

1 **Glutamate dehydrogenase (Gdh2)-dependent alkalization is**  
2 **dispensable for escape from macrophages and virulence of**  
3 ***Candida albicans***

4

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26 repression

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28

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30 **Abstract**

31

32 *Candida albicans* cells depend on the energy derived from amino acid catabolism to

33 induce and sustain hyphal growth inside phagosomes of engulfing macrophages.

34 The concomitant deamination of amino acids is thought to neutralize the acidic

35 microenvironment of phagosomes, a presumed requisite for survival and initiation of

36 hyphal growth. Here, in contrast to an existing model, we show that mitochondrial-

37 localized NAD<sup>+</sup>-dependent glutamate dehydrogenase (*GDH2*) catalyzing the

38 deamination of glutamate to  $\alpha$ -ketoglutarate, and not the cytosolic urea amidolyase

39 (*DUR1,2*), accounts for the observed alkalization of media when amino acids are the

40 sole sources of carbon and nitrogen. *C. albicans* strains lacking *GDH2* (*gdh2*<sup>-/-</sup>) are

41 viable and do not extrude ammonia on amino acid-based media. Environmental

42 alkalization does not occur under conditions of high glucose (2%), a finding

43 attributable to glucose-repression of *GDH2* expression and mitochondrial function.

44 Consistently, inhibition of oxidative phosphorylation or mitochondrial translation by

45 antimycin A or chloramphenicol, respectively, prevents alkalization. *GDH2*

46 expression and mitochondrial function are derepressed as glucose levels are

47 lowered from 2% (~110 mM) to 0.2% (~11 mM), or when glycerol is used as carbon

48 source. Using time-lapse microscopy, we document that *gdh2*<sup>-/-</sup> cells survive,

49 filament and escape from primary murine macrophages at rates indistinguishable

50 from wildtype. Consistently, *gdh2*<sup>-/-</sup> strains are as virulent as wildtype in fly and

51 murine models of systemic candidiasis. Thus, although Gdh2 has a critical role in

52 central nitrogen metabolism, Gdh2-catalyzed deamination of glutamate is

53 surprisingly dispensable for escape from macrophages and virulence,

54 demonstrating that amino acid-dependent alkalization is not essential for hyphal

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55 growth, survival in macrophages and hosts. An accurate description of the  
56 microenvironment within the phagosomal compartment of macrophages and the  
57 metabolic events underlying the survival of phagocytosed *C. albicans* cells and their  
58 escape are critical to understanding the host-pathogen interactions that ultimately  
59 determine the pathogenic outcome.

60

61 **Author Summary**

62 *Candida albicans* is a commensal component of the human microflora and the most  
63 common fungal pathogen. The incidence of candidiasis is low in healthy  
64 populations. Consequently, environmental factors, such as interactions with innate  
65 immune cells, play critical roles. Macrophages provide the first line of defense and  
66 rapidly internalize *C. albicans* cells within specialized intracellular compartments  
67 called phagosomes. The microenvironment within phagosomes is dynamic and ill  
68 defined, but has a low pH, and contains potent hydrolytic enzymes and oxidative  
69 stressors. Despite the inhospitable conditions, phagocytized *C. albicans* cells  
70 catabolize amino acids to obtain energy to survive and grow. Here, we have critically  
71 examined amino acid catabolism and ammonia extrusion in *C. albicans*, the latter is  
72 thought to neutralize the phagosomal pH and induce the switch of morphologies  
73 from round “yeast-like” to elongated hyphal cells that can pierce the phagosomal  
74 membrane leading to escape from macrophages. We report that Gdh2, which  
75 catalyzes the deamination of glutamate to  $\alpha$ -ketoglutarate, is responsible for the  
76 observed environmental alkalization when *C. albicans* catabolize amino acids.  
77 Strikingly, Gdh2 is dispensable for escape from macrophages and virulent growth.

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78 These results provide new insights into host-pathogen interactions that determine  
79 the pathogenic outcome of *C. albicans* infections.

80

81 **INTRODUCTION**

82 *Candida albicans* is a benign member of mucosal microbiota of most humans.

83 However, in individuals with an impaired immune response, *C. albicans* can cause

84 serious systemic infections associated with high rates of mortality (1, 2). In

85 establishing virulent infections, *C. albicans* cells overcome potential obstacles

86 inherent to the microenvironments in the host. Consistently, the capacity of *C.*

87 *albicans* to establish a wide spectrum of pathologies is attributed to multiple

88 virulence factors, one of which involves morphological switching from the yeast to

89 filamentous forms (i.e., hyphae and pseudohyphae), reviewed in (3-5). The ability to

90 switch from yeast to filamentous growth is required for tissue invasion and escape

91 from innate immune cells, such as macrophages, whereas, the yeast form facilitates

92 dissemination via the bloodstream. In addition to escaping from innate immune

93 cells, fungal cells must successfully compete with host cells and even other

94 constituents of the microbiome to take up necessary nutrients for growth (6).

95 Phagocytes, such as macrophages, are among the first line of host defenses

96 encountered by *C. albicans* (reviewed in (7)). These innate immune cells recognize

97 specific fungal surface antigens via specific plasma membrane-bound receptors (8).

98 Once recognized, fungal cells are enveloped by membrane protrusions that form the

99 phagosomal compartment. The phagosome matures by fusing with discrete

100 intracellular organelles, resulting in a compartment with potent hydrolytic enzymes,

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101 oxidative stressors and low pH (8-10). Acidification is important to optimize the  
102 activity of the hydrolytic enzymes that target to the phagosome.

103 *C. albicans* can survive and even escape macrophage engulfment. This is thought  
104 to depend on the ability of fungal cells to raise the phagosomal pH via ammonia  
105 extrusion. It has been suggested that urea amidolyase (Dur1,2), localized to the  
106 cytoplasm, catalyzes the reactions generating the ammonia extruded from cells by  
107 the plasma membrane-localized Ato proteins (11, 12). In addition to impairing the  
108 activity of pH-sensitive proteolytic enzymes, phagosomal alkalization is thought to  
109 initiate and promote hyphal growth (11, 13). Consistent with this notion, *C. albicans*  
110 lacking *STP2*, encoding one of the SPS (Ssy1-Ptr3-Ssy5) sensor controlled effector  
111 factors governing amino acid permease gene transcription (14), fail to form hyphae  
112 and escape macrophages (13). These observations led to a model that the reduced  
113 capacity of *stp2Δ* strains to take up amino acids limits the supply of substrates of  
114 Dur1,2 catalyzed deamination reactions, which would result in the reduced capacity  
115 to alkalinize the phagosome (12, 13).

116 We have recently shown that the mitochondrial proline catabolism is required for  
117 hyphal growth and macrophage evasion. The proline catabolic pathway is the  
118 primary route of arginine utilization (15) and operates independently of the cytosolic  
119 Dur1,2-catalyzed urea-CO<sub>2</sub> pathway (15, 16). In contrast to the proposed model (12),  
120 we observed that *dur1,2-/-* cells retain the capacity to alkalinize a basal medium  
121 containing arginine as sole nitrogen and carbon source (15). Furthermore, strains  
122 carrying *put1-/-* or *put2-/-* mutations exhibit strong growth defects and  
123 consequently, are incapable of alkalinizing the same medium, suggesting that  
124 alkalization is linked to proline catabolism.

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125 A potential source of ammonia responsible for alkalization is the deamination of  
126 glutamate, a metabolic step downstream of Put2. In *Saccharomyces cerevisiae*, the  
127 primary source of free ammonia is generated by the mitochondrial-localized NAD<sup>+</sup>-  
128 dependent glutamate dehydrogenase (Gdh2) catalyzed deamination of glutamate to  
129  $\alpha$ -ketoglutarate, a reaction that generates NADH and NH<sub>3</sub> (17). Importantly, the  
130 reaction is anaplerotic and replenishes the tricarboxylic acid (TCA) cycle with  $\alpha$ -  
131 ketoglutarate, a key TCA cycle intermediate between isocitrate and succinyl CoA,  
132 and an important precursor for amino acid biosynthesis.

133 Here, we have examined the role of Gdh2 in morphological switching under *in*  
134 *vitro* conditions in filament-inducing media, *in situ* in the phagosome of primary  
135 murine macrophages, and in virulence in two model host systems. We show that  
136 when *C. albicans* utilize amino acids as sole nitrogen- and carbon-sources they  
137 extrude ammonia, which originates from Gdh2-catalyzed deamination of glutamate.  
138 In contrast to current understanding regarding the importance of phagosomal  
139 alkalization, we report that *C. albicans* strains lacking *GDH2* filament and escape the  
140 phagosome of engulfing macrophages at rates indistinguishable to wildtype.  
141 Furthermore, we also report that the Gdh2-catalyzed reaction is dispensable for  
142 virulence in both fly and murine models of systemic candidiasis.

143

## 144 RESULTS

### 145 ***C. albicans* GDH2 is responsible for amino acid-dependent alkalization *in vitro***

146 Arginine is rapidly converted to proline and then catabolized to glutamate in the  
147 mitochondria through the concerted action of two enzymes, proline oxidase (Put1;

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148 proline to  $\Delta^1$ -pyrroline-5-carboxylate or P5C) and P5C dehydrogenase (Put2; P5C to  
149 glutamate) (**Fig. 1A**). *C. albicans* strains lacking *PUT1* (*put1*<sup>-/-</sup>) and/or *PUT2* (*put2*<sup>-/-</sup>)  
150 are unable to grow efficiently in minimal medium containing 10 mM of arginine as  
151 sole nitrogen and carbon source (YNB+Arg, pH = 4.0), and fail to alkalize the  
152 medium (15). In contrast, cells carrying null alleles of *DUR1,2* (*dur1,2*<sup>-/-</sup>) grow  
153 robustly and alkalize the media (15). To test if the catabolism of amino acids other  
154 than arginine and proline can be used as sole carbon source we examined the  
155 growth characteristics of the strains in YNB containing 1% casamino acids, a  
156 medium containing high levels of all proteinogenic amino acids (**Fig. 1B**). In this  
157 media, *dur1,2*<sup>-/-</sup> cells grew as wildtype and readily alkalized the media (compare  
158 tube 5 with 6). In contrast, *put1*<sup>-/-</sup> cells exhibited poor growth and weakly alkalized  
159 the medium (tube 3). Cells lacking Put2 activity (*put2*<sup>-/-</sup>) did not grow and the culture  
160 media remained acidic (tube 2). Interestingly, a *put1*<sup>-/-</sup> *put2*<sup>-/-</sup> double mutant strain  
161 grew better than the single *put2*<sup>-/-</sup> mutant (compare tube 4 with 2). The severe  
162 growth impairment associated with the loss of Put2 is likely due to the accumulation  
163 of P5C, which is known to cause mitochondrial dysfunction (18). These results  
164 indicate that the amino acids metabolized via the proline catabolic pathway are  
165 preferentially used as carbon sources when mixtures of amino acids are present,  
166 e.g., in casamino acid preparations. The catabolism of these non-preferred amino  
167 acids contribute only modestly to alkalization, consistent with reports that not all  
168 amino acids can serve as carbon sources and contribute to environmental  
169 alkalization (12).

170 The requirement of proline catabolism for growth suggested that the downstream  
171 deamination of glutamate to  $\alpha$ -ketoglutarate, catalyzed by glutamate

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172 dehydrogenase, provided the metabolite responsible for alkalinizing the media. In *S.*  
173 *cerevisiae*, mitochondrial glutamate dehydrogenase (*GDH2*) is the primary source of  
174 free ammonia (17). The *C. albicans* genome has one gene predicted to encode  
175 glutamate dehydrogenase (*GDH2*, C5\_02600W), and using CRISPR/Cas9 we  
176 inactivated both alleles of this gene in SC5314. Two independent clones were  
177 analyzed (**Fig. S1A and S1B**). The *gdh2*<sup>-/-</sup> strains were viable on YPD or YPG (**Fig.**  
178 **S1C**), however, they showed strong growth and alkalization defects when amino  
179 acids were used as sole nitrogen and carbon sources, such as in YNB+Arg (**Fig.**  
180 **S1A**) and YNB+CAA media (**Fig. 1B, 1C, and S2A**). Consistent with what is known  
181 for *S. cerevisiae*, the *gdh2*<sup>-/-</sup> mutant showed a modest growth defect in media  
182 containing glutamate or proline as sole nitrogen source (**Fig. S1C**).

183 To further test whether Gdh2 is responsible for environmental alkalization we  
184 assessed the capacity of the *gdh2*<sup>-/-</sup> mutant to grow and alkalinize the external  
185 growth milieu on solid YNB+CAA (**Fig. 1C**). In the absence of an additional carbon  
186 source, cells lacking *GDH2* did not grow appreciably, and failed to form colonies. By  
187 contrast, on YNB+CAA supplemented with 2% glucose the *gdh2*<sup>-/-</sup> strain formed  
188 colonies of similar size as WT, indicating that Gdh2 is dispensable for growth in  
189 media with high levels of glucose. Consistent with a requirement of a fermentable  
190 carbon source, the *gdh2*<sup>-/-</sup> strain exhibited reduced growth on media with 0.2%  
191 glucose, or the non-fermentable carbon source glycerol (1%). Although able to grow  
192 in the presence of an added carbon source, the *gdh2*<sup>-/-</sup> stain failed to alkalinize the  
193 media. In contrast, wildtype (WT), *dur1,2*<sup>-/-</sup> and CRISPR control cells formed  
194 colonies of equal size on all media and exhibited identical capacities to alkalinize the



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195 media (**Fig. 1C**, compare columns 3 and 4 with 1). These observations confirm that  
196 Gdh2 is responsible for alkalization of the external growth environment.

