/Δ, cas5Δ/Δ,*
206 *cup9Δ/Δ*) had decreased farnesol accumulation either in the whole culture or supernatant fraction with
207 a $\log_2$ fold change of <1 or greater compared to the SN152$^+$ parent strain (Table 1). Three of these low
208 farnesol accumulating mutants (*msn4Δ/Δ, sko1Δ/Δ, cas5Δ/Δ*) have known roles in stress response
209 (Nicholls et al. 2004; Bruno et al. 2006; Rauceo et al. 2008; Xie et al. 2017) and two mutants (*sko1Δ/Δ,*
210 *cup9Δ/Δ*) have known roles in farnesol response (Lu et al. 2014).
211 Three of the four mutants chosen because of literature precedent had elevated farnesol, but did
212 not reach statistical significance (*csr1Δ/Δ, upc2Δ/Δ, tac1Δ/Δ*) (Table 1). One mutant (*tup1Δ/Δ*) did not
213 appear to over accumulate farnesol in these assay conditions (Table 1). At present we have no
214 explanation why the *tup1Δ/Δ* mutant did not over produce farnesol at the levels we had reported
215 previously (Kebaara et al. 2008).
216 **Mutants altered in the farnesol supernatant/pellet ratio**
217 The supernatant/pellet (S/P) ratios for each analyte were determined for each mutant in
218 comparison to those for their SN152$^+$ control (Fig. 3). The value for SN152$^+$ (n= 22 batches) was 0.12 for
219 farnesol. As we had observed previously (Boone et al. 2022), farnesol was predominantly retained in the
220 cell pellet. The S/P ratios were all tightly clustered from 0.11-0.16 except for four (*msn4Δ/Δ, fcr1Δ/Δ,*
221 *gzf3Δ/Δ, and orf19.6874Δ/Δ*) which exhibited less secretion (0.05-0.08) and seven (*swi4Δ/Δ, pho4Δ/Δ,*
222 *cas5Δ/Δ, hap5Δ/Δ, ssn6Δ/Δ, tye7Δ/Δ, and rap1Δ/Δ*) which exhibited greater secretion (0.19-0.30) (Fig.
223 6C).
224 Among these mutants, *hap5Δ/Δ* and *cas5Δ/Δ* are of special interest in that they exhibit greater
225 secretion without being accompanied by greater total farnesol production (Fig. 3). Thus, they target
226 secretion specifically. Mutants such as *swi4Δ/Δ, ahr1Δ/Δ, and rap1Δ/Δ* with much greater farnesol
227 production (Fig. 6A, Table 1) could exhibit a higher supernatant to pellet ratio because internal and
228 external farnesol are in equilibrium or there is a need to keep internal farnesol levels below a critical

229 threshold value. In both cases, the greater secretion would be an indirect consequence of greater
230 production.
231
232 **Mutants altered in aromatic fusel alcohol accumulation**
233 Nineteen mutants were identified with statistically significant differences in aromatic fusel
234 alcohol accumulation (Table 2, Fig. 7); 15 exhibited decreased production while only four exhibited
235 increased production. The four with increased production were *CR_02120CΔ/Δ, fgr15Δ/Δ, gzf3Δ/Δ,* and
236 *hap5Δ/Δ*. *CR_02120CΔ/Δ* and *fgr15Δ/Δ* were increased for all three aromatic fusel alcohols, *gzf3Δ/Δ* for
237 only phenethyl alcohol, and *hap5Δ/Δ* for only tyrosol (Table 2, Fig. 7A). Gzf3 has a known role in
238 negatively regulating nitrogen catabolite repression (Soussi-Boudekou et al. 1997).
239 Fifteen mutants had statistically significant decreased accumulation (*tup1Δ/Δ, ssn6Δ/Δ, stp2Δ/Δ,*
240 *rap1Δ/Δ, dal81Δ/Δ, nrg1Δ/Δ, stp4Δ/Δ, swi4Δ/Δ, ahr1Δ/Δ, czf1Δ/Δ, ndt80Δ/Δ, cph2Δ/Δ, bcr1Δ/Δ,*
241 *cas5Δ/Δ, aro80Δ/Δ).* Of these, 5 mutants (*tup1Δ/Δ, ssn6Δ/Δ, rap1Δ/Δ, stp4Δ/Δ, ndt80Δ/Δ*) were
242 decreased for all three aromatic fusel alcohols and five mutants (*stp2Δ/Δ, dal81Δ/Δ, nrg1Δ/Δ, swi4Δ/Δ,*
243 *cph2Δ/Δ*) were decreased for phenethyl alcohol and tryptophol (Table 2, Fig. 7B). A plot of phenethyl
244 alcohol accumulation vs tryptophol accumulation for $SN152^{+}$ and all 164 TR mutants (Fig. 4) showed that
245 these two fusel alcohols were closely proportional to one another, at least when grown on YPD. Tup1
246 and Ssn6 form a corepressor complex in yeast and have a known role in regulating amino acid
247 permeases (Tanaka and Mukai 2015), while Stp2 is an amino acid regulated TR that regulates amino acid
248 permeases (Martínez and Ljungdahl 2005). Dal81 has a known role in positively regulating nitrogen
249 degradation in *S. cerevisiae* (Bricmont et al. 1991), but has a different role in regulating arginine
250 synthesis in *C. parapsilosis* (Turner et al. 2018).
251 Finally, two mutants (*ahr1Δ/Δ, czf1Δ/Δ*) were decreased in phenethyl alcohol only, one mutant
252 *aro80Δ/Δ* in tyrosol only, one mutant *cas5Δ/Δ* in tryptophol only, and one mutant *bcr1Δ/Δ* in both

253 tryptophol and tyrosol (Table 2). Aro80 has a known role in regulating the Ehrlich pathway (Ghosh et al.
254 2008). An intriguing suggestion from these data is that despite their close structural similarity, phenethyl
255 alcohol and tyrosol accumulation are regulated differently. This idea is further supported by comparison
256 of their respective syntheses in all the TR mutants (Fig. 5).

257 **Mutants altered in the aromatic fusel alcohol supernatant/pellet ratio**

258 The three fusel alcohols were mostly secreted. The S/P ratios for SN152^{+} (n=22) were 13.9, 8.9,
259 and 6.6 for phenethyl alcohol, tyrosol, and tryptophol, respectively. For phenethyl alcohol, the S/P ratios
260 were all tightly clustered from 11-20 except for *tup1Δ/Δ* at 2.34, *nrg1Δ/Δ* at 5.45, and *ssn6Δ/Δ* at 9.53. In
261 contrast, the S/P values for tryptophol were more diverse; 36 mutants were in the 5-8 range, close to
262 SN152^{+}, while 10 exhibited much less secretion (1.1-3.8) and another 10 exhibited much greater
263 secretion (11.6-34) (File S2).