197

#### 198 **GDH2 is required for ammonia extrusion**

199 Next, we analyzed whether the alkalization defect of the *gdh2*<sup>-/-</sup> mutant was due to  
200 the lack of ammonia extrusion. The levels of volatile ammonia produced was  
201 measured by colonies growing on solid YNB+CAA with 0.2% glucose medium  
202 buffered with MOPS (pH = 7.4); the standard acidic growth medium (pH = 4.0) traps  
203 ammonia (NH<sub>3</sub>) as ammonium (NH<sub>4</sub><sup>+</sup>), decreasing the level of volatile ammonia and  
204 thereby interfering with the assay. As shown in **Fig. 1D**, the *gdh2*<sup>-/-</sup> strain did not  
205 release measurable ammonia. Consistent with their ability to alkalinize the growth  
206 media (**Fig. 1C**), wildtype, *dur1,2*<sup>-/-</sup> and CRISPR control strains released substantial  
207 and indistinguishable levels of ammonia. Together, these results indicate that the  
208 reaction catalyzed by Gdh2 generates the ammonia that alkalinizes the growth  
209 environment when *C. albicans* uses amino acids as the primary energy source.

210

#### 211 **Environmental alkalization originates in the mitochondria**

212 We recently confirmed that mitochondrial activity in *C. albicans* can be repressed by  
213 glucose (15), a finding that is consistent with existing transcriptional profiling data  
214 (19). Consequently, the glucose repressible nature of extracellular alkalization in the  
215 presence of amino acids could be linked to glucose repressed mitochondrial  
216 function. To examine this notion, we first sought to confirm that Gdh2 localizes to  
217 mitochondria. Cells (CFG273) expressing the functional *GDH2-GFP* reporter were

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218 grown in synthetic glutamate media with 0.2% glucose (SED<sub>0.2%</sub>) and YNB+CAA.  
219 The GFP fluorescence in cells grown under both conditions clearly localized to the  
220 mitochondria as determined by the precise overlapping pattern of fluorescence with  
221 the mitochondrial marker MitoTracker Deep Red (MTR) (**Fig. 2A**).

222 To independently assess the role of mitochondrial activity in the alkalization  
223 process, we grew the wildtype cells in standard YNB+CAA medium (without  
224 glucose), in the presence of Antimycin A, a potent inhibitor of respiratory complex III.  
225 No alkalization was observed in the medium even after 24 h of growth (**Fig. 2B**,  
226 upper left panel). Antimycin A clearly impeded the growth of wildtype cells, which  
227 phenocopies the *gdh2*<sup>-/-</sup> growth in YNB+CAA. To ascertain whether the failure to  
228 alkalize the medium was due to inhibiting mitochondrial respiration and not due to  
229 cell death, we harvested the cells from antimycin-treated cultures and suspended  
230 them in fresh medium; the cells regained their capacity to alkalize the medium  
231 (**Fig. 2B**, lower left panel). To further test that the inhibitory effect of antimycin A on  
232 alkalization is specific to mitochondrial function and not an indirect effect of growth  
233 inhibition, we performed the same experiment but starting with a high cell density  
234 (OD<sub>600</sub> of 5). As shown in **Fig. 2B** (right panel), the color endpoint indicating  
235 alkalization in wildtype control occurred in a span of 2.5 h following inoculation  
236 whereas all antimycin A-treated cells failed to neutralize the pH of the medium. This  
237 clearly demonstrates that mitochondrial function is essential for environmental  
238 alkalization. We also grew the cells in the presence of chloramphenicol, a potent  
239 mitochondrial inhibitor that targets mitochondrial translation by reversibly binding to  
240 the 50S subunit of the 70S ribosome in yeast (20). In the presence of this inhibitor  
241 and a low starting cell density, a delay in alkalization was observed initially (**Fig.**

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242 **S2A**), but then alkalization was virtually indistinguishable after 48 h, or when a high  
243 starting cell density was used (data not shown). These results, together with our  
244 observation that glucose availability influences Gdh2-dependent growth and  
245 alkalization (**Fig. 1C**), support our conclusion that alkalization originates from  
246 metabolism localized to mitochondria.

247 **Gdh2 expression is repressed by glucose**

248 To follow up on the observations that glucose negatively affects Gdh2 activity and  
249 Gdh2 is a component of mitochondria (**Fig. 2A**), we sought to visualize Gdh2  
250 expression in living cells when shifted from repressing YPD (2% glucose) to non-  
251 repressing YNB+CAA. To do this, we used the same Gdh2-GFP reporter strain  
252 described earlier (**Fig. 2A**). This enabled us to observe Gdh2 expression in single  
253 cells growing on a thin YNB+CAA agar slab over a period of 6 h. The Gdh2-GFP  
254 signal was initially weak ( $t = 0$  h), becoming more intense as time progressed and as  
255 cells underwent several rounds of cell division, some leading to filamentous  
256 pseudohyphal growth (**Fig. 3A**).

257 To relate this observation with the actual alkalization process, we analyzed the  
258 levels of Gdh2-GFP in cells grown in liquid culture taken at similar time points. Cells,  
259 pre-grown in YPD (2% glucose), were shifted to YNB+CAA and the levels of Gdh2-  
260 GFP were assessed by immunoblot analysis. To enable the recovery of adequate  
261 amounts of cells for subsequent extract preparation, we increased the starting cell  
262 density of the culture (i.e.,  $OD_{600} \approx 2.0$ ). As shown in **Fig. 3B** (left panel) the Gdh2-  
263 GFP level in YPD-grown cells was initially low ( $t = 0$  h) but within 2 h the level was  
264 greatly enhanced and remained so during the entire 6 hr incubation. During the  
265 course of growth, the media became successively alkaline, increasing from the

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266 starting pH of 4 to 7 (**Fig. 3B**, right panel). The finding that Gdh2 expression is  
267 induced in cells growing in media rich in amino acids (i.e., YNB+CAA or YPG)  
268 indicates that *GDH2* expression in *C. albicans*, in contrast to *S. cerevisiae* (21), is not  
269 subject to NCR.

270 Next, we examined the expression and stability of Gdh2-GFP in cells shifted from  
271 YPD to YPG (**Fig. 3C**). Again, the level of Gdh2-GFP rapidly increased (lanes 1-3)  
272 and remained high following the addition of glucose (2% final concentration) (**Fig.**  
273 **3C**, lanes 5-6), an observation reminiscent of isocitrate lyase (*Icl1*), a glyoxylate  
274 cycle enzyme that is not subject to catabolite inactivation in *C. albicans* (22, 23). To  
275 further illustrate the effect of glucose on Gdh2-GFP expression, we shifted YPD  
276 grown cells to YNB+CAA in the presence of 0.2% glucose or 1% glycerol,  
277 conditions that are not repressive to mitochondrial function (15). As shown in **Fig.**  
278 **3C**, the level of Gdh2-GFP substantially increased, clearly demonstrating that Gdh2  
279 expression is sensitive to repression by glucose.

280

### 281 **Inactivation of Gdh2 does not impair morphogenesis**

282 Based on current understanding that the ability of *C. albicans* to alkalinize their  
283 growth environments contributes to the induction of hyphal growth, and the  
284 observation that *gdh2*<sup>-/-</sup> cells formed smooth macrocolonies on YNB+CAA (pH =  
285 4.0) with non-repressing 0.2% glucose and 1% glycerol (**Fig. 1C, column 2**), we  
286 examined whether the inactivation of Gdh2 would negatively affect morphogenesis.  
287 To test this notion, we examined growth on Spider and Lee's media, two standard  
288 media used to assess filamentation. These media contain amino acids, have a

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289 neutral pH, and are known to promote filamentous growth of wildtype cells. Similar  
290 to wildtype and CRISPR control strains, the macrocolonies formed by the *gdh2*<sup>-/-</sup>  
291 strain were wrinkled and surrounded by an extensive outgrowth of hyphal cells (**Fig.**  
292 **4A**). This indicates that Gdh2 function is dispensable for filamentation. This is  
293 supported by the recent paper showing the capacity of *gdh2* $\Delta/\Delta$  to switch in amino  
294 acid-based medium (24).

295 This unexpected result led us to evaluate the capacity of *gdh2*<sup>-/-</sup> cells to filament  
296 within phagosomes of engulfing macrophages. FITC-stained WT (SC5314) and  
297 *gdh2*<sup>-/-</sup> (CFG279) cells were individually co-cultured with RAW264.7 macrophages.  
298 Non-phagocytosed fungal cells were removed and then co-cultures were imaged  
299 after 1.5 h of incubation. We readily observed macrophages containing *gdh2*<sup>-/-</sup> cells  
300 that had formed hyphal extensions (**Fig. 4B**), clearly suggesting that amino acid-  
301 dependent alkalization of the phagosome is not a requisite for the induction of  
302 filamentous growth.

303

304 **Gdh2-GFP expression is rapidly induced upon phagocytosis by macrophages.**

305 To assess the time-course of Gdh2-GFP expression in phagocytized *C. albicans*  
306 cells, we co-cultured the Gdh2-GFP reporter strain CFG273 with RAW264.7 (RAW)  
307 macrophages and followed the interaction by time-lapse microscopy. The Gdh2-  
308 GFP signal significantly increased after phagocytosis (**Fig. 5A**, see Video V1,  
309 Supporting Information). We repeated the experiment using primary murine bone  
310 marrow-derived macrophage (BMDM) and obtained a similar result. However, due to  
311 the inherent green autofluorescence of BMDM, the GFP fluorescence appeared less

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312 pronounced (**Fig. 5B**, see Video V2, Supporting Information). These results  
313 demonstrate that although Gdh2 expression is induced rapidly following  
314 phagocytosis, presumably the reflection of limiting glucose availability and the  
315 subsequent release from glucose repression.

316

317 **Gdh2 activity is not required to escape macrophages**

318 We directly compared the ability of wildtype and the *gdh2*<sup>-/-</sup> mutant cells to survive  
319 and escape after being phagocytized by primary BMDM using a competition assay  
320 (**Fig. 6**). To carry out the experiment we created a wildtype strain constitutively  
321 expressing GFP (*ADH1/P<sub>ADH1</sub>-GFP*) and a *gdh2*<sup>-/-</sup> mutant strain constitutively  
322 expressing RFP (*gdh2*<sup>-/-</sup> *ADH1/P<sub>ADH1</sub>-RFP*). Both strains exhibited unaltered growth  
323 characteristics (see **Fig. S2B**). Equal numbers of WT and *gdh2*<sup>-/-</sup> cells were mixed  
324 (green:red; 1:1), and the fungal cell suspension was incubated with BMDM at a MOI  
325 of 3:1 in HBSS for 30 min before washing non-phagocytosed fungal cells away. The  
326 co-cultures were monitored by time-lapse microscopy.

327 Again, contrary to what we expected, the *gdh2*<sup>-/-</sup> mutant remained fully  
328 competent to initiate hyphal growth in the phagosome of BMDM (**Fig. 6**).  
329 Furthermore, given the perceived importance of environmental alkalization in the  
330 onset of phagosomal escape by *C. albicans*, we anticipated that the *gdh2*<sup>-/-</sup> mutant  
331 would be killed more efficiently than the wildtype. To test this notion, we performed  
332 a colony forming unit (CFU) assay to quantify the survival of phagocytized cells. The  
333 results show that Gdh2-mediated alkalization is not essential for survival in BMDM  
334 (**Fig. 6**, lower right panel), a result that is consistent to the recent report by Westman

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335 et al. (25). Together, our data indicate that ammonia extrusion and environmental  
336 alkalization are not requisites for the initiation of hyphal formation, growth and  
337 survival in the phagosome of primary BMDM.

338

339 **Gdh2 activity is dispensable for virulence in intact host**

340 Next, we examined the role of Gdh2-dependent alkalization in the capacity of *C.*  
341 *albicans* to successfully infect an intact living host. We used an improved fruit fly  
342 (*Drosophila melanogaster*) infection model with the *Bom*<sup>A55C</sup> flies that lack 10  
343 Bomanin genes on chromosome 2 that encode for secreted peptides with  
344 antimicrobial property (26). As shown in the survival curve, the *gdh2*<sup>-/-</sup> mutant  
345 remained competent to infect *Bom*<sup>A55C</sup> flies similar to wildtype control (**Fig. 7A**). The  
346 data indicate that Gdh2-dependent alkalization is not required for virulence in a  
347 *Drosophila* infection model.

348 To further assess the importance of Gdh2 in virulence in a more complex host, we  
349 used a tail vein infection model using C57BL/6 mice. Two groups of mice (n = 10)  
350 were challenged with 3 x 10<sup>5</sup> wildtype or *gdh2*<sup>-/-</sup> cells and survival was monitored  
351 for a period of up to 8 days. Similar to the fly model, we observed that the loss of  
352 Gdh2 activity did not attenuate virulence (**Fig. 7B**); the *gdh2*<sup>-/-</sup> mutant exhibited  
353 survival indistinguishable from wildtype. Consistently, the fungal burden of *gdh2*<sup>-/-</sup>  
354 cells in the brain, kidney and spleen of infected mice 3 days post-infection did not  
355 significantly differ to mice infected with wildtype (**Fig. 7C**). Next we performed a  
356 competition assay; equal numbers of wildtype and *gdh2*<sup>-/-</sup> cells were intravenously  
357 injected in mice and the ratio (R) of wildtype to *gdh2*<sup>-/-</sup> cells recovered from kidneys

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358 3 days post-infection was determined. Consistent to our findings in mice individually  
359 infected with each strain, the ratio of recovered cells did not significantly differ to  
360 that of the inoculum ratio (I) (**Fig. 7C**). Together, our results indicate that Gdh2 is not  
361 required for virulence, and that the loss of Gdh2 activity does not create a selective  
362 disadvantage or significantly impair growth in infected model host systems.

363

## 364 **DISCUSSION**

365 In this work, we identified a major metabolic step that endows *C. albicans* with the  
366 capacity to increase the extracellular pH by ammonia extrusion in the presence of  
367 amino acids. We have shown that under *in vitro* growth conditions, environmental  
368 alkalization is dependent upon *GDH2*, a gene that encodes the mitochondrial-  
369 localized glutamate dehydrogenase. Strikingly, the data clearly show that despite its  
370 unambiguous role in alkalization *in vitro*, *GDH2* remained dispensable for the  
371 induction of hyphal growth and escape of *C. albicans* from macrophages, and also  
372 dispensable for virulence in intact hosts. Our results, consistent with a recent report  
373 (25), suggest that phagosomal alkalization is unlikely to be a defining event required  
374 for hyphal initiation of *C. albicans* in phagosomes of engulfing macrophages.