264 **Discussion**

265 We examined each of the 164 independent transcriptional regulator knockout mutants for their
266 accumulation and secretion of farnesol and the three aromatic fusel alcohols. This project was
267 undertaken to define the genes needed for their synthesis, secretion, and regulation. For logistical
268 reasons, all cultures were grown at 30°C in liquid YPD medium and sampled after 24 hours when the
269 cells were in early stationary phase. The 24 hour time point was chosen based on our prior
270 measurements with wild type *C. albicans* SC5314 over 72 hours of culture which showed that the time
271 courses for farnesol and fusel alcohol production differed dramatically (Boone et al. 2022). Farnesol
272 generally followed increased cell mass, peaked in early stationary phase, and then declined sharply after
273 ~ 30 hours. In contrast, accumulation of the aromatic fusel alcohols only started about 12 hours after
274 inoculation, just as the cells were entering stationary phase, and continued throughout stationary phase
275 (Boone et al. 2022). Thus, the 24 hour time point was intended to cover the four analytes of interest.

276 Similarly, our choice of YPD as a growth medium was to insure that all of the TR mutants grew well by
277 24hrs and indeed 164 out of 165 had done so.
278 The choice of a single 24 hour time point also impacts the observed fusel alcohol production
279 ratios. The SN152^{+} ratios at 24 hours were 12:3:1 for phenethyl alcohol, tryptophol, and tyrosol,
280 respectively (Figs. 4 and 5), but they would likely be different if we had chosen a 36 or 48 hour time
281 point instead. In an earlier study with *C. albicans* SC5314, we found that the appearance of phenethyl
282 alcohol and tryptophol closely paralleled one another, plateauing in accumulation by 36 hours, but the
283 appearance of tyrosol was delayed, with much of it accumulating after 36 hours (Boone et al. 2022).
284 With the advantage of now having reliable measures for analyte presence in whole cultures, cell
285 pellets, and supernatants, we can now approach the genetic basis for their synthesis, secretion,
286 regulation, and turnover. As expected, most of the TR mutants were unchanged for these parameters
287 (Figs. 1-5) while a few potentially useful mutants gave significantly higher and lower levels of the
288 analytes of interest. Significantly, no mutants were entirely devoid of farnesol; its levels often varied but
289 they never dropped to zero. A tentative conclusion from this observation is that farnesol has an
290 essential physiological function over and above acting as a quorum sensing molecule and a virulence
291 factor or is the product of a redundant biosynthetic pathway.
292 There are many questions which can now be approached via the genetic information gleaned
293 from this screen. Among them are: 1/ How is carbon flow through the farnesyl pyrophosphate (FPP)
294 branch point regulated? 2/ Is farnesol made directly from FPP by an appropriate pyrophosphatase (e.g.
295 Dpp1p, Dpp2p, or Dpp3p) or are there other more easily regulated and/or safer biosynthetic pathways?
296 A method suggested from *Arabidopsis* invokes the necessary turnover of farnesylated proteins, where
297 proteolytic degradation of those proteins releases farnesylated cysteine which can be cleaved by a ligase
298 to release farnesaldehyde which is then converted to farnesol by a farnesol dehydrogenase (Bhandari et
299 al. 2010). 3/ Is the disappearance of farnesol later in stationary phase due strictly to evaporation of a

300 volatile molecule or to enzymatic conversion of farnesol to, for instance, 2,3-dihydrofarnesol (Brasch et
301 al. 2013; Costa et al. 2020). Significantly, regarding its ability to block germ tube formation and hyphal
302 development, 2,3-dihydrofarnesol is inactive; with only 0.34% of the activity of farnesol (Shchepin et al.
303 2003). 4/ Large amounts of the aromatic fusel alcohols are produced, usually in the ratios of phenethyl
304 alcohol >> tyrosol and tryptophol. Are these fusel alcohols synthesized via the well-known Ehrlich
305 pathway as in *S. cerevisiae* (Hazelwood et al. 2008), through the biosynthetic shikimic acid pathway
306 using prephenate dehydratase or dehydrogenase (Dai et al. 2021), or as in Arabidopsis from tyrosine to
307 tyrosol via a tyramine intermediate (Chung et al. 2017; Rodrigues and Černáková 2020)? These are just
308 four examples. Likely many more will surface following a careful examination of the collected data now
309 available in this mutant screen.

310 **Author contributions:**

311 DJG: conceptualization, methodology, validation, investigation, writing — original draft, writing —
312 review and editing, visualization, formal analysis, data curation, project administration; JJK: validation,
313 investigation, writing — review and editing; BMT: validation, investigation, writing — review and editing;
314 CHTB: methodology, validation, writing – review and editing; ALA: conceptualization, methodology,
315 resources, supervision, writing — review and editing, project administration; KWN: conceptualization,
316 methodology, resources, supervision, writing — original draft, writing — review and editing, project
317 administration, funding acquisition.

318 **Data availability:**

319 The datasets generated during and/ or analyzed during the current study are available from the
320 corresponding author on reasonable request. The initial screen (File S1) and rescreen dataset (File S2)
321 are available at Figshare.

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Compliance with ethical standards:

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493 **Tables**

494 Table 1. Summary of transcription regulator mutants with statistically significant differences in farnesol
495 accumulation.[a/]

Mutant	*S. cerevisiae* ortholog or best hit from CGD (Skrzypek et al. 2017)	Mean whole culture E,E-farnesol log_2 fold change vs WT control ± SD (n=6)	Mean supernatant fraction E,E-farnesol log_2 fold change vs WT control ± SD (n=6)	Predicted or known biological process(es) in *Candida albicans* adapted from CGD (Skrzpek et al. 2017)
E,E-farnesol over accumulating mutants				
swi4Δ/Δ	*SWI4*	2.57*** ± 0.39	3.11*** ± 0.50	Component of SBF complex, G1/S progression
ahr1Δ/Δ	*LYS14*	2.27*** ± 0.42	2.56 ***± 0.32	Regulation of adhesion genes, white/opaque switch, repressor of START
nrg1Δ/Δ	*NRG2*	1.73*** ± 0.45	1.93*** ± 0.49	Regulator of hyphal gene induction, virulence, chlamydospore formation, stress response
rap1Δ/Δ	*RAP1*	1.15*** ± 0.62	2.42*** ± 0.48	Telomere maintenance
pho4Δ/Δ	*PHO4*	1.07*** ± 0.53	1.68*** ± 0.23	Phosphate acquisition
tye7Δ/Δ	*TYE7*	0.95** ± 0.21	1.83*** ± 0.07	Regulator of glycolysis and carbohydrate metabolism
ash1Δ/Δ	*ASH1*	0.82* ± 0.43	0.92** ± 0.28	GATA-like transcription factor required for WT virulence and filamentous growth
aaf1Δ/Δ	*none*	0.76* ± 0.31	1.21*** ± 0.19	Expression in *S. cerevisiae* increases adherence
rtg1Δ/Δ	*RTG1*	0.63 ± 0.49	0.96** ± 0.20	Regulation of sphingolipid homeostasis, galactose catabolism
ssn6Δ/Δ	*CYC8*	0.20 ± 0.90	0.99*** ± 0.73	Forms a corepressor complex with Tup1, regulates a distinct set of

				genes different than Tup1 in *C. albicans*
wor2Δ/Δ	*UME6*	0.43 ± 0.82 (p=0.999)	0.59 ± 0.83 (p=0.217)	Regulator of white/opaque switching, maintenance of opaque state
tac1Δ/Δ #	*HAL9*	0.23 ± 0.56 (p=0.999)	0.36 ± 0.26 (p=0.919)	Transcriptional activator of drug-responsive genes *CDR1* and *CDR2*
csr1Δ/Δ #	*ZAP1*	0.32 ± 0.31 (p=0.984)	0.60 ± 0.13 (p=0.195)	Transcription factor, role in zinc homeostasis
upc2Δ/Δ #	*UPC2*	0.11 ± 0.43 (p=0.999)	0.31 ± 0.46 (p=0.980)	Regulator of ergosterol biosynthetic genes and sterol uptake
tup1Δ/Δ #	*TUP1*	0.0 ± 0.15 (p=0.999)	-0.02 ± 0.47 (p=0.999)	Transcriptional corepressor with *Ssn6p*, regulates morphological switching, farnesol response, germ tube formation
E,E-farnesol under accumulating mutants				
orf19.6874 Δ/Δ (C2_05640 W_A)	*BAS1*	-2.10*** ± 0.73	-2.65*** ± 1.03	Transcription factor, null mutant exhibits increase in invasive growth
msn4Δ/Δ	*MSN2*	-1.89*** ± 0.37	-2.40*** ± 0.65	Zinc finger transcription factor, not a significant stress response regulator in *C. ablicans*
cph2Δ/Δ	*HMS1*	-1.93*** ± 0.63	-2.04*** ± 0.91	Transcriptional activator of hyphal growth
fcr1Δ/Δ	*CAT8*	-1.97*** ± 0.88	-3.68*** ± 3.83	Negative regulator of fluconazole/ketoconazole/brefeldin A resistance
sko1Δ/Δ	*SKO1*	-1.93*** ± 0.39	-1.41*** ± 0.46	Cell wall damage response, morphological switching, oxidative stress response
gzf3Δ/Δ	*GZF3*	-1.32*** ± 0.32	-1.63*** ± 0.54	GATA type transcription factor, ortholog regulates nitrogen catabolic gene expression

cas5Δ/Δ	*MOT3*	-1.16** ± 0.13	-0.51 ± 0.32	Cell wall homeostasis, adherence, stress response, cell cycle
cup9Δ/Δ	*TOS8*	-1.08** ± 0.63	-0.85 ± 0.86	Represses SOK1 expression in response to farnesol inhibition
tec1Δ/Δ	*TEC1*	-0.75* ± 0.66	-0.58 ± 0.76	Hyphal gene regulator, white cell pheromone response

496 a/ Log_2 fold change production values ± SD represent the mean farnesol accumulation value of 3 X and 3
497 Y (n=6) independently derived mutant compared to the mean farnesol accumulation value of the paired
498 SN152 parent (n=22. Both over and under accumulating mutants are ordered by how much they exceed
499 the SN152 parent. The four mutants added because of literature precedent are indicated by #.
500 Differences between mutants (n=6) and the $SN152^+$ parent (n=22 batches) were accessed by One-way
501 ANOVA with Dunnett's multiple comparisons test using GraphPad Prism. Differences were considered
502 significant at $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

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520 Table 2. Summary of transcription regulator mutants with statistically significant differences in fusel
521 alcohol accumulation.[a/]

Mutant	*S. cerevisiae* ortholog or best hit from CGD (Skrzypek et al. 2017)	Mean supernatant fraction log_2 fold change vs WT control ± SD (n=6)			Predicted or known biological process(es) in *Candida albicans* adapted from CGD (Skrzpek et al. 2017)
		Phenethyl alcohol	Tyrosol	Tryptophol	
Aromatic fusel alcohol over accumulating mutants					
19.2612Δ/Δ (CR_02120C)	*SWI5*	1.38*** ± 0.32	1.14* ± 0.62	1.39*** ± 0.40	zinc finger transcription factor of unknown function
fgr15Δ/Δ	*none*	1.06*** ± 0.37	0.63 ± 1.27	1.01** ± 0.22	Transposon mutation affects filamentous growth
gzf3Δ/Δ	*GZF3*	0.76*** ± 0.26	-0.24 ± 0.34	-0.04 ± 1.62	Ortholog negatively regulates nitrogen catabolic gene expression
hap5Δ/Δ	*HAP5*	0.13 ± 0.17	1.49*** ± 0.25	-0.15 ± 0.26	Regulator of genes involved in filamentous and respiratory growth; iron homeostasis
Aromatic fusel alcohol under accumulating mutants					
tup1Δ/Δ	*TUP1*	-4.90*** ± 0.20	-3.45*** ± 2.70	-3.56*** ± 0.45	Transcriptional corepressor with Ssn6, regulates morphological switching, farnesol response, germ tube formation
ssn6Δ/Δ	*CYC8*	-3.82*** ± 0.18	-2.26*** ± 1.61	-3.25*** ± 0.57	Forms a corepressor complex with Tup1, regulates a distinct set of genes different than Tup1 in *C. albicans*
stp2Δ/Δ	*STP2*	-3.10*** ± 0.19	0.20 ± 0.49	-2.58*** ± 0.38	Amino-acid-regulated; activator of amino acid permease genes

rap1Δ/Δ	*RAP1*	-2.01*** ± 0.61	-1.31* ± 1.05	-1.71*** ± 0.45	Telomere maintenance
dal81Δ/Δ	*DAL81*	-1.75*** ± 0.70	-1.17 ± 1.00	-1.87*** ± 0.89	Ortholog involved in activation of nitrogen-degradation genes
nrg1Δ/Δ	*NRG2*	-1.67*** ± 0.44	-0.24 ± 0.16	-2.39*** ± 1.31	Regulator of hyphal gene induction, virulence, chlamydospore formation, stress response
stp4Δ/Δ	*STP3*	-1.44*** ± 0.47	-1.37* ± 0.86	-1.32*** ± 0.64	transcription factor, paralogs have a role in amino acid metabolism
swi4Δ/Δ	*SWI4*	-1.33*** ± 0.30	0.00 ± 0.33	-0.94* ± 0.38	Component of SBF complex, G1/S progression
ahr1Δ/Δ	*LYS14*	-0.87*** ± 0.26	-0.70 ± 0.13	0.52 ± 0.17	Regulation of adhesion genes, white/opaque switch, repressor of START
czf1Δ/Δ	*UME6*	-0.81*** ± 0.28	-1.07 ± 0.73	-0.86 ± 0.31	Regulator of white/opaque switching and hyphal growth
ndt80Δ/Δ	*NDT80*	-0.81*** ± 0.22	-1.56** ± 0.81	-1.05** ± 0.21	meiosis-specific transcription factor; activator of *CDR1* induction by antifungal drugs
cph2Δ/Δ	*HMS1*	-0.78** ± 0.54	-1.12 ± 0.71	-1.16** ± 0.32	Transcriptional activator of hyphal growth
bcr1Δ/Δ	*USV1*	-0.58 ± 0.47	-1.51** ± 0.19	-1.01* ± 0.43	Regulator of a/α biofilm formation, adherence, cell surface genes, fluconazole resistance
cas5Δ/Δ	*MOT3*	-0.49 ± 0.28	0.12 ± 0.41	-1.11** ± 0.39	Cell wall homeostasis, adherence, stress response, cell cycle
aro80Δ/Δ [#]	*ARO80*	0.10 ± 0.19	-1.45** ± 0.82	0.16 ± 0.27	Activator of aromatic amino acid catabolism; regulator of aromatic alcohol biosynthesis

522 a/ Log_2 fold change production values ± SD represent the mean aromatic fusel alcohol accumulation
523 value of 3 X and 3 Y (n=6) independently derived mutant compared to the mean aromatic fusel alcohol
524 accumulation value of the paired SN152 parent (n=22). False positives are excluded. Both over and
525 under accumulating phenethyl alcohol mutants are ordered by how much they exceed the SN152
526 parent. Differences between mutants (n=6) and the $SN152^+$ parent (n=22 batches) were accessed by
527 One-way ANOVA with Dunnett's multiple comparisons test using GraphPad Prism. Differences were
528 considered significant at $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$). Note that *fgr15Δ/Δ, stp2Δ/Δ,*
529 *dal81Δ/Δ, and aro80Δ/Δ* only had a 1 independent mutant, so these were not accessed in the Y plate,
530 but instead 6 replicates in the X plate.