375 Despite its role as a key enzyme of central nitrogen metabolism, the *gdh2*<sup>-/-</sup>  
376 mutant exhibited only a modest growth defect on synthetic glucose or glycerol  
377 media containing, glutamate as sole nitrogen source (**Fig. S1C**). Consistent with  
378 proline being catabolized to glutamate in a linear pathway mediated by Put1 and  
379 Put2, a similar modest growth defect of *gdh2*<sup>-/-</sup> was observed when proline was the  
380 sole source of nitrogen (**Fig. S1C**). However, when glucose or glycerol is removed



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381 from the media (i.e., YNB+CAA), and amino acids serve as both carbon and nitrogen  
382 sources, the *gdh2*<sup>-/-</sup> mutant demonstrated a striking growth defect (**Fig. 1B, 1C,**  
383 **S2A**). Under these conditions, the impaired growth of cells lacking Gdh2 is likely the  
384 consequence of diminished levels of  $\alpha$ -ketoglutarate. In the absence of Gdh2, cells  
385 must rely entirely on the TCA cycle to generate  $\alpha$ -ketoglutarate required to support  
386 *de novo* biosynthetic needs, e.g., amino acid biosynthesis. In the absence of  
387 glucose or glycerol, the supply of acetyl-CoA derived from pyruvate becomes  
388 limiting, stalling the TCA cycle, and consequently, the growth of *gdh2*<sup>-/-</sup> cells.

389 Similar to *S. cerevisiae*, the Gdh2-catalyzed reaction is the primary source of  
390 extruded ammonia in *C. albicans*. This conclusion is based on the following key  
391 observations: 1) a mutant strain lacking *GDH2* (*gdh2*<sup>-/-</sup>) is unable to alkalinize media  
392 with amino acids as carbon and nitrogen source (i.e., YNB+CAA) and under non-  
393 repressing glucose conditions (**Fig. 1C**); 2) ammonia extrusion is impaired in *gdh2*<sup>-/-</sup>  
394 mutant (**Fig. 1D**); and 3) the Gdh2-catalyzed reaction responsible for alkalization  
395 occurs in the mitochondria, and is subject to glucose repression and inhibited by  
396 inhibitors of mitochondrial respiration (**Fig. 2**).

397 The capacity of glucose to repress mitochondrial activity (15) and Gdh2  
398 expression (**Fig. 3C**) may explain why alkalization is only observed when glucose  
399 becomes limited or replaced by glycerol. The induced levels of Gdh2 expression  
400 observed upon phagocytosis is consistent with the apparent low levels of glucose in  
401 phagosomes (27). Together with an expected surge in glutamate coming from the  
402 catabolism of arginine or proline (15); Put2 levels are upregulated in phagocytized *C.*  
403 *albicans*, which requires proline binding to the transcription factor Put3 (15, 28). In  
404 the presence of arginine as sole nitrogen and carbon source (i.e., YNB+Arg), the role

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405 of proline catabolism in alkalization is essential since it is the primary route to  
406 generate glutamate in the mitochondria. However, when other amino acids are  
407 present (i.e., YNB+CAA) such as glutamine, alanine, and aspartate (29), these amino  
408 acids can be converted directly to glutamate bypassing the requirement for the  
409 proline catabolic pathway. The fact that the *put1*<sup>-/-</sup> strain still showed a weaker  
410 alkalization compared to wildtype in YNB+CAA indicates that in the absence of  
411 glucose, proline functions a preferred carbon source, which is likely due its  
412 catabolism is efficiently coupled to the generation of ATP.

413 An unanswered question is how ammonia generated in the mitochondria is  
414 extruded to the external environment. It is possible that ammonia (NH<sub>3</sub>), known to be  
415 membrane permeable, diffuses across the inner mitochondrial membrane, moving  
416 towards the more acidic inner membrane space where it likely becomes protonated  
417 to ammonium. Ammonium then moves to the cytosol. Although the dissociation of  
418 ammonium to ammonia is not favored at the pH of the cytosol (pH ~7), the small  
419 amount of ammonia that forms can rapidly diffuse across the PM out of cells as long  
420 as the external environment is acidic. Hence, the ability of the ammonia generated  
421 by Gdh2 will likely be the consequence of Pma1 activity, the major proton pumping  
422 ATPase in the PM. Alternatively, and according to several reports, putative ammonia  
423 transport proteins, the Ato family of plasma membrane proteins, are thought to  
424 facilitate ammonia export in *C. albicans* (11). Supporting this notion, the deletion of  
425 *ATO5* significantly delays alkalization. Interestingly, the requirement for the Ato  
426 proteins suggests that the species traversing the plasma membrane from within the  
427 cell is either charged or polar, thus, it is likely that the transported species is  
428 ammonium (NH<sub>4</sub><sup>+</sup>) and coupled to H<sup>+</sup> import as previously suggested in yeast (30,

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429 31). Since cytoplasmic pH is tightly regulated, the conundrum persists as to how  
430 extruding ammonium can facilitate steady-state alkalization. The underlying  
431 mechanism of how Ato proteins facilitate alkalization needs to be precisely defined  
432 and placed in context to the fact that ammonia can readily diffuse through  
433 membranes, and in so doing, is expected to move directionally towards acidic  
434 environments.

435 Due to its central role in nitrogen metabolism, it was surprising that the  
436 inactivation of *GDH2* did not affect the capacity of *C. albicans* to form hyphae in the  
437 phagosome of macrophage, which is thought to contain amino acids as primary  
438 energy sources. This is opposite to what we observed in strains lacking *PUT1*  
439 and/or *PUT2*, which show phagosome-specific defect in hyphal growth (15). On  
440 amino acid-rich Spider and Lee's medium, containing 1% mannitol and 1.25%  
441 glucose as primary carbon sources, respectively, the *gdh2*<sup>-/-</sup> mutant also did not  
442 show a filamentation defect. On Spider, but not on Lee's media, both *put1*<sup>-/-</sup> and  
443 *put2*<sup>-/-</sup> mutants have noticeable defects on formation of invasive filaments despite  
444 forming wrinkled colonies (our unpublished data). Together, these observations  
445 suggest that when glucose is limiting, the energy obtained by the catabolism of  
446 proline to glutamate suffices to induce and support hyphal growth; the additional  
447 energy derived from the NADH generated by the Gdh2-catalyzed deamination of  
448 glutamate is not required. The recent work by Westman et al., demonstrating that  
449 phagosomal alkalization is an effect of hyphal expansion and not the underlying  
450 trigger that causes filamentation (25), aligns well with our observations. Accordingly,  
451 Westman et al. proposed that the step-wise alkalization of the phagosome could be  
452 attributed to proton leakage out of the compartment due to the transient physical

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453 stress imposed by hyphal expansion. Also, hyphal formation was found to start prior  
454 to a measurable change in pH (pH ~ 5-6), suggesting that alkalization is not the  
455 primary stimulus triggering hyphal formation in the phagosome. Beyond the realm of  
456 the phagosome, our work also suggests that environmental alkalization via ammonia  
457 extrusion, a mechanism that is thought to facilitate virulence of fungal pathogens  
458 (32), is dispensable for pathogenesis of *C. albicans* (**Fig. 7**) requiring us to rethink  
459 the specific role of alkalization in fungal virulence.

460 We confirmed that *DUR1,2* does not significantly contribute to alkalization.  
461 *DUR1,2* is under tight regulatory control by NCR, and thus is not expressed under  
462 growth conditions in the presence of preferred amino acids. Our results are  
463 inconsistent with previous suggestions that Dur1,2 activity significantly contributes  
464 to alkalization (12). The usual growth media (i.e., YPD or SD) used for *C. albicans*  
465 propagation and the standard mammalian cell culture medium (i.e., DMEM or RPMI)  
466 used for co-culturing fungal cells with macrophages are all very rich in amino acids,  
467 certainly conditions that repress *DUR1,2* expression (33). In co-culture experiments,  
468 we observed that hyphal initiation (i.e., germ tube formation) following phagocytosis  
469 is very rapid, occurring as early as 15-20 min following phagocytosis even in  
470 experiments that were carried out in a neutral buffer (i.e., HBSS) suggesting  
471 signaling cascades driving this switch are activated at a much earlier time.

472 Finally, we note that time-lapse microscopy has a distinct advantage over  
473 endpoint microscopy of fixed co-cultures, since it enables the spatio-temporal  
474 dynamics of hyphal formation of both wildtype and *gdh2*<sup>-/-</sup> mutant to be accurately  
475 followed inside the same macrophage. Time-lapse microscopy allowed us to  
476 observe that non-phagocytized fungal cells that remained external even after

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477 excessive washing can filament resulting in the false impression that the fungal cells  
478 are escaping from the macrophage.

479 Counteracting the antimicrobial assault in the macrophage phagosome is crucial  
480 for *C. albicans* survival and dissemination. Due to acidic phagosomal  
481 microenvironment, phagosomal alkalization via ammonia extrusion is not surprising  
482 at all. However, given that *C. albicans* is retained in a hostile microenvironment  
483 essentially devoid of key nutrients required for growth, it is of paramount importance  
484 for *C. albicans* to be able to synthesize the cellular components required to  
485 counteract these stresses. For example, the highly polarized hyphal growth requires  
486 a lot of ATP to drive actin polymerization and also the proton extrusion process  
487 mediated by Pma1 to regulate intracellular pH when extracellular pH is low requires  
488 ATP. Thus, on a bioenergetic standpoint, and considering the energy-demanding  
489 nature of hyphal function, the alkalization process does not seem adequate for an  
490 explanation as to how *C. albicans* is able to support hyphal formation in a hostile  
491 microenvironment with restricted nutrient content. The ability to undergo dimorphic  
492 transitions to hyphal growth and the distention of phagosomal membrane apparently  
493 has major physiological consequence that affects the capacity of macrophage  
494 killing. The findings documented here illuminate and further the understanding of a  
495 major feature of the innate immune surveillance arsenal required for integrity of the  
496 human host, however, more work is clearly needed to understand the spatio-  
497 temporal dynamics of hyphal formation of *C. albicans* in the macrophage  
498 phagosome.

499 **METHODOLOGY**

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500 **Organisms, culture media, and chemicals.** Strains listed in Table S1 were  
501 routinely cultivated in YPD agar medium (1% yeast extract, 2% peptone, 2%  
502 glucose, 2% Bacto agar) at 30 °C after recovery from -80 °C glycerol stock. Where  
503 needed, YPD medium was supplemented with 25, 100 or 200 µg/ml nourseothricin  
504 (Nou; Jena Biosciences, Jena, Germany). Also, where indicated, the glucose in YPD  
505 is lowered to 0.2% (YPD<sub>0.2%</sub>) or replaced with 1% glycerol (YPG) or 2% mannitol  
506 (YPM). Specific growth assays were carried out in synthetic minimal medium  
507 containing 0.17% yeast nitrogen base without ammonium sulfate and amino acids  
508 (YNB; Difco), supplemented with the indicated amino acid (10 mM) as sole nitrogen  
509 source and the indicated carbon source, and buffered (pH = 6.0, 50 mM MES).

510 **Alkalization assays.** Alkalization was assessed using YNB+CAA medium (0.17%  
511 YNB, 1% of casamino acids (CAA; Sigma) containing 0.01% Bromocresol Purple  
512 (BCP; Sigma) as pH indicator; the pH was set at 4.0 using 1 M HCl. Where  
513 indicated, YNB+Arg was used, which contains 10 mM arginine instead of 1% CAA  
514 as sole nitrogen and carbon source. Also, where indicated, YNB+CAA medium was  
515 supplemented with glycerol (1%) or glucose (2% or 0.2%). Cells from overnight YPD  
516 cultures were harvested, washed at least twice in ddH<sub>2</sub>O, and then suspended at an  
517 OD<sub>600</sub> ≈ 0.05 unless otherwise indicated. Cultures were grown with vigorous  
518 agitation at 37 °C. Where appropriate Antimycin A or Chloramphenicol was added at  
519 the concentrations indicated. Assays on solid media (2% agar), 5 µl aliquots of  
520 washed cell suspensions (OD<sub>600</sub> ≈ 1) were spotted onto the surface of media in a 6-  
521 well microplate and then grown at 37 °C for up to 72 h.

522 **CRISPR/Cas9 Mediated Gene Inactivation.** CRISPR/Cas9 was used to  
523 simultaneously inactivate both alleles of *GDH2* (C5\_02600W) (34, 35). Synthetic

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524 guide RNAs (sgRNAs), repair templates (RT), and verification primers used for gene  
525 editing are listed in Table S2. Briefly, 20-bp sgRNAs primers (p1/p2), designed  
526 according to (36), were ligated to *Esp3I* (*BsmBI*)-restricted and dephosphorylated  
527 pV1524 creating pFS108. The CRISPR/Cas9 cassette (100 ng/μl) of pFS108, with  
528 sgRNA targeting *GDH2*, was released by *KpnI* and *SacI* restriction and introduced  
529 into *C. albicans* together with a PCR-amplified RT (p3/p4; 100 ng/μl) containing  
530 multiple stop codons and a diagnostic *XhoI* restriction site (plasmid:repair template  
531 volume ratio of 1:3). *C. albicans* transformation was performed using the hybrid  
532 lithium acetate/DTT-electroporation method by Reuss, et al. (37). After applying the  
533 1.5 kV electric pulse, cells were immediately recovered in YPD medium  
534 supplemented with 1 M sorbitol for at least 4 hours, and then plated on YPD-Nou  
535 plates (200 μg/ml). Nou-resistant (Nou<sup>R</sup>) transformants were re-streaked on YPD-  
536 Nou plates (100 μg/ml) and screened for the ability to alkalize YNB+Arg media.  
537 DNA was isolated from transformants exhibiting an alkalization defect and subjected  
538 to PCR-restriction digest (PCR-RD) verification using primers (p5/p6) and *XhoI*  
539 restriction enzyme. The *gdh2*<sup>-/-</sup> clones (CFG277 and CFG278) were grown overnight  
540 in YPM to pop-out the CRISPR/Cas9 cassette. Nou sensitive (Nou<sup>S</sup>) cells were  
541 identified by plating on YPD supplemented with 25 μg/ml Nou (37), resulting in  
542 strains CFG279 and CFG281.