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568 **Figure Captions**

569 **Fig. 1.** Distribution of wild-type and transcription regulator mutant whole culture analytes from the
570 initial screen. Phenethyl alcohol, E,E-farnesol, tyrosol, and tryptophol were measured 24 hours post
571 inoculation following growth at 30°C in YPD. Data shown are the means of the initial screen
572 accumulation values for the $SN152^+$ parent (n=34) and transcription regulator mutants (n=164).

573 **Fig. 2.** Initial screen whole culture accumulation of phenethyl alcohol vs E,E-farnesol. Analytes were
574 measured 24 hours post inoculation at 30°C in YPD. Accumulation values are for each transcription
575 regulator mutant (black) (n=164) and each $SN152^+$ replicate (red) (n=34). The red X indicates the mean
576 value for the $SN152^+$ replicates (n=34).Dotted lines represent a log2 fold change increase or decrease, of
577 1 for each analyte from the means for $SN152^+$.

578 **Fig. 3.** Initial screen E,E-farnesol supernatant to pellet ratio vs E,E- farnesol whole culture accumulation
579 at 24 hours post inoculation. Data represents the accumulation values for each transcription regulator
580 mutant (black) (n=164) and each $SN152^+$ replicate (red) (n=34). The red X indicates the mean value for
581 the $SN152^+$ replicates (n=34). Dotted lines represent a log2 fold change increase or decrease of 1 for
582 each analyte from the mean for $SN152^+$.

583 **Fig. 4.** Initial screen whole culture accumulation of phenethyl alcohol vs tryptophol. Values were
584 measured 24 hours post inoculation at 30°C in YPD for each transcription regulator mutant (black)
585 (n=164) and each $SN152^+$ replicate (red) (n=34). The red X indicates the mean value for the $SN152^+$
586 replicates (n=34).

587 **Fig. 5.** Initial screen whole culture accumulation of phenethyl alcohol vs tyrosol. Values were measured
588 at 24 hours post inoculation at 30°C in YPD medium for each transcription regulator mutant (black)
589 (n=164) and each $SN152^+$ replicate (red) (n=34). The red X indicates the mean value for the $SN152^+$
590 replicates (n=34).