543 **Reporter Strains.** For C-terminal GFP tagging of Gdh2, an approximately 2.8 kB of  
544 PCR cassette was amplified from plasmid pFA-GFPγ-*URA3* (38) using primers  
545 (p7/p8). The amplicon was purified and then introduced into CAI4 (*ura3/ura3*).  
546 Transformants were selected on synthetic complete dextrose (CSD) plate lacking  
547 uridine. The correct integration of the GFP reporter was assessed using PCR

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548 (p5/p9), immunoblotting, and fluorescence microscopy. CFG275, a *gdh2*<sup>-/-</sup> strain  
549 constitutively expressing RFP, was constructed by introducing a *KpnI/SacI* fragment  
550 from pJA21 containing the P<sub>ADH1</sub>-RFP-Nou construct (39) into CFG279;  
551 transformants were selected on YPD agar with 200 µg/ml Nou. RFP-positive clones  
552 were verified by PCR (p11/p12) and fluorescence microscopy.

553 **Ammonia release assay.** Quantification of volatile ammonia release was performed  
554 in accordance to the modified acid trap method by Morales et al. (40). Briefly, a 2 µl  
555 aliquot of OD<sub>600</sub> ≈ 1 cell suspension was spotted onto each well of a 96-well  
556 microplate containing 150 µl of YNB+CAA solid medium supplemented with 0.2%  
557 glucose and buffered to pH = 7.4 with 50 mM MOPS. The spotted microplate was  
558 inverted and then precisely positioned on top of another microplate in which each  
559 well contains 100 µl of 10 % (w/v) citric acid. Plates were sealed by parafilm and  
560 then incubated at 37°C for 72 h after which, the citric acid solution was sampled for  
561 ammonia analysis using Nessler's reagent (Sigma-Aldrich). The solution was diluted  
562 10-fold in 10% citric acid and then a 20-µl aliquot was added to 80 µl Nessler's  
563 reagent on another microplate. After a 30 min incubation period at room  
564 temperature, OD<sub>400</sub> was measured using Enspire microplate reader. The level of  
565 ammonia entrapped in the citric acid solution was calculated based on ammonium  
566 chloride (NH<sub>4</sub>Cl) standard curve.

567 **Immunoblotting.** For Gdh2 level analysis, cells expressing Gdh2-GFP (CFG273)  
568 were grown in liquid YPD for overnight at 30°C and then washed 3X with ddH<sub>2</sub>O.  
569 Cells were diluted in the indicated alkalization media at OD<sub>600</sub> ≈ 2 and then  
570 incubated continuously in a rotating drum for 6 h at 37°C with sampling performed  
571 every 2 h. In each sampling point, cells were harvested, washed once with ice-cold



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572 ddH<sub>2</sub>O, and then adjusted to OD<sub>600</sub> ≈ 2. Whole cell lysates were prepared using  
573 sodium hydroxide/ trichloroacetic acid (NaOH/TCA) method as described previously  
574 with minor modifications (41). Briefly, 500 µl of adjusted cell suspension were added  
575 to tube containing 280 µl of ice-cold 2 M NaOH with 7% β-Mercaptoethanol (β-Me)  
576 for 15 min. Proteins were then precipitated overnight at 4°C by adding the same  
577 volume of cold 50% TCA. Protein pellets were collected by high-speed  
578 centrifugation at 13,000 rpm for 10 min (4°C) and then the NaOH/TCA solution  
579 completely removed. The pellets were resuspended in 50 µl of 2X SDS sample  
580 buffer with additional 5 µl of 1 M Tris Base (pH = 11) to neutralize the excess TCA.  
581 Samples were denatured at 95-100°C for 5 min before resolving the proteins in  
582 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4-  
583 12% pre-cast gels (Invitrogen). Proteins were analyzed by immunoblotting on  
584 nitrocellulose membrane according to standard procedure. After transfer,  
585 membranes were blocked using 10% skimmed milk in TBST (TBS + 0.1% Tween)  
586 for 1 h at room temperature. For Gdh2-GFP detection, membranes were first  
587 incubated with mouse anti-GFP primary antibody at 1:2,000 dilution (JL8, Takara) for  
588 overnight at 4°C. For the detection of the primary antibody, an HRP-conjugated goat  
589 anti-mouse secondary antibody (Pierce) was used. For loading control, α-tubulin  
590 was detected with rat monoclonal antibody conjugated to HRP [YOL1/34] (Abcam).  
591 For both secondary antibody and loading control, antibodies were used at 1:10,000  
592 dilution in 5% skimmed milk in TBST incubated for 1 h at room temperature.  
593 Immunoreactive bands were visualized by enhanced chemiluminescent detection  
594 system (SuperSignal Dura West Extended Duration Substrate; Pierce) using  
595 ChemiDoc MP system (BioRad).

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596 **Filamentation assay.** Filamentation in solid Spider (42) or Lee's (43) media was  
597 performed as described (44). Cells from overnight YPD liquid cultures were  
598 harvested, washed 3X with sterile PBS, adjusted to OD<sub>600</sub> ≈ 1 and then 5 µl of cell  
599 suspensions were spotted onto the indicated media. Plates were allowed to dry at  
600 room temperature before incubating at 37 °C as indicated.

601 **Macrophage culture.** RAW264.7 murine macrophage cells (ATCC TIB-71) and  
602 primary bone marrow-derived macrophages (BMDM) were cultured and passaged in  
603 complete RPMI medium supplemented with 10% fetal bovine serum (FBS), 100  
604 U/ml penicillin and 100 mg/ml streptomycin (referred to as R10 medium in the text)  
605 in a humidified chamber set at 37°C with 5% CO<sub>2</sub>. For BMDM differentiation, bone  
606 marrows collected from mouse femurs of C57BL/6 wildtype mice (7- to 9- week old)  
607 were mechanically homogenized and resuspended in R10 medium supplemented  
608 with 20% L929 conditioned media (LCM). Differentiation was carried out initially for  
609 3 days before boosting the cells with another dose of 20% LCM until harvested.  
610 BMDM were used 7-10 days after differentiation.

611 ***C. albicans* killing assay.** To assess candidacidal activity by BMDM, we co-  
612 cultured *C. albicans* wildtype and *gdh2*<sup>-/-</sup> mutant with BMDM and then assessed  
613 colony forming units (CFU) following co-incubation. About 16-24 h prior to co-  
614 culture, differentiated BMDM were collected by scraping, counted, and then seeded  
615 at 1 x 10<sup>6</sup> cells/well into a 24-well microplate. *C. albicans* cells from overnight YPD  
616 cultures were collected by centrifugation, washed 3X with sterile PBS, and then  
617 added to macrophages at MOI 3:1 (C:M). The plates were briefly centrifuged at 500  
618 x g for 5 min to collect the fungal cells at the bottom of each well and then co-  
619 cultured for 2 h in a humidified chamber. After co-culture, each well was treated with

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620 0.1% Triton X-100 for 2 min followed by vigorous pipetting to lyse the macrophage  
621 and release the fungal cells. Each well was rinsed seven times (7X) with ice-cold  
622 ddH<sub>2</sub>O and collected in a 15-ml conical tube. Lysates were serially diluted and then  
623 plated onto YPD. Plates containing colonies between 30-300 were counted. The  
624 candidacidal activity (% killing) of BMDM was defined as [1 - (CFU with  
625 macrophage/CFU of initial fungal inoculum)] x 100 (45).

626 **Confocal microscopy.** For subcellular localization of Gdh2, cells expressing Gdh2-  
627 GFP (CFG273) from log-phase YPD cultures were harvested, washed 3X with  
628 ddH<sub>2</sub>O, and then grown in SED<sub>0.2%</sub> (10 mM glutamate and 0.2% glucose) and  
629 YNB+CAA for 24 h at 37°C at a starting OD<sub>600</sub> ≈ 0.05. Cells from each culture were  
630 harvested, washed once with PBS, and then stained with 200 nM (in PBS) of the  
631 mitochondrial marker, MitoTracker Red (MTR; Molecular Probes) for 30 min at 37°C.  
632 After staining, the cells were collected again and resuspended in PBS before  
633 viewing the cells using confocal microscope (LSM800, 63x oil) in the green and red  
634 channels.

635 For the investigation of hyphal formation in the phagosome, we co-cultured  
636 individual strains with RAW264.7 macrophages. For easier visualization, we pre-  
637 stained the fungal cells with fluorescein isothiocyanate (FITC). Briefly, fungal cells  
638 were harvested from YPD overnight cultures, washed once with PBS, and then  
639 adjusted to OD<sub>600</sub> ≈ 10. Cells from 1 ml of adjusted cell suspension were collected  
640 and then incubated with FITC solution (1 mg/ml in 0.1 M NaHCO<sub>3</sub> buffer, pH = 9.0)  
641 for 15 min at 30 °C before extensive washing in PBS (3X). Macrophages were  
642 seeded at 1 x 10<sup>6</sup> cells into a 35 mm glass bottom imaging dish (ibidi) and were  
643 allowed to adhere overnight (16-24 h). FITC stained fungal cells were added at a

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644 MOI of 1:1 (C:M), and after 20 min, the co-cultures were washed extensively with  
645 HBSS (5X) to remove non-phagocytosed fungal cells before adding CO<sub>2</sub>-  
646 independent medium (Gibco). Co-cultures were further incubated for 1.5 h in  
647 temperature-controlled chamber (37 °C) of the LSM800 confocal microscope prior  
648 to imaging cells (63x objective).

649 **Time lapse microscopy (TLM).** Unless otherwise indicated, all TLM experiments  
650 performed in this paper used the Zeiss Cell Observer inverted microscope equipped  
651 with temperature control chamber (37 °C) and appropriate filters to detect  
652 fluorophores. For the analysis of Gdh2 expression during alkalization on solid media,  
653 cells expressing Gdh2-GFP (CFG273) were collected from overnight YPD culture,  
654 washed 3X with ddH<sub>2</sub>O, and then adjusted to a cell density of OD<sub>600</sub> ≈ 0.05. To make  
655 a YNB+CAA agar slab on which to grow the cells, a 100 µl molten YNB+CAA agar  
656 was placed on top of a flame-sterilized slide and then spread evenly to make a thin  
657 agar film. The agar was allowed to congeal at room temperature before spotting a 2  
658 µl aliquot of adjusted cell suspension and then covered with a coverslip. Single cells  
659 were located and then the GFP expression was followed every hour in the green  
660 (GFP) channel alongside DIC for 6 h.

661 For Gdh2-GFP expression during macrophage interaction, the same strain  
662 (CFG273) was co-cultured with either RAW264.7 or BMDM macrophage pre-stained  
663 with LysoTracker Red DND-99 (LST; Thermo Scientific) that marks the acidic  
664 compartments inside the macrophage. Macrophages were seeded at 1 x 10<sup>6</sup> cells  
665 into a 35 mm glass bottom imaging dish and were allowed to adhere overnight (16-  
666 24 h). Prior to co-culture, medium was removed and then replaced with CO<sub>2</sub>-  
667 independent medium containing 200 nM of LST. Macrophages were stained for at

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668 least 30 min at 37°C. For fungal cell preparation, cells from overnight YPD cultures  
669 were harvested, washed 3X with sterile PBS, and then added to macrophages at  
670 MOI of 1:1 (C:M). Interaction was followed every 1.5 min for ~3 h (with RAW cells)  
671 and 2 min for ~4 h (with BMDM) in the DIC, green (Gdh2-GFP) and red (LST)  
672 channels. Movies were saved at 10 fps.

673 For competition assay in the same macrophage (BMDM) co-culture system,  
674 wildtype cells constitutively expressing GFP (SCADH1G4A) and *gdh2*<sup>-/-</sup> strain  
675 expressing RFP (CFG275) from overnight YPD cultures were collected by  
676 centrifugation, washed 3X with sterile PBS, and then diluted to OD<sub>600</sub> ≈ 1. Cells were  
677 mixed 1:1 (v/v) in a sterile Eppendorf tube and then vortexed. Prior to co-culture,  
678 BMDM (1 × 10<sup>6</sup>) seeded on imaging dish were washed 2X with HBSS to remove the  
679 growth medium. A 100 μl aliquot of mixed fungal cells (~3 × 10<sup>6</sup> cells) were added to  
680 the dish (MOI of 3:1, C:M) and phagocytosis was carried out in HBSS for  
681 approximately 30 min in the humidified chamber. Co-cultures were washed at least  
682 5X with HBSS and 1X with CO<sub>2</sub>-independent medium to remove non-phagocytosed  
683 fungal cells. CO<sub>2</sub>-independent medium was added to the dish and TLM was carried  
684 out at 37°C in the DIC, green (wildtype) and red (*gdh2*<sup>-/-</sup>) channels. Images were  
685 acquired every 2 min for ~5 h and then saved as movie at 10 fps.

686 **Murine systemic infection model.** Groups of female C57BL/6 mice, aged 6–8  
687 weeks, were purchased from Beijing Vital River Laboratory Animal Technology Co.,  
688 Ltd. These mice were housed in individual ventilated cages in a pathogen-free  
689 animal facility at Institut Pasteur of Shanghai, Chinese Academy of Sciences. In  
690 each of the mouse studies, the animals were assigned to the different experimental  
691 groups. Infections were performed under SPF conditions. All animal experiments

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692 were carried out in strict accordance with the Regulations for the Administration of  
693 Affairs Concerning Experimental Animals issued by the Ministry of Science and  
694 Technology of the People's Republic of China, and approved by IACUC at the  
695 Institut Pasteur of Shanghai, Chinese Academy of Science with an approval number  
696 P2018050. Wildtype SC5314 and *gdh2*<sup>-/-</sup> mutant *C. albicans* cells were inoculated  
697 into YPD broth and grown overnight at 30 °C. Cells were harvested and washed  
698 three times with phosphate-buffered saline (PBS), and counted using  
699 hemocytometer. For each strain, mice (n=10) were injected via the lateral tail vein  
700 with 3x10<sup>5</sup> CFU of *C. albicans* cells. The mice were monitored once daily for weight  
701 loss, disease severity and survival. The fungal burden was assessed by counting  
702 CFU. The survival curves were statistically analyzed by the Kaplan-Meier method (a  
703 log-rank test, GraphPad Prism). Competitive bloodstream infections were performed  
704 using equal numbers of SC5314 and *gdh2*<sup>-/-</sup> mutant cells, i.e., with an inoculum (I)  
705 ratio of 1:1. At 3 days post infection the abundance and genotype of fungal cells  
706 recovered from kidneys was determined and the ratio of wildtype:*gdh2*<sup>-/-</sup> in kidneys  
707 was calculated (R). Cells lacking *gdh2*<sup>-/-</sup> cannot grow on selective YNB+Arg  
708 medium. The log<sub>2</sub>(R/I) values was compared using unpaired t-test.