591 **Fig. 6.** Transcription regulator mutants differing in farnesol accumulation and localization. Accumulation
592 values were measured 24 hours post inoculation at 30°C in YPD for each transcription regulator mutant
593 in both the X (3) and Y (3) independent mutants (total n=6) and the SN152^{+} parent (n=22 batches). Data
594 represents the mean accumulation value ± SD (A,B) or mean farnesol supernatant to pellet ratio ± SD
595 (C). The mutants added because of literature precedent are indicated by #. Differences between groups
596 that were normally distributed and that had equal variances (C) were accessed by One-way ANOVA with
597 Dunnett's multiple comparisons test. Differences between groups that were not normally distributed or
598 heteroscedastic (A,B) were accessed by Kruskal-Wallis test with Dunn's multiple comparisons tests.
599 Differences were considered significant at $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$).
600 **Fig. 7.** Transcription regulator mutants differing in aromatic fusel alcohol accumulation. Accumulation
601 values were measured 24 hours post inoculation at 30°C in YPD for each transcription regulator mutant
602 in both the X (3) and Y (3) independent mutants (total n=6) and the SN152^{+} parent (n=22 batches). Data
603 represents the mean accumulation value ± SD for mutants over-accumulating (A) or under-accumulating
604 (B) aromatic fusel alcohols. The mutants added because of literature precedent are indicated by #.
605 Differences between groups that were not normally distributed or heteroscedastic (A,B) were accessed
606 by Kruskal-Wallis test with Dunn's multiple comparisons tests. Differences were considered significant at
607 $p<0.05$ (*$p<0.05$, **$p<0.01$, ***$p<0.001$).
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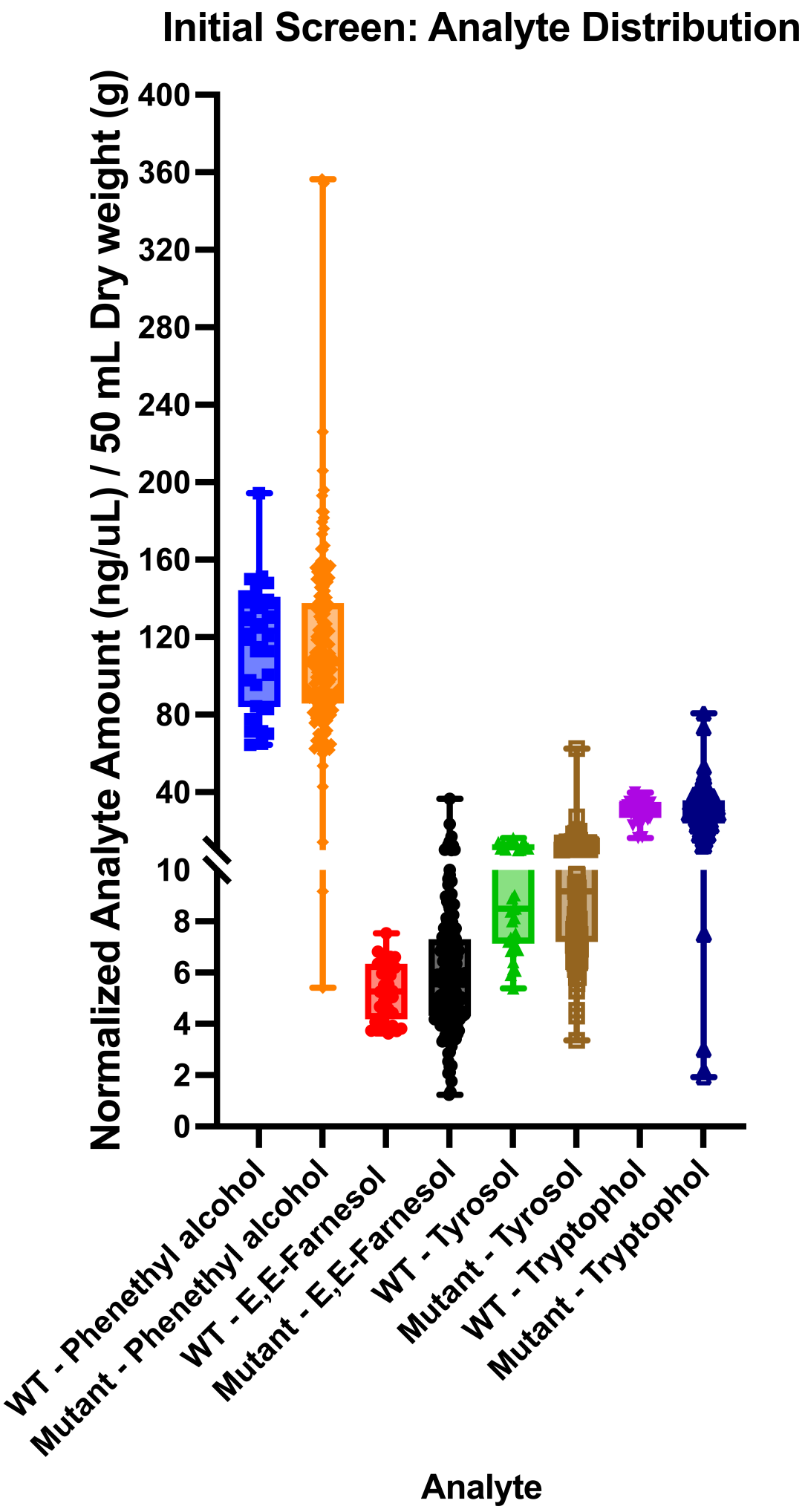
Initial Screen: Analyte Distribution
Normalized Analyte Amount (ng/uL) / 50 mL Dry weight (g)
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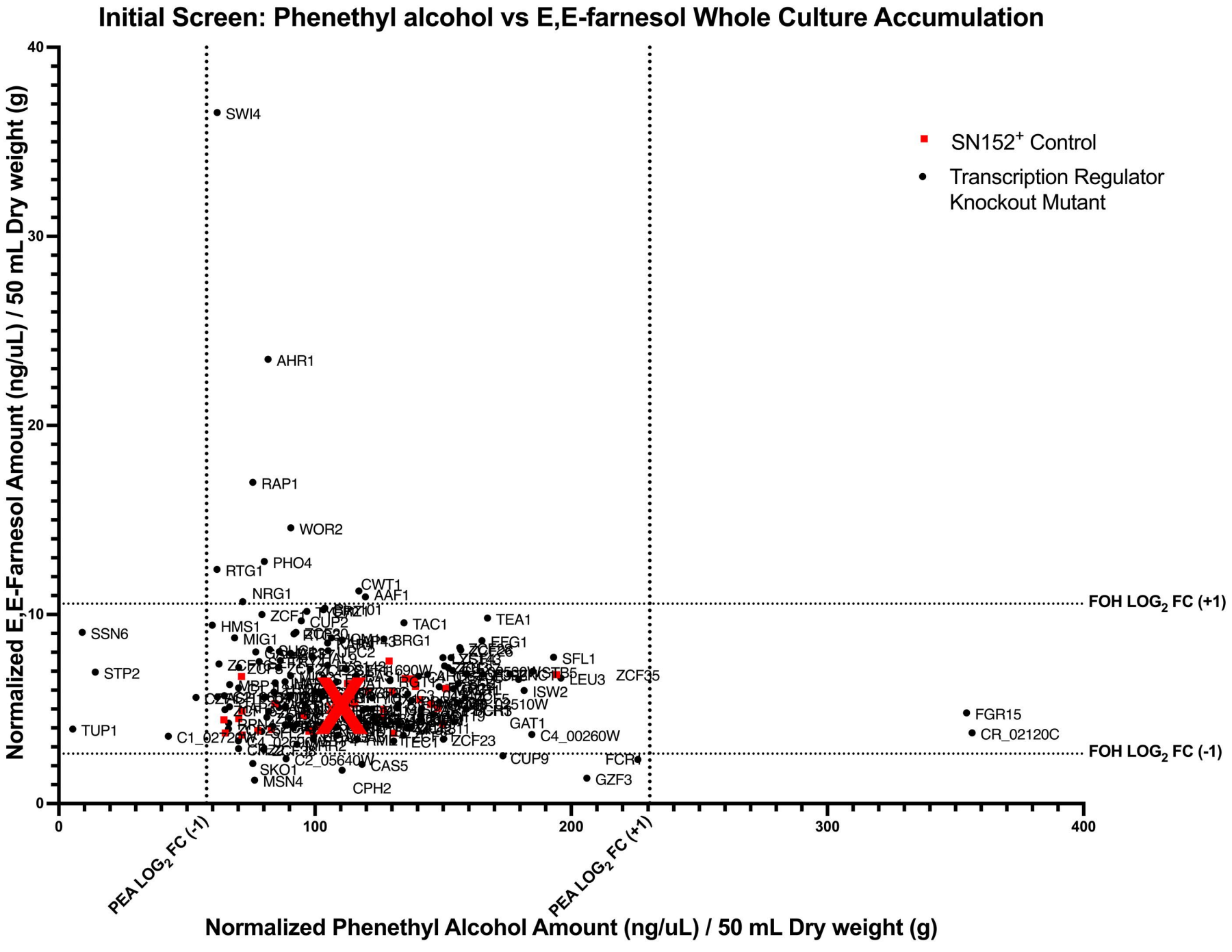
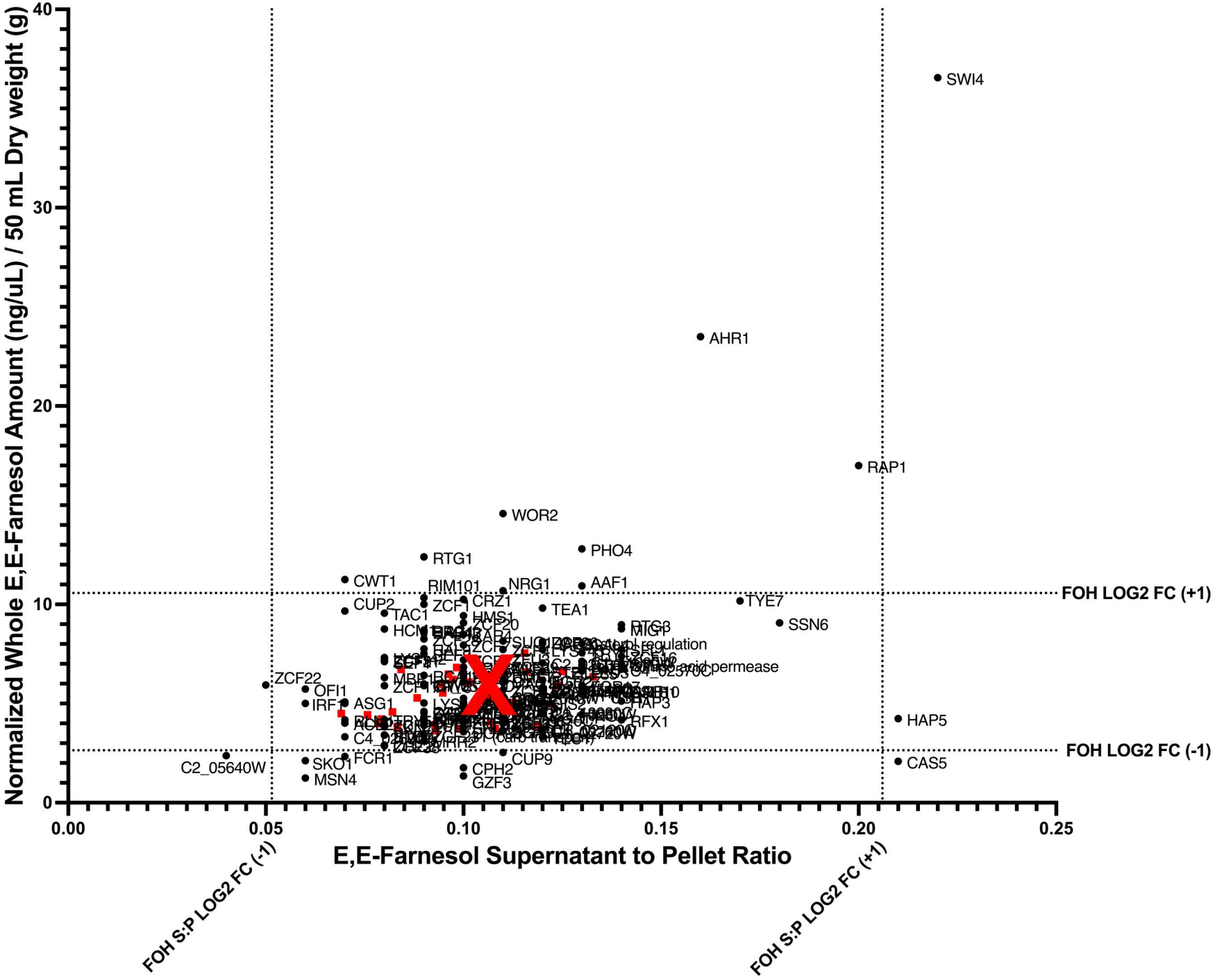
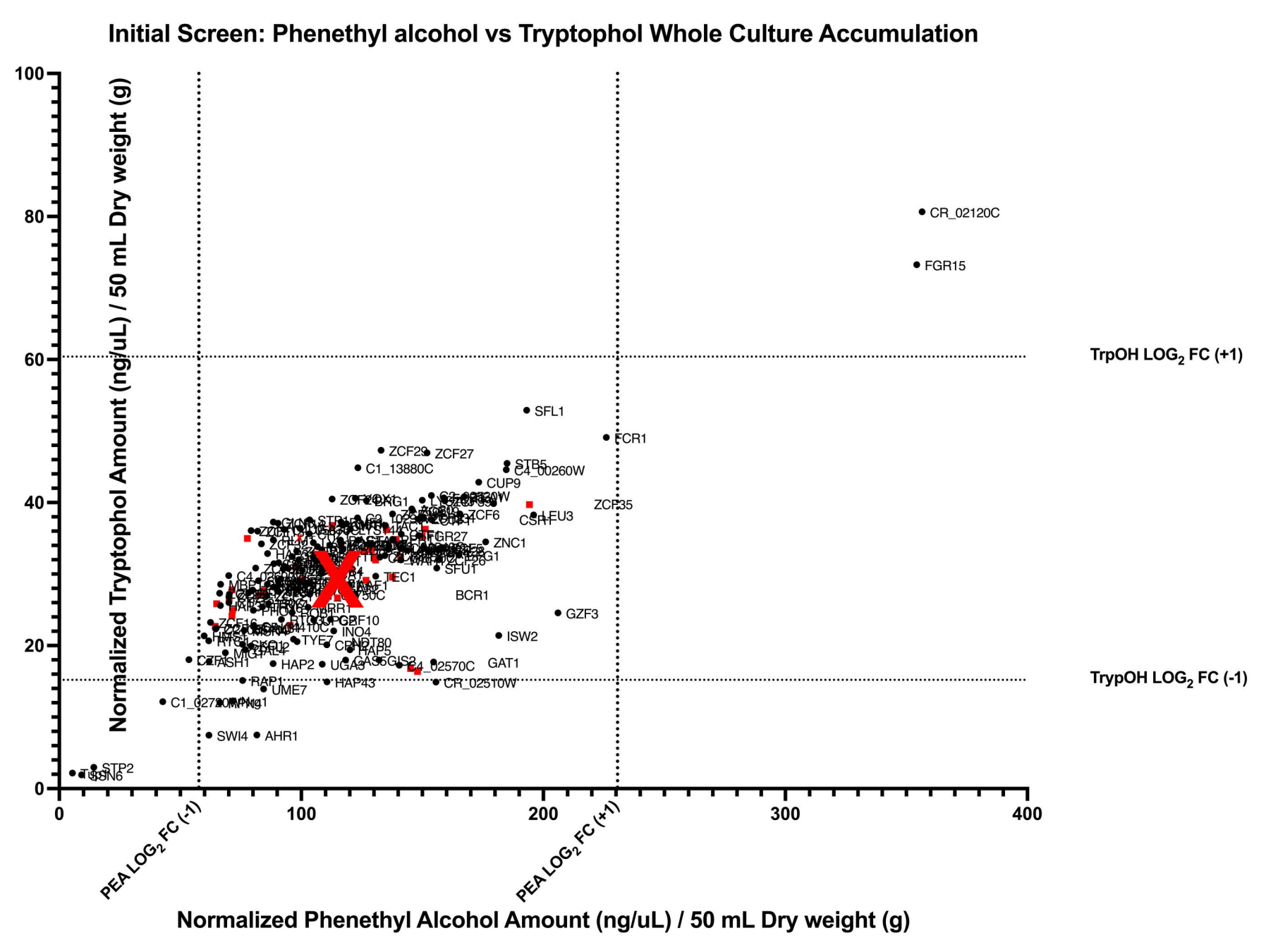
10
8
6
4
2
0
WT - Phenethyl alcohol
Mutant - Phenethyl alcohol
WT - E,E-Farnesol
Mutant - E,E-Farnesol
WT - Tyrosol
Mutant - Tyrosol
WT - Tryptophol
Mutant - Tryptophol
Analyte



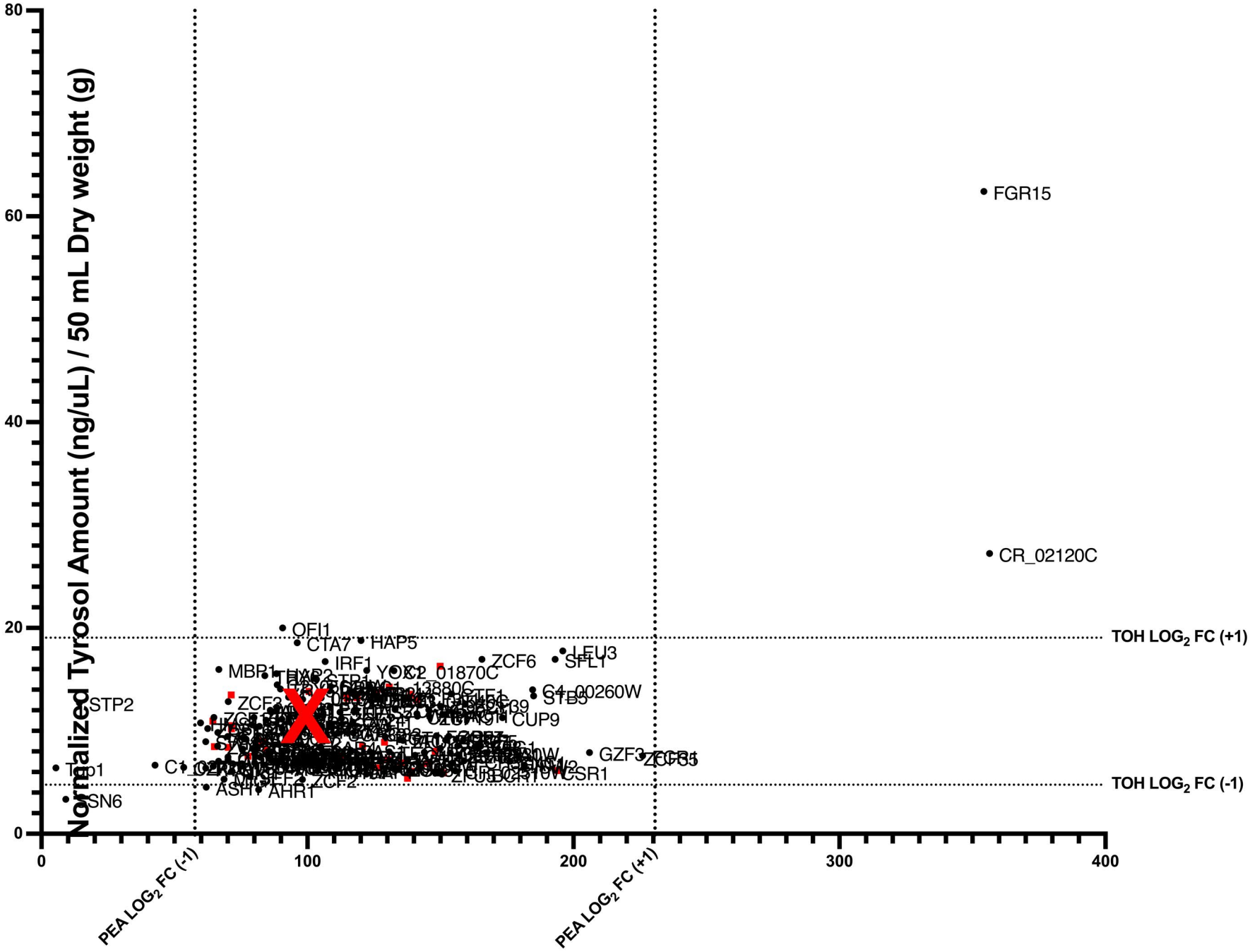
Initial Screen: Phenethyl alcohol vs E,E-farnesol Whole Culture Accumulation
Normalized E,E-Farnesol Amount (ng/uL) / 50 mL Dry weight (g)
Normalized Phenethyl Alcohol Amount (ng/uL) / 50 mL Dry weight (g)
SN152+ Control
Transcription Regulator Knockout Mutant
FOH LOG2 FC (+1)
FOH LOG2 FC (-1)
PEA LOG2 FC (-1)
PEA LOG2 FC (+1)
SWI4
AHR1
RAP1
WOR2
PHO4
RTG1
NRG1
CWT1
AAF1
TEA1
TAC1
HMS1
MIG1
SSN6
STP2
TUP1
SFL1
LEU3
ZCF35
ISW2
GAT1
C4_00260W
ZCF23
CUP9
FCR4
GZF3
CAS5
CPH2
SKO1
MSN4
FGR15
CR_02120C