709

710 **Author contribution statements**

711 F.G.S.S. and P.O.L. conceived and designed the experiments. F.G.S.S., K.R., T.J.,  
712 M.W., and N.H. performed experiments. F.G.S.S., C.C., and N.N.L. and P.O.L.  
713 supervised the experimental work. F.G.S.S., K.R., M.W., N.H., C.C., T.J., N.N.L.,  
714 and P.O.L. analyzed the data and prepared the figures. F.G.S.S. and P.O.L. wrote

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715 the paper. P.O.L. acquired the main funding and all authors critically reviewed and  
716 approved the manuscript.

717

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732 **REFERENCES**

- 733 1. Kullberg BJ, Arendrup MC. Invasive Candidiasis. *N Engl J Med.*  
734 2016;374(8):794-5.
- 735 2. Moyes DL, Naglik JR. Mucosal immunity and *Candida albicans* infection. *Clin*  
736 *Dev Immunol.* 2011;2011:346307.
- 737 3. Berman J. Morphogenesis and cell cycle progression in *Candida albicans*. *Curr*  
738 *Opin Microbiol.* 2006;9(6):595-601.
- 739 4. Sudbery P, Gow N, Berman J. The distinct morphogenic states of *Candida*  
740 *albicans*. *Trends Microbiol.* 2004;12(7):317-24.
- 741 5. Sudbery PE. Growth of *Candida albicans* hyphae. *Nat Rev Microbiol.*  
742 2011;9(10):737-48.
- 743 6. Brown AJ, Brown GD, Netea MG, Gow NA. Metabolism impacts upon *Candida*  
744 immunogenicity and pathogenicity at multiple levels. *Trends Microbiol.*  
745 2014;22(11):614-22.
- 746 7. Erwig LP, Gow NA. Interactions of fungal pathogens with phagocytes. *Nat Rev*  
747 *Microbiol.* 2016;14(3):163-76.
- 748 8. Russell DG, Vanderven BC, Glennie S, Mwandumba H, Heyderman RS. The  
749 macrophage marches on its phagosome: dynamic assays of phagosome  
750 function. *Nat Rev Immunol.* 2009;9(8):594-600.
- 751 9. Sun-Wada GH, Tabata H, Kawamura N, Aoyama M, Wada Y. Direct recruitment  
752 of H<sup>+</sup>-ATPase from lysosomes for phagosomal acidification. *J Cell Sci.*  
753 2009;122(Pt 14):2504-13.
- 754 10. Canton J, Khezri R, Glogauer M, Grinstein S. Contrasting phagosome pH  
755 regulation and maturation in human M1 and M2 macrophages. *Mol Biol Cell.*  
756 2014;25(21):3330-41.
- 757 11. Danhof HA, Lorenz MC. The *Candida albicans* ATO gene family promotes  
758 neutralization of the macrophage phagolysosome. *Infect Immun.*  
759 2015;83(11):4416-26.
- 760 12. Vylkova S, Carman AJ, Danhof HA, Collette JR, Zhou H, Lorenz MC. The fungal  
761 pathogen *Candida albicans* autoinduces hyphal morphogenesis by raising  
762 extracellular pH. *MBio.* 2011;2(3):e00055-11.
- 763 13. Vylkova S, Lorenz MC. Modulation of phagosomal pH by *Candida albicans*  
764 promotes hyphal morphogenesis and requires Stp2p, a regulator of amino acid  
765 transport. *PLoS Pathog.* 2014;10(3):e1003995.



*Amino acid-dependent alkalization is linked to mitochondrial function*

- 766 **14.** Martínez P, Ljungdahl PO. Divergence of Stp1 and Stp2 transcription factors in  
767 *Candida albicans* places virulence factors required for proper nutrient acquisition  
768 under amino acid control. *Mol Cell Biol.* 2005;25(21):9435-46.
- 769 **15.** Silao FGS, Ward M, Ryman K, Wallström A, Brindefalk B, Udekwu K, et al.  
770 Mitochondrial proline catabolism activates Ras1/cAMP/PKA-induced  
771 filamentation in *Candida albicans*. *PLoS Genet.* 2019;15(2):e1007976.
- 772 **16.** Ghosh S, Navarathna DH, Roberts DD, Cooper JT, Atkin AL, Petro TM, et al.  
773 Arginine-induced germ tube formation in *Candida albicans* is essential for  
774 escape from murine macrophage line RAW 264.7. *Infect Immun.*  
775 2009;77(4):1596-605.
- 776 **17.** Miller SM, Magasanik B. Role of NAD-linked glutamate dehydrogenase in  
777 nitrogen metabolism in *Saccharomyces cerevisiae*. *J Bacteriol.*  
778 1990;172(9):4927-35.
- 779 **18.** Nishimura A, Nasuno R, Takagi H. The proline metabolism intermediate Delta1-  
780 pyrroline-5-carboxylate directly inhibits the mitochondrial respiration in budding  
781 yeast. *FEBS Lett.* 2012;586(16):2411-6.
- 782 **19.** Rodaki A, Bohovych IM, Enjalbert B, Young T, Odds FC, Gow NA, et al. Glucose  
783 promotes stress resistance in the fungal pathogen *Candida albicans*. *Mol Biol*  
784 *Cell.* 2009;20(22):4845-55.
- 785 **20.** Balbi HJ. Chloramphenicol: a review. *Pediatr Rev.* 2004;25(8):284-8.
- 786 **21.** Daugherty JR, Rai R, el Berry HM, Cooper TG. Regulatory circuit for responses  
787 of nitrogen catabolic gene expression to the GLN3 and DAL80 proteins and  
788 nitrogen catabolite repression in *Saccharomyces cerevisiae*. *J Bacteriol.*  
789 1993;175(1):64-73.
- 790 **22.** Childers DS, Raziunaite I, Mol Avelar G, Mackie J, Budge S, Stead D, et al. The  
791 rewiring of ubiquitination targets in a pathogenic yeast promotes metabolic  
792 flexibility, host colonization and virulence. *PLoS Pathog.* 2016;12(4):e1005566.
- 793 **23.** Sandai D, Yin Z, Selway L, Stead D, Walker J, Leach MD, et al. The evolutionary  
794 rewiring of ubiquitination targets has reprogrammed the regulation of carbon  
795 assimilation in the pathogenic yeast *Candida albicans*. *MBio.* 2012;3(6).
- 796 **24.** Han TL, Cannon RD, Gallo SM, Villas-Boas SG. A metabolomic study of the  
797 effect of *Candida albicans* glutamate dehydrogenase deletion on growth and  
798 morphogenesis. *NPJ Biofilms Microbiomes.* 2019;5:13.
- 799 **25.** Westman J, Moran G, Mogavero S, Hube B, Grinstein S. *Candida albicans*  
800 hyphal expansion causes phagosomal membrane damage and luminal  
801 alkalization. *MBio.* 2018;9(5).
- 802 **26.** Clemmons AW, Lindsay SA, Wasserman SA. An effector peptide family required  
803 for *Drosophila* toll-mediated immunity. *PLoS Pathog.* 2015;11(4):e1004876.

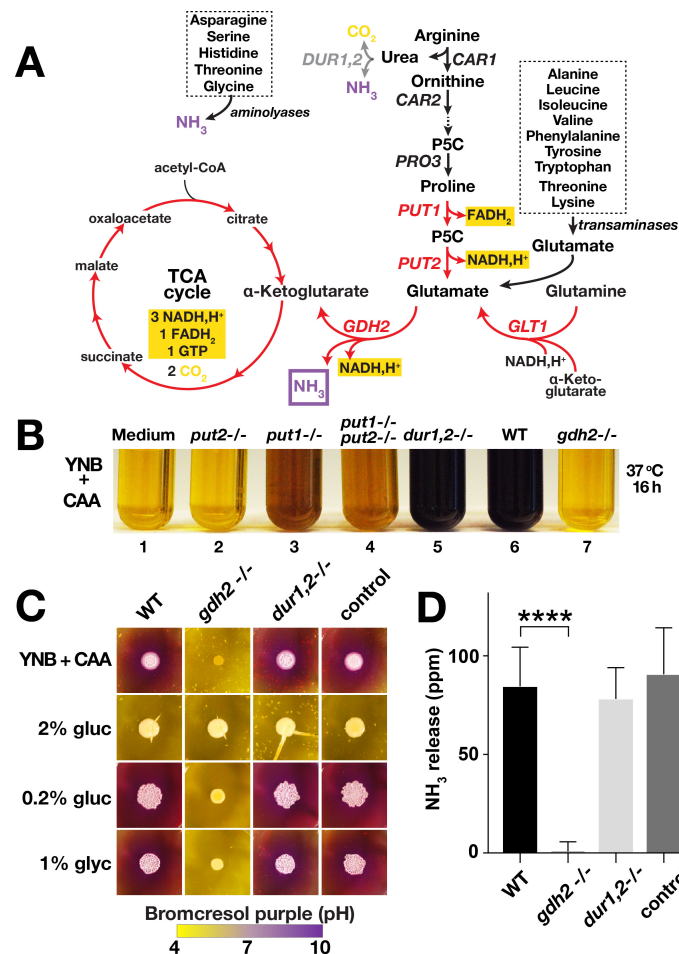
*Amino acid-dependent alkalization is linked to mitochondrial function*

- 804 **27.** Lorenz MC, Bender JA, Fink GR. Transcriptional response of *Candida albicans*  
805 upon internalization by macrophages. *Eukaryot Cell*. 2004;3(5):1076-87.
- 806 **28.** Tebung WA, Omran RP, Fulton DL, Morschhäuser J, Whiteway M. Put3  
807 positively regulates proline utilization in *Candida albicans*. *mSphere*. 2017;2(6).
- 808 **29.** Ljungdahl PO, Daignan-Fornier B. Regulation of amino acid, nucleotide, and  
809 phosphate metabolism in *Saccharomyces cerevisiae*. *Genetics*.  
810 2012;190(3):885-929.
- 811 **30.** Palkova Z, Devaux F, Ilicova M, Minarikova L, Le Crom S, Jacq C. Ammonia  
812 pulses and metabolic oscillations guide yeast colony development. *Mol Biol*  
813 *Cell*. 2002;13(11):3901-14.
- 814 **31.** Rícicova M, Kucerova H, Vachova L, Palkova Z. Association of putative  
815 ammonium exporters Ato with detergent-resistant compartments of plasma  
816 membrane during yeast colony development: pH affects Ato1p localisation in  
817 patches. *Biochim Biophys Acta*. 2007;1768(5):1170-8.
- 818 **32.** Fernandes TR, Segorbe D, Prusky D, Di Pietro A. How alkalization drives  
819 fungal pathogenicity. *PLoS Pathog*. 2017;13(11):e1006621.
- 820 **33.** Liao WL, Ramon AM, Fonzi WA. GLN3 encodes a global regulator of nitrogen  
821 metabolism and virulence of *C. albicans*. *Fungal Genet Biol*. 2008;45(4):514-26.
- 822 **34.** Vyas VK, Barrasa, M.I., Fink, G.R. A *Candida albicans* CRISPR system permits  
823 genetic engineering of essential genes and gene families. *Sci Adv*. 2015;1(3):1-6.
- 824 **35.** Vyas VK, Bushkin GG, Bernstein DA, Getz MA, Sewastianik M, Barrasa MI, et al.  
825 New CRISPR mutagenesis strategies reveal variation in repair mechanisms  
826 among fungi. *mSphere*. 2018;3(2).
- 827 **36.** Farboud BMBJ. Dramatic enhancement of genome editing by CRISPR:Cas9  
828 through improved guide RNA design. *Genetics*. 2015;199:959-71.
- 829 **37.** Reuss O, Vik A, Kolter R, Morschhäuser J. The *SAT1* flipper, an optimized tool  
830 for gene disruption in *Candida albicans*. *Gene*. 2004;341:119-27.
- 831 **38.** Tumusiime S, Zhang C, Overstreet MS, Liu Z. Differential regulation of  
832 transcription factors Stp1 and Stp2 in the Ssy1-Ptr3-Ssy5 amino acid sensing  
833 pathway. *J Biol Chem*. 2011;286(6):4620-31.
- 834 **39.** Davis MM, Alvarez FJ, Ryman K, Holm AA, Ljungdahl PO, Engström Y. Wild-  
835 type *Drosophila melanogaster* as a model host to analyze nitrogen source  
836 dependent virulence of *Candida albicans*. *PLoS One*. 2011;6(11):e27434.
- 837 **40.** Morales DK, Grahl N, Okegbe C, Dietrich LE, Jacobs NJ, Hogan DA. Control of  
838 *Candida albicans* metabolism and biofilm formation by *Pseudomonas*  
839 *aeruginosa* phenazines. *MBio*. 2013;4(1):e00526-12.

*Amino acid-dependent alkalization is linked to mitochondrial function*

- 840 **41.** Silve S, Volland C, Garnier C, Jund R, Chevallier MR, Haguenaer-Tsapis R.  
841 Membrane insertion of uracil permease, a polytopic yeast plasma membrane  
842 protein. *Mol Cell Biol.* 1991;11(2):1114-24.
- 843 **42.** Liu H, Kohler J, Fink GR. Suppression of hyphal formation in *Candida albicans*  
844 by mutation of a *STE12* homolog. *Science.* 1994;266(5191):1723-6.
- 845 **43.** Lee KL, Buckley HR, Campbell CC. An amino acid liquid synthetic medium for  
846 the development of mycelial and yeast forms of *Candida albicans*. *Sabouraudia.*  
847 1975;13(2):148-53.
- 848 **44.** Martínez P, Ljungdahl PO. An ER packaging chaperone determines the amino  
849 acid uptake capacity and virulence of *Candida albicans*. *Mol Microbiol.*  
850 2004;51(2):371-84.
- 851 **45.** Vonk AG, Wieland CW, Netea MG, Kullberg BJ. Phagocytosis and intracellular  
852 killing of *Candida albicans* blastoconidia by neutrophils and macrophages: a  
853 comparison of different microbiological test systems. *J Microbiol Methods.*  
854 2002;49(1):55-62.
- 855 **46.** Fonzi WA, Irwin MY. Isogenic strain construction and gene mapping in *Candida*  
856 *albicans*. *Genetics.* 1993;134(3):717-28.
- 857 **47.** Dabas N, Morschhäuser J. Control of ammonium permease expression and  
858 filamentous growth by the GATA transcription factors *GLN3* and *GAT1* in  
859 *Candida albicans*. *Eukaryot Cell.* 2007;6(5):875-88.  
860

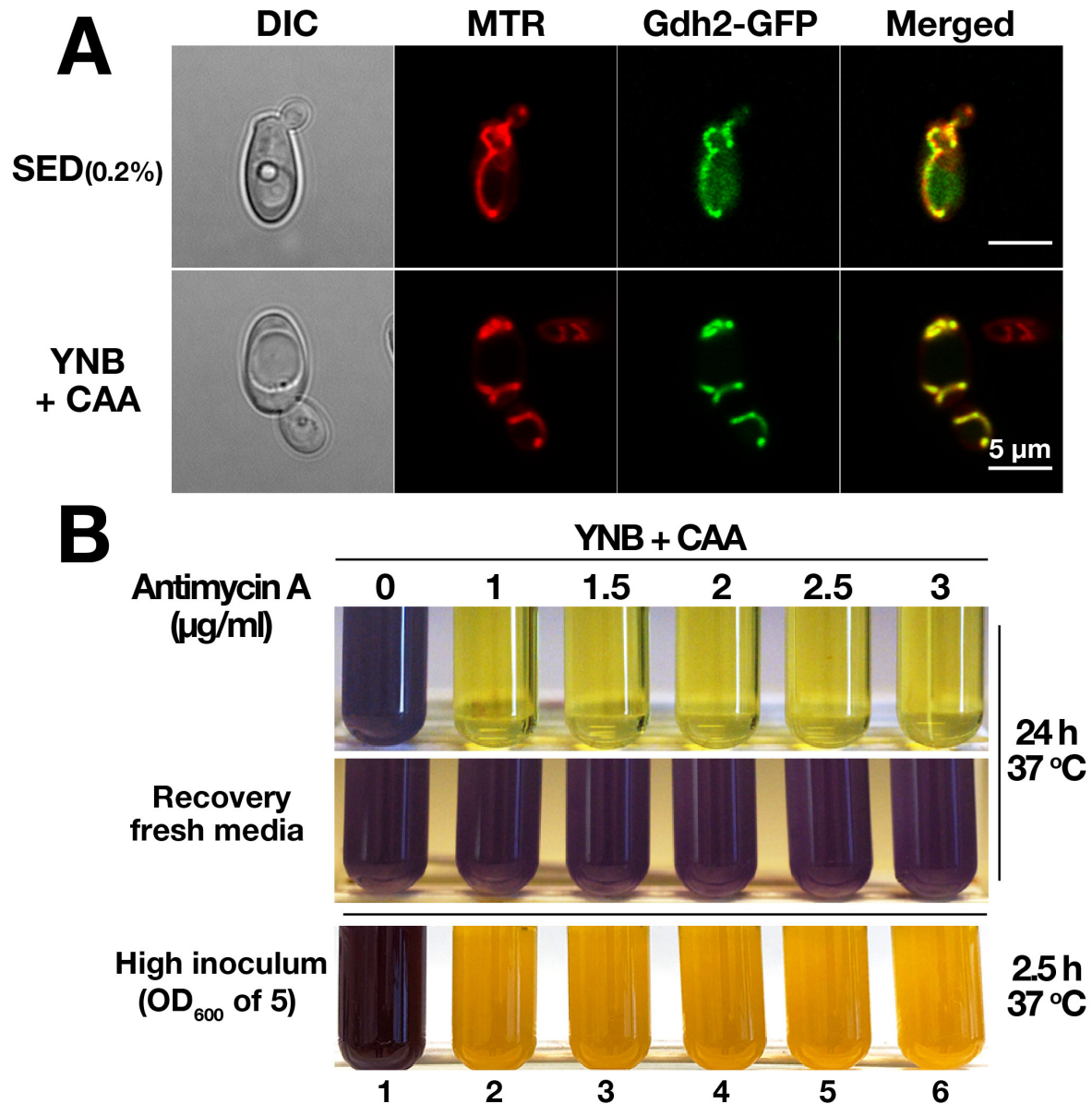
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861 **Figure 1. *C. albicans* GDH2 is required for growth using amino acids as sole**  
 862 **carbon and nitrogen sources**

863 (A) Schematic diagram of arginine/proline catabolism. Arginine is catabolized to  
 864 proline in the cytoplasm, proline is transported into mitochondria, proline is  
 865 catabolized to glutamate in two enzymatic reactions, catalyzed by FAD-dependent  
 866 proline oxidase (*PUT1*) and NAD<sup>+</sup>-linked Δ<sup>1</sup>-pyrroline-5-carboxylate (P5C)  
 867 dehydrogenase (*PUT2*), respectively. The two central reactions for nitrogen source  
 868 utilization are catalyzed by NADH-dependent glutamate synthase (*GLT1*) and NAD<sup>+</sup>-  
 869 linked glutamate dehydrogenase (*GDH2*). The gene products and metabolic steps  
 870 marked in red are localized to the mitochondria. (B) YPD grown *put2*<sup>-/-</sup> (CFG318),  
 871 *put1*<sup>-/-</sup> (CFG154), *put1*<sup>-/-</sup> *put2*<sup>-/-</sup> (CFG159), *dur1,2*<sup>-/-</sup> (CFG246), wildtype (WT,  
 872 SC5314) and *gdh2*<sup>-/-</sup> (CFG279) cells were washed, resuspended at an OD<sub>600</sub> of 0.05  
 873 in YNB+CAA containing the pH indicator bromocresol purple, and the cultures were  
 874 incubated shaking at 37 °C for 16 h. (C) Wildtype (WT, SC5314), *gdh2*<sup>-/-</sup> (CFG279),  
 875 *dur1,2*<sup>-/-</sup> (CFG246) and CRISPR control (CFG182) cells were pre-grown in YPD,  
 876 washed, resuspended at an OD<sub>600</sub> of 1, and 5 μl were spotted onto the surface of  
 877 solid YNB + CAA bromocresol purple without and with the indicated carbon source.  
 878 The plates were incubated at 37 °C for 72 h. The images are representative of at  
 879 least 3 independent experiments. (D) Volatile ammonia released from strains as in  
 880 (C); the results are the average of at least 3 independent experiments (Ave. ± CI; \*\*\*\*  
 881 p ≤ 0.0001).

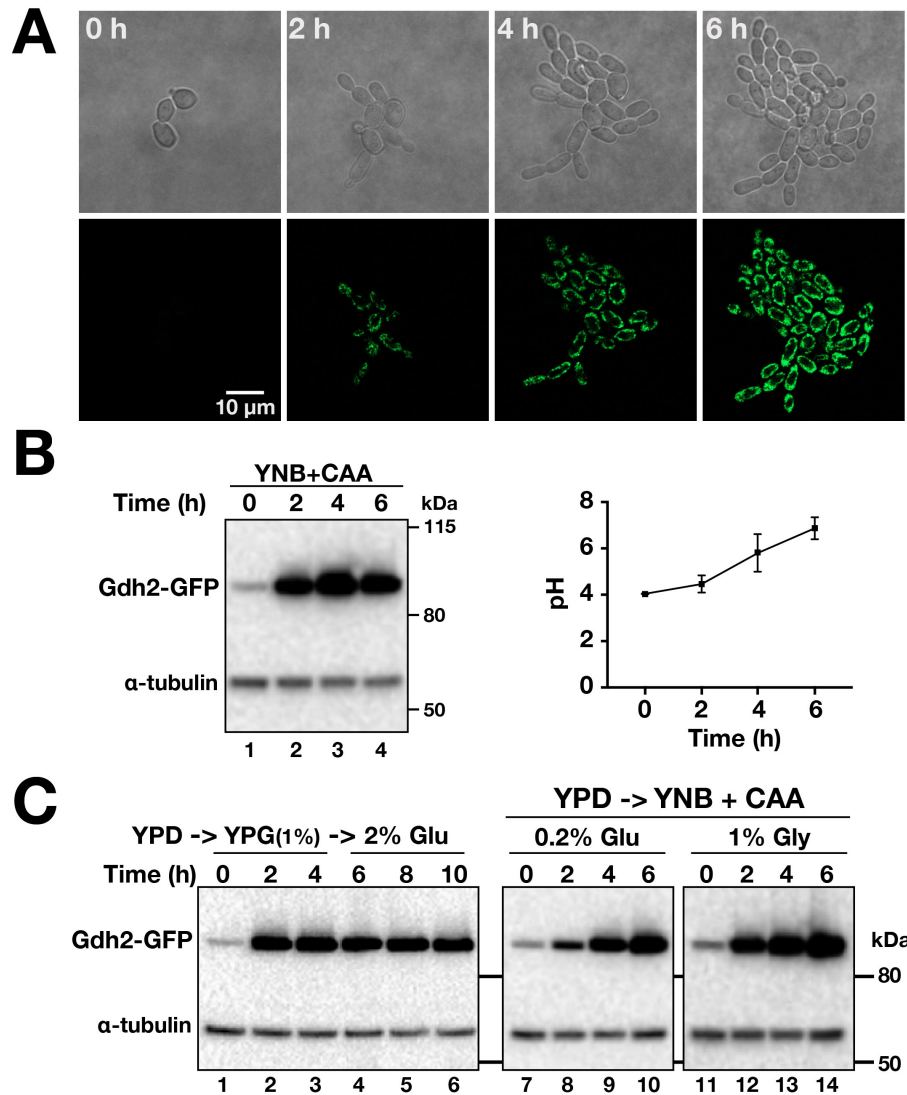
*Amino acid-dependent alkalization is linked to mitochondrial function*



882 **Figure 2. *C. albicans* Gdh2 localizes to the mitochondria and environmental**  
883 **alkalization requires mitochondrial function**

884 (A) Gdh2-GFP co-localizes with the mitochondrial marker MitoTracker Red (MTR).  
885 YPD grown cells expressing *GDH2-GFP* (CFG273) were harvested, washed, grown  
886 in SED (0.2% glucose) or YNB + CAA at 37 °C for 24 h, and stained with 200 nM  
887 MTR prior to imaging by differential interference contrast (DIC) and confocal  
888 fluorescence microscopy; the scale bar = 5 μm. (B) Wildtype cells (SC5314) from  
889 overnight YPD cultures were washed and then diluted to either OD<sub>600</sub> ≈ 0.1 (top  
890 panel) or ≈ 5 (bottom panel) in liquid YNB+CAA with the indicated concentrations of  
891 mitochondrial complex III inhibitor antimycin A. Cultures were grown at 37 °C under  
892 constant aeration for 24 h and 2.5 h, respectively, and photographed. To assess  
893 viability after Antimycin A treatment, inhibited cells from 24 h old culture (top panel)  
894 were harvested, washed, and then resuspended in fresh YNB+CAA media and  
895 incubated for 24 h (up to 48 h) at 37 °C (middle panel). Images are representative of  
896 at least 3 independent experiments.

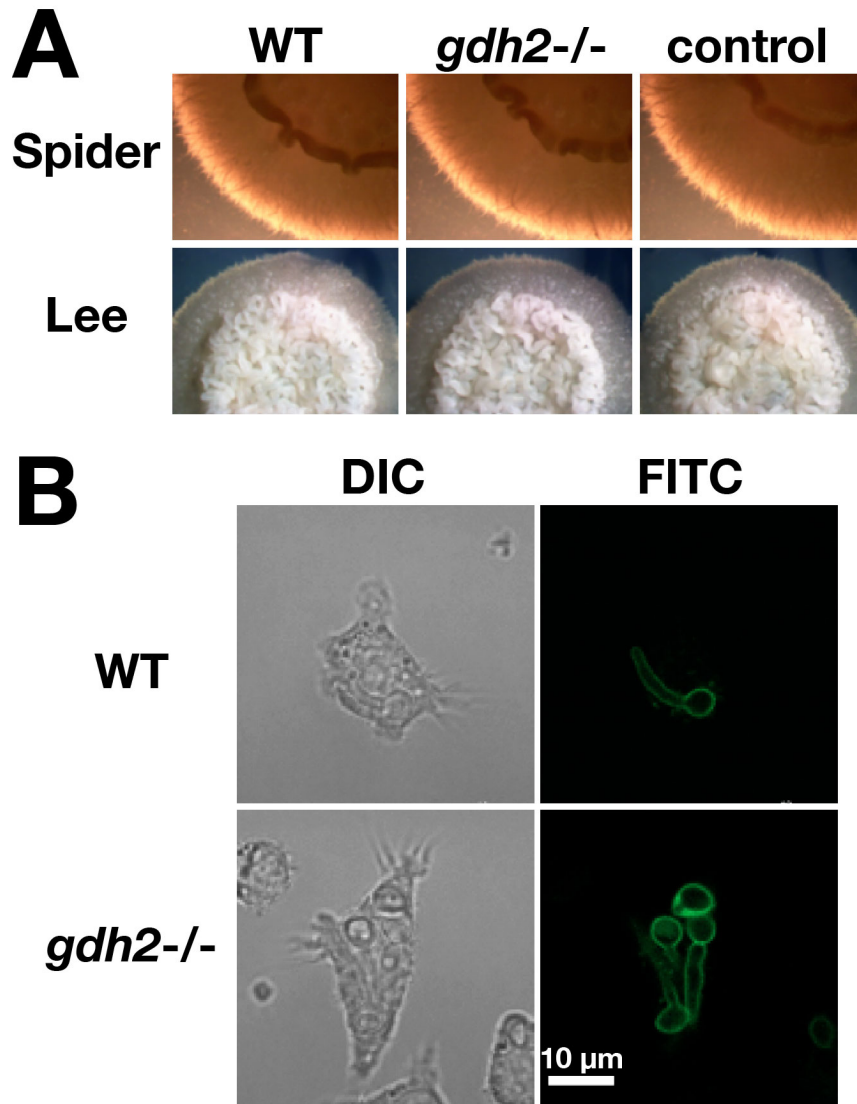
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897 **Figure 3. GDH2 expression is repressed by glucose**