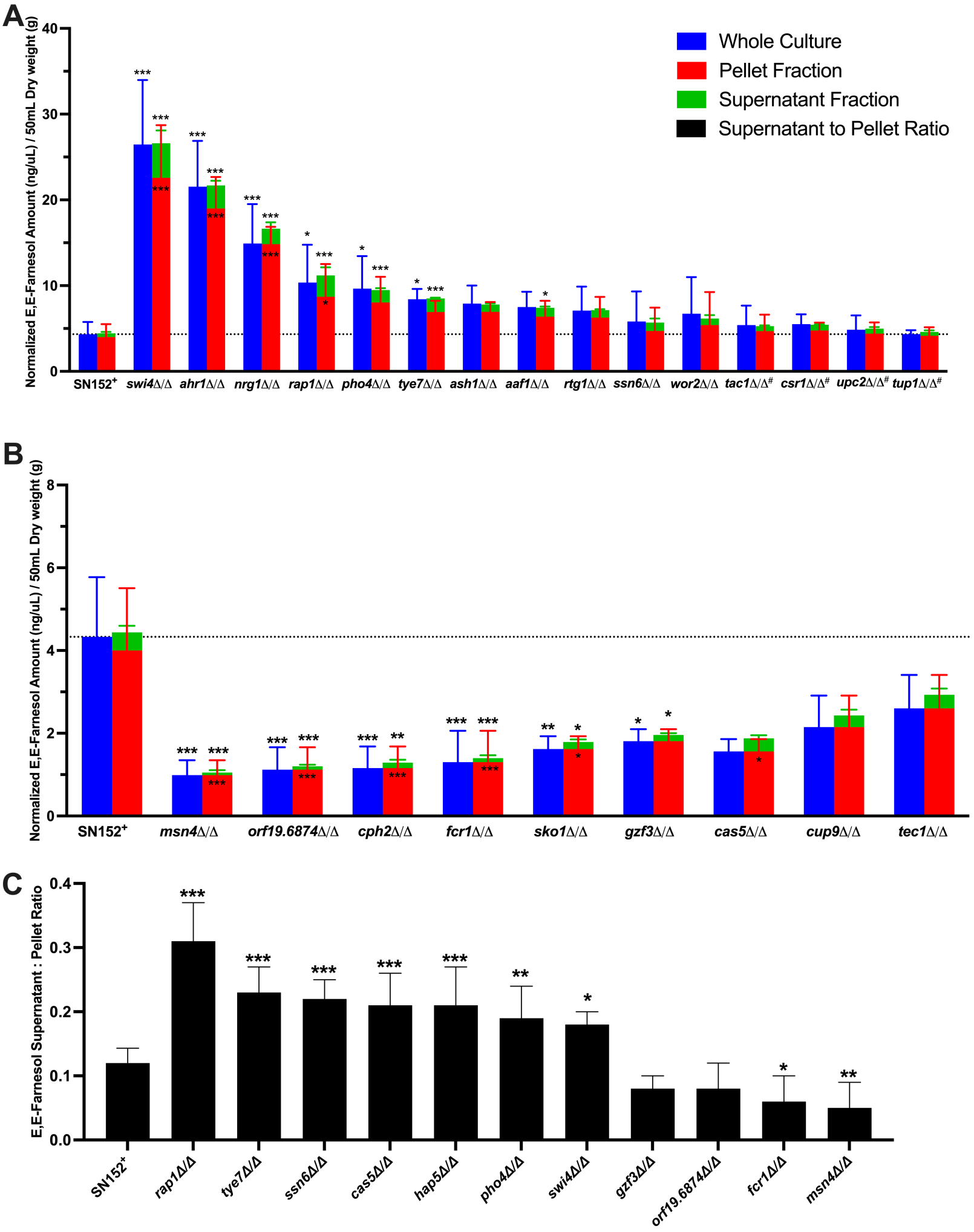
Initial Screen: E,E-Farnesol Supernatant to Pellet Ratio VS Whole Culture Accumulation