898 (A) Live cell imaging of Gdh2-GFP expression in cells shifted from YPD to  
 899 YNB+CAA. CFG273 (Gdh2-GFP) cells were pre-grown in YPD and transferred to a  
 900 thin agar slab of YNB+CAA medium. Growth at 37 °C was monitored every hour for  
 901 6 h. (B) Gdh2-GFP expression is rapidly induced in cells shifted from YPD to  
 902 YNB+CAA. Cells (same strain as in A) were pre-grown in YPD and used to inoculate  
 903 liquid YNB+CAA (OD<sub>600</sub> of 2.0); at the times indicated, the pH was measured (right  
 904 panel; average of 3 independent experiments) and the levels of Gdh2-GFP  
 905 expression (left panel) were monitored by immunoblot analysis. (C) Gdh2 expression  
 906 is carbon source dependent. Cells (same strain as in A) grown in YPD were  
 907 harvested, transferred to YPG (YP + 1% glycerol; lanes 1-3) and after subsampling  
 908 at 6 hr, 2% glucose was added to cultures (lanes 5-6) (left panel); YPD grown cells  
 909 were shifted to YNB + CAA with 0.2% glucose (lanes 7-10) or 1% glycerol (lanes 11-  
 910 14) and grown at 37 °C (right panels). Extracts were prepared at the times indicated  
 911 and the levels of Gdh2-GFP and tubulin (loading control) were assessed by  
 912 immunoblotting using primary  $\alpha$ -GFP and  $\alpha$ -tubulin antibodies.

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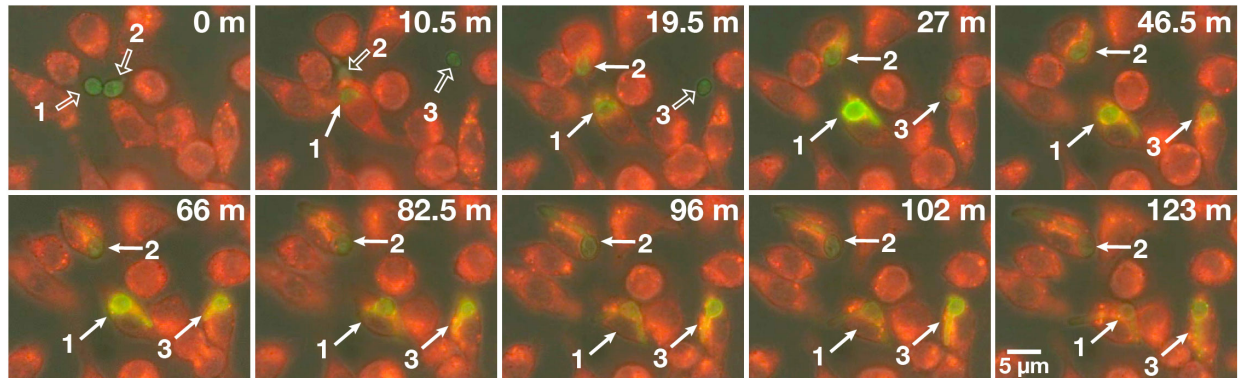
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914 **Figure 4. Gdh2 is dispensable for filamentous growth on solid media and inside**  
915 **phagosomes of engulfing macrophages**

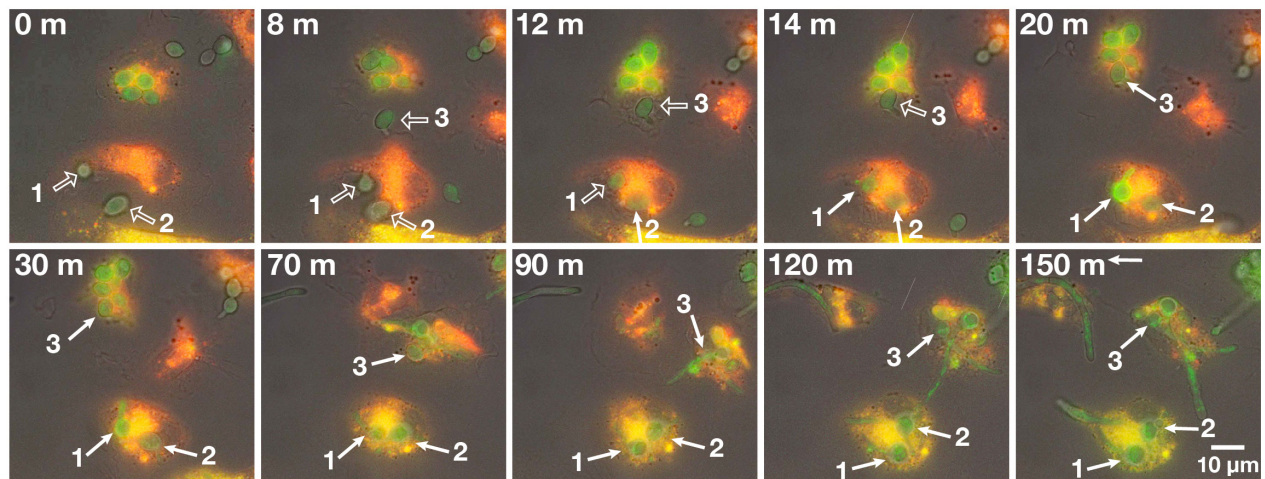
916 (A) Wildtype (WT, SC5314), *gdh2*<sup>-/-</sup> (CFG279), and CRISPR control (CFG182)  
917 strains, pre-grown in YPD, were washed, resuspended at an OD<sub>600</sub> of 1 in water, and  
918 5  $\mu$ l aliquots were spotted on solid Spider and Lee's media. Representative colonies  
919 were photographed 5 days after incubation at 37 °C. (B) FITC-stained WT (SC5314)  
920 and *gdh2*<sup>-/-</sup> (CFG279) cells were individually co-cultured with RAW264.7  
921 macrophages. Non-phagocytosed fungal cells were removed by washing and the  
922 co-cultures were monitored by live cell imaging for 1.5 h; scale bar = 10  $\mu$ m.

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**A** RAW264.7 murine macrophages (LST) : WT *GDH2-GFP* (C:M 1:1)



**B** murine BMDM (LST): WT *GDH2-GFP* (C:M 1:1)



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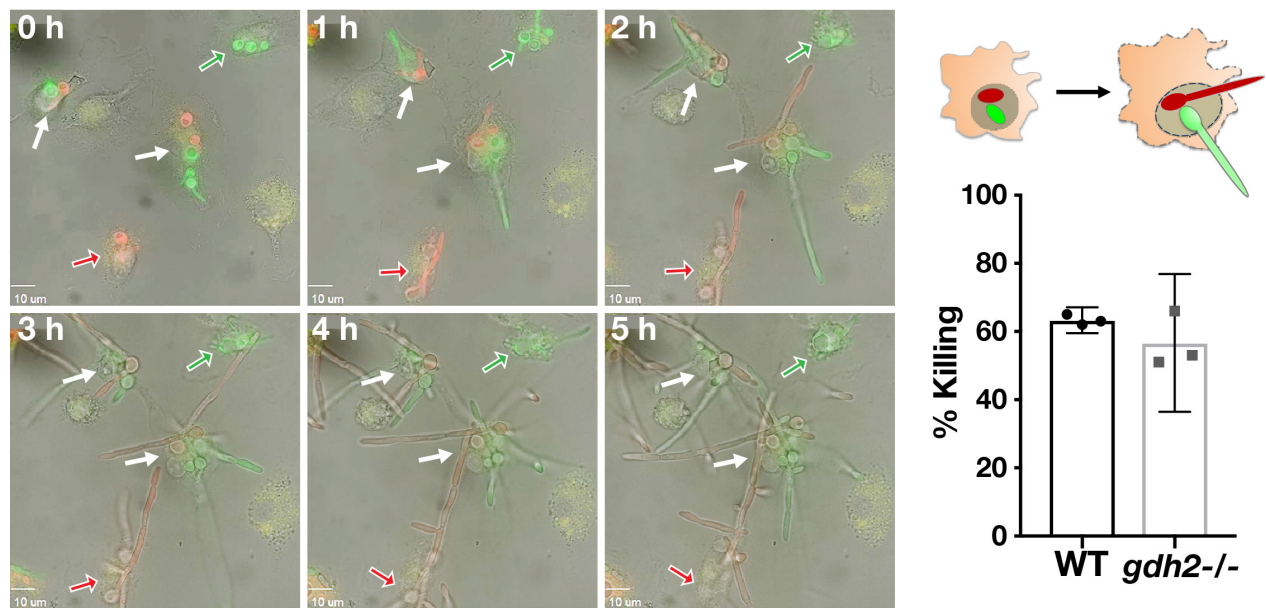
924 **Figure 5. Gdh2-GFP expression is induced in *C. albicans* cells phagocytized by**  
925 **murine macrophages**

926 CFG273 cells were co-cultured in CO<sub>2</sub>-independent medium with (A) RAW264.7  
927 macrophages, or (B) primary murine bone marrow-derived macrophages (BMDM)  
928 pre-stained with Lysotracker Red (LST) at MOI of 1:1 (C:M). The co-cultures were  
929 followed by live cell imaging. Micrographs were taken at the times indicated (see  
930 Videos V1 and V2, Supporting Information). In each series, three CFG273 cells are  
931 marked prior to (open arrows) and after (closed arrows) being phagocytized.



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murine BMDM : WT  $P_{ADH1}$ -GFP ::  $gdh2\Delta$   $P_{ADH1}$ -RFP (C:M 3:1; C::C 1:1)



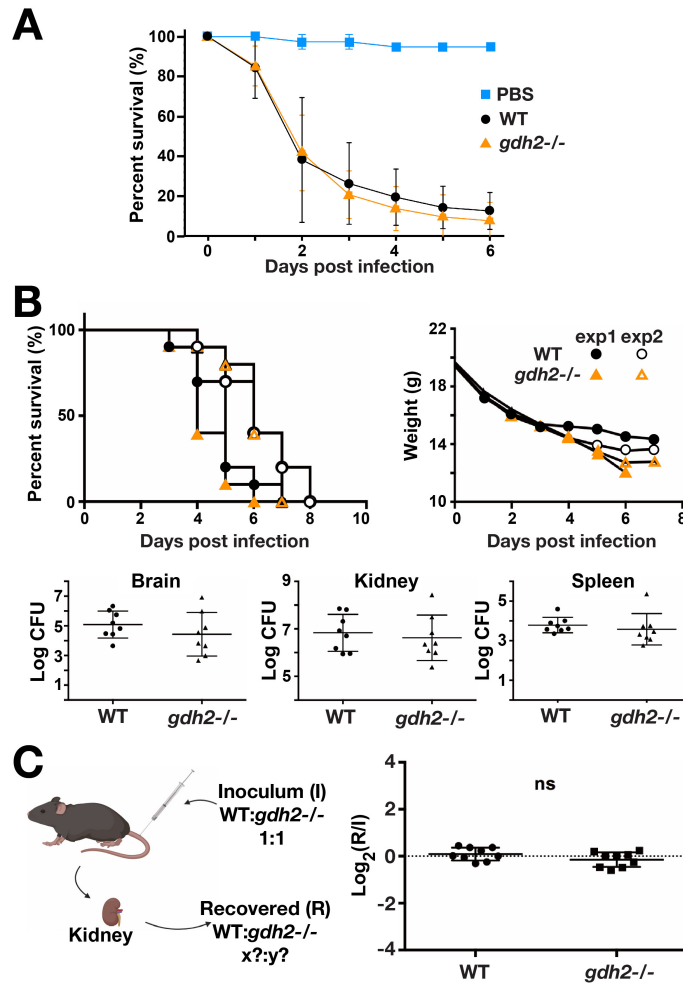
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933 **Figure 6. Competition assay to compare wildtype and  $gdh2^{-/-}$  filamentation**  
934 **and survival upon phagocytosis by primary BMDM**

935 Wildtype (WT;  $P_{ADH1}$ -GFP; SCADH1G4A) and  $gdh2^{-/-}$  ( $P_{ADH1}$ -RFP, CFG275) cells (1:1)  
936 were co-cultured with primary BMDM (MOI of 3:1; C:M) for 30 min in HBSS. Non-  
937 phagocytosed fungal cells were removed by washing and the co-culture was  
938 monitored by live cell imaging for 5 h (see video V3, Supporting Information). Solid  
939 arrows indicate macrophages with phagosomes containing both WT and  $gdh2^{-/-}$ -  
940 cells; open arrows indicate macrophages with phagosomes containing either WT  
941 (green) or  $gdh2^{-/-}$  (red) cells. The observed growth of WT and  $gdh2^{-/-}$  cells within a  
942 single macrophage is schematically illustrated (right upper panel). Candidacidal  
943 activity of BMDM (right lower panel); CFUs recovered at 2 h were compared to the  
944 CFUs in the starting inoculum.