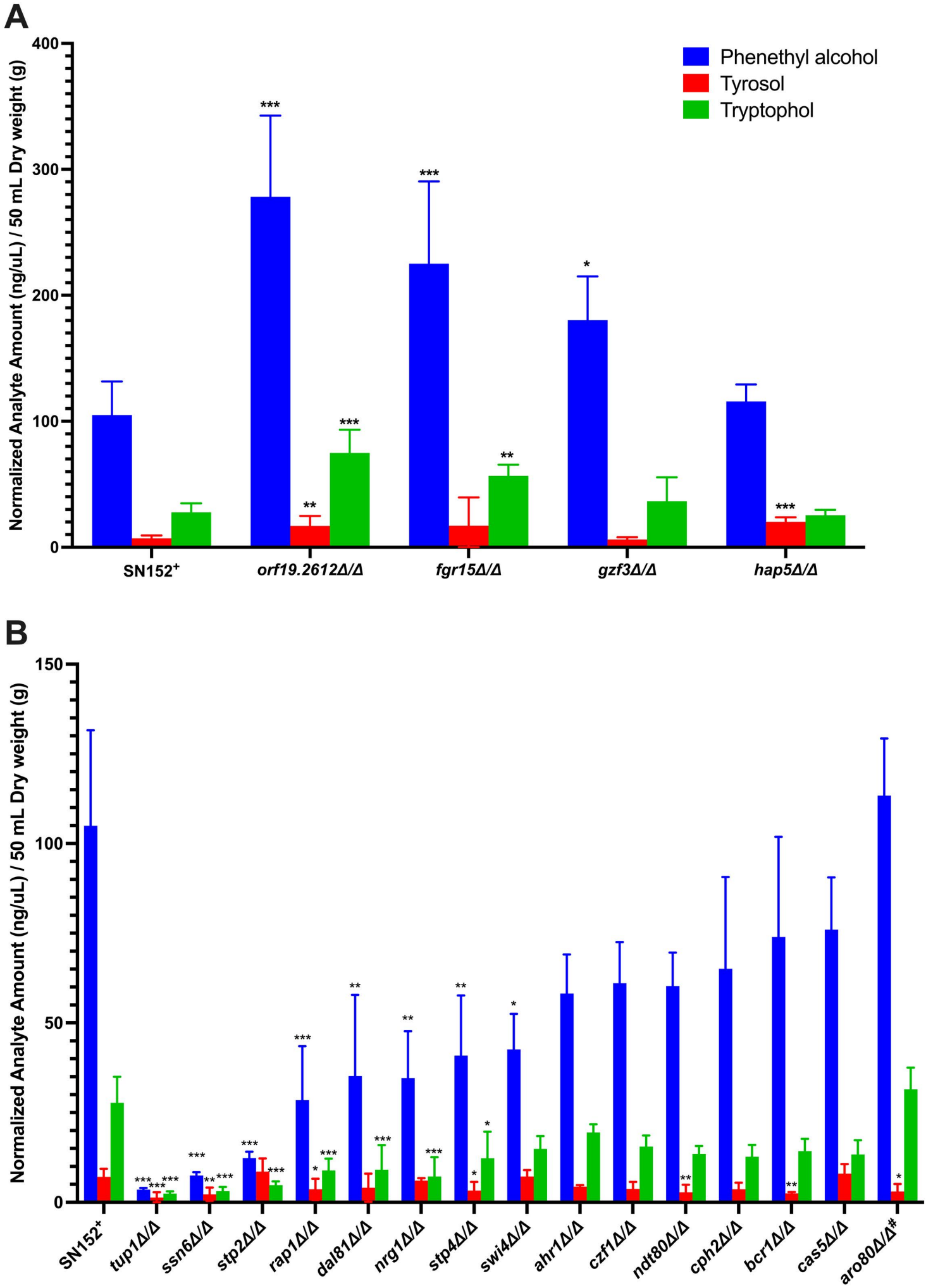



Initial Screen: Phenethyl alcohol vs Tryptophol Whole Culture Accumulation
Normalized Tryptophol Amount (ng/uL) / 50 mL Dry weight (g)
Normalized Phenethyl Alcohol Amount (ng/uL) / 50 mL Dry weight (g)
TrpOH LOG2 FC (+1)
TrypOH LOG2 FC (-1)
PEA LOG2 FC (-1)
PEA LOG2 FC (+1)
0
20
40
60
80
100
0
100
200
300
400
CR_02120C
FGR15
SFL1
FCR1
ZCF29
ZCF27
C1_13880C
STR5
C4_00260W
CUP9
ZCF35
LEU3
CSH1
ZNC1
SFU1
TEC1
BCR1
GZF3
ISW2
GAT1
C4_02570C
CR_02510W
HAP43
UME7
HAP2
UGA3
INO4
SWI4
AHR1
STP2

Initial Screen: Phenethyl alcohol vs Tyrosol Whole Culture Accumulation




A
Normalized E,E-Farnesol Amount (ng/uL) / 50mL Dry weight (g)
Whole Culture
Pellet Fraction
Supernatant Fraction
Supernatant to Pellet Ratio
SN152+
swi4Δ/Δ
ahr1Δ/Δ
nrg1Δ/Δ
rap1Δ/Δ
pho4Δ/Δ
tye7Δ/Δ
ash1Δ/Δ
aaf1Δ/Δ
rtg1Δ/Δ
ssn6Δ/Δ
wor2Δ/Δ
tac1Δ/Δ#
csr1Δ/Δ#
upc2Δ/Δ#
tup1Δ/Δ#
B
Normalized E,E-Farnesol Amount (ng/uL) / 50mL Dry weight (g)
SN152+
msn4Δ/Δ
orf19.6874Δ/Δ
cph2Δ/Δ
fcr1Δ/Δ
sko1Δ/Δ
gzf3Δ/Δ
cas5Δ/Δ
cup9Δ/Δ
tec1Δ/Δ
C
E,E-Farnesol Supernatant : Pellet Ratio
SN152+
rap1Δ/Δ
tye7Δ/Δ
ssn6Δ/Δ
cas5Δ/Δ
hap5Δ/Δ
pho4Δ/Δ
swi4Δ/Δ
gzf3Δ/Δ
orf19.6874Δ/Δ
fcr1Δ/Δ
msn4Δ/Δ


A
Phenethyl alcohol
Tyrosol
Tryptophol
Normalized Analyte Amount (ng/uL) / 50 mL Dry weight (g)
400
300
200
100
0
SN152+
orf19.2612Δ/Δ
fgr15Δ/Δ
gzf3Δ/Δ
hap5Δ/Δ
B
Normalized Analyte Amount (ng/uL) / 50 mL Dry weight (g)
150
100
50
0
SN152+
tup1Δ/Δ
ssn6Δ/Δ
stp2Δ/Δ
rap1Δ/Δ
dal81Δ/Δ
nrg1Δ/Δ
stp4Δ/Δ
swi4Δ/Δ
ahr1Δ/Δ
czf1Δ/Δ
ndt80Δ/Δ
cph2Δ/Δ
bcr1Δ/Δ
cas5Δ/Δ
aro80Δ/Δ#