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### 947 **Figure 7. Virulence of wildtype and *gdh2*<sup>-/-</sup> *C. albicans* in *Drosophila* and** 948 **murine systemic infection models**

949 (A) *D. melanogaster* *Bom*<sup>A55C</sup> flies were infected with wildtype (SC5314) or *gdh2*<sup>-/-</sup>  
950 (CFG279) cells as indicated, and the survival of flies was followed for six days. Each  
951 curve represents the average of a minimum of three independent infection  
952 experiments (20 flies/strain) performed on different days. (B) Groups of C57BL/6  
953 mice (n=10) were infected via the lateral tail vein with 3x10<sup>5</sup> CFU of *C. albicans*  
954 wildtype or *gdh2*<sup>-/-</sup> cells (upper panels) and survival (left) and weight loss (right) was  
955 monitored at the timepoints indicated. Survival curves from two independent  
956 experiments were statistically analyzed by the Kaplan-Meier method (a log-rank test,  
957 GraphPad Prism), no significant difference. The fungal burden (lower panels) in brain  
958 (left), kidney (middle), and spleen (right) extracted from mice 3 days post infection.  
959 Each symbol represents a sample from an individual mouse and results were  
960 compared by Student *t*-test, no significant difference. (C) Competition assay; mice  
961 were infected via the tail vein with an inoculum (I) comprised of an equal number of  
962 wildtype (SC5314) and *gdh2*<sup>-/-</sup> (CFG279), 1:1. At 3 days post infection, the  
963 abundance and genotype of fungal cells recovered from kidneys was quantitated  
964 and the ratio of wildtype:*gdh2*<sup>-/-</sup> recovered (R) was determined. The significance of  
965 the log<sub>2</sub>(R/I) values was assessed using an unpaired *t*-test, no significant difference  
966 (ns).

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967 **Supporting Information**

968 **Figures:**

969 **Fig S1.** CRISPR/Cas9-Mediated Inactivation of *GDH2* in *C. albicans*

970 **Fig S2.** Growth characteristics of wildtype and *gdh2*<sup>-/-</sup> strains in liquid YNB+CAA  
971 with and without glucose and chloramphenicol (Cm)

972

973 **Videos:**

974 **V1.** Gdh2-GFP is induced upon phagocytosis by murine RAW264.7 macrophages  
975 (**Fig. 5A**).

976 **V2.** Gdh2-GFP is induced upon phagocytosis by primary murine BMDM (**Fig. 5B**).

977 **V3.** Competition assay to compare wildtype and *gdh2*<sup>-/-</sup> filamentation and  
978 survival upon phagocytosis by BMDM (**Fig. 6**).

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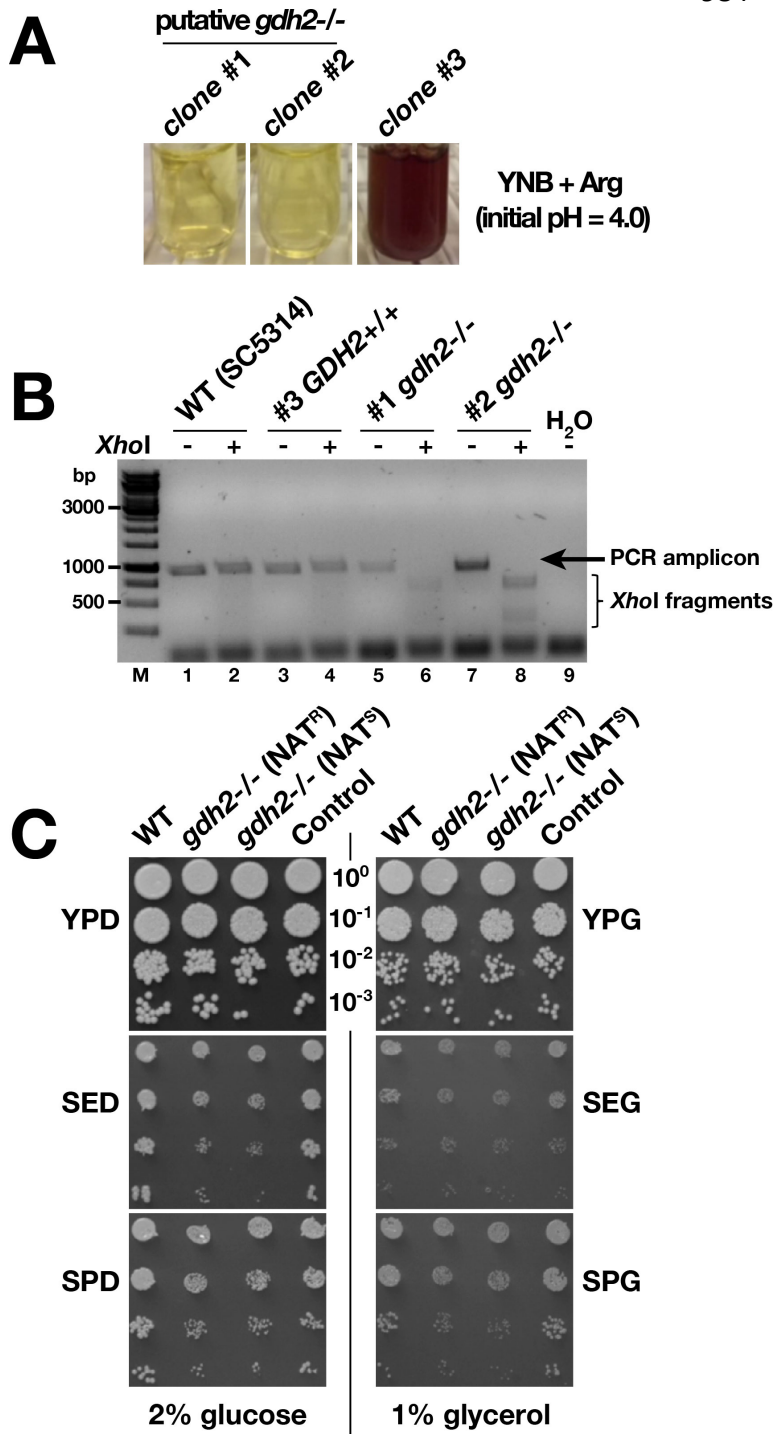
980 **Tables:**

981 **Table S1.** Strains used in this study

982 **Table S2.** Primers used in this study

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983 Supporting Information – Figures



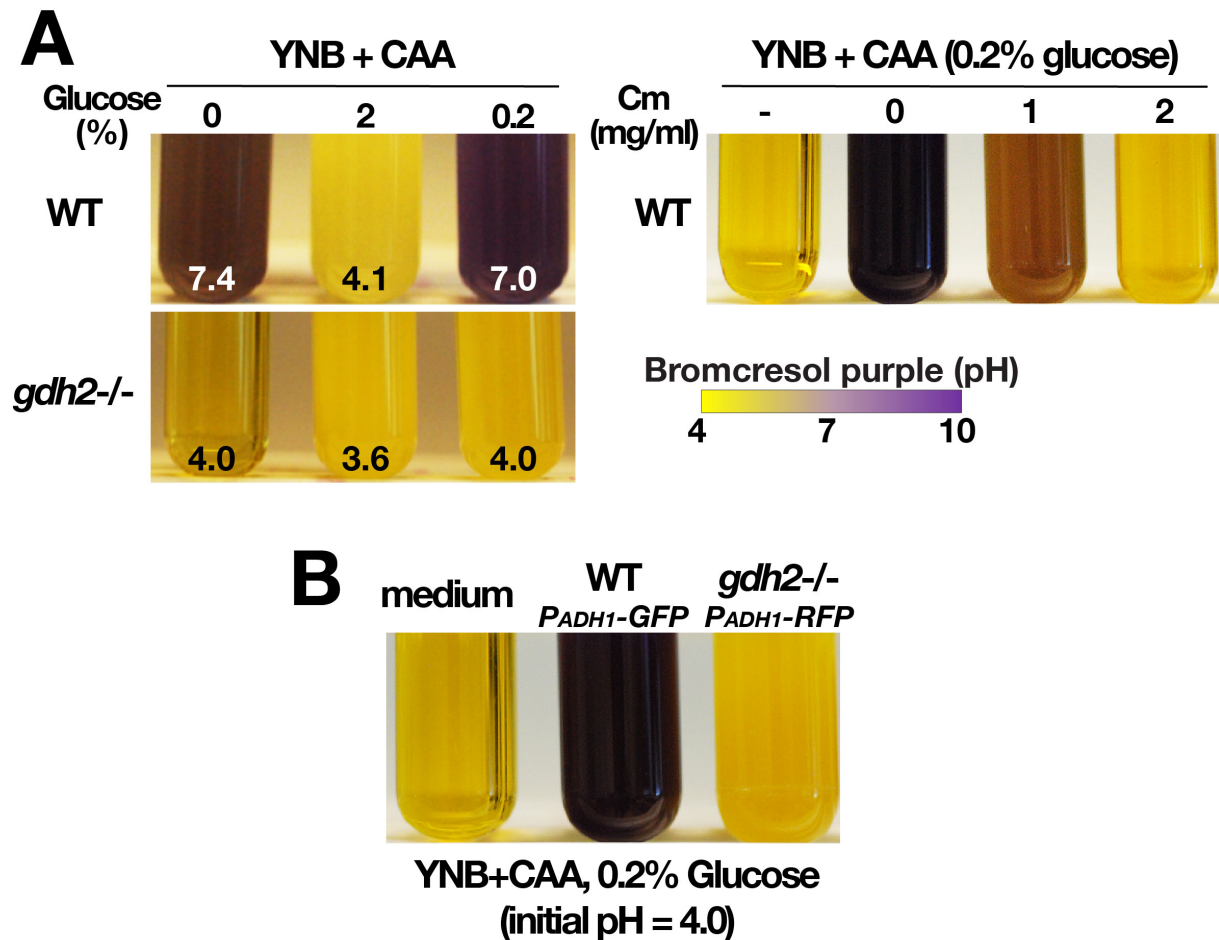
984 **Fig. S1. CRISPR/Cas9-mediated gene inactivation of *GDH2* in *C. albicans***

(A) A purified *Kpn*I/*Sac*I fragment from pFS108, harboring *GDH2*-specific sgRNA, and PCR generated repair template (RT) were introduced into wildtype strain SC5314 by electroporation. *Nou*<sup>R</sup> transformants were pre-screened in YNB+Arg medium containing the pH indicator bromocresol purple; the initial pH was 4.0. Three *Nou*<sup>R</sup> colonies were picked for further analysis. Clones #1 and #2 grew poorly and were unable to alkalize the media; clone #3 grew and alkalized the media. (B) Genomic DNA, isolated from the three clones, was used as template for PCR amplification of the targeted *GDH2* locus; ddH<sub>2</sub>O was used as negative control. Restriction of the amplified ~900 bp fragment by *Xho*I is diagnostic for successful mutagenesis. Strains, clone #1 (CFG277) and clone #2 (CFG278) carry inactivated *gdh2*<sup>-/-</sup> alleles. (C) *GDH2* is not essential but required for robust growth on glutamate or proline as sole nitrogen source. Five microliters of serially diluted wildtype (SC5314), *gdh2*<sup>-/-</sup> NAT<sup>R</sup> (CFG277), *gdh2*<sup>-/-</sup> NAT<sup>S</sup> (CFG279), and control (CFG182) cells were spotted on yeast peptone (YP), synthetic glutamate (SE) and synthetic proline (SP) media containing either 2% glucose (D) or 1% glycerol (G) as carbon source. The plates were incubated for 48 h at 30 °C and photographed.

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1038 **Fig. S2. Growth characteristics of wildtype and *gdh2*<sup>-/-</sup> strains in liquid**  
1039 **YNB+CAA with and without glucose and chloramphenicol (Cm)**

1040 (A) Gdh2-dependent alkalization is sensitive to glucose (Left panel). YPD grown  
1041 wildtype (WT, SC5314) and *gdh2*<sup>-/-</sup> (CFG279) cells were collected, washed, and  
1042 diluted to an OD<sub>600</sub> of 0.05 in YNB+CAA with 0, 2 or 0.2% glucose as indicated. The  
1043 cultures were grown under vigorous agitation at 37 °C for 16 h and the pH was  
1044 measured (the initial pH was 4.0; the values indicated are the average of three  
1045 replicate cultures). Alkalization is linked to mitochondrial function (Right panel).  
1046 Wildtype cells (SC5314) from overnight YPD cultures were washed and diluted to  
1047 OD<sub>600</sub> ≈ 0.1 in liquid YNB+CAA (0.2% glucose) with the indicated concentrations of  
1048 mitochondrial translation inhibitor chloramphenicol. Cultures were grown at 37 °C  
1049 under vigorous agitation for 16 h. (B) Phenotypic validation of the reporter strains  
1050 used in macrophage co-cultures. Growth of wildtype (WT; *P<sub>ADH1</sub>-GFP*; SCADH1G4A)  
1051 and *gdh2*<sup>-/-</sup> (*P<sub>ADH1</sub>-RFP*, CFG275) cells in YNB+CAA supplemented with 0.2%  
1052 glucose. Cultures were grown for 16 h at 37 °C.

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*Amino acid-dependent alkalization is linked to mitochondrial function*

1054 **Supporting Information - Tables**

**Table S1. Strains used in this study.**

Strain	Genotype	Reference
CAI4-derived strains		
CAI4	<i>ura3Δ::imm434/ura3Δ::imm434</i>	(46)
CFG273	<i>ura3Δ::imm434/ura3Δ::imm434 GDH2/GDH2-GFP-URA3</i>	This work
SC5314-derived strains		
SC5314	<i>Prototrophic wild type</i>	
CFG154	<i>NEUT5L::FRT put1-/put1-</i>	(15)
CFG159	<i>NEUT5L::FRT put1-/put1- ENO1/eno1::P<sub>ENO1</sub>-CC9-pFS083 put2-/put2-</i>	(15)
CFG318	<i>NEUT5L::FRT put2-/put2-</i>	(15)
CFG182	<i>NEUT5L::pV1524</i>	(15)
CFG246	<i>ENO1/eno1::P<sub>ENO1</sub>-CC9-pFS039 dur1,2-/-</i>	(15)
CFG275	<i>NEUT5L::FRT gdh2-/- ADH1/adh1::P<sub>ADH1</sub>-RFP-NAT</i>	This work
CFG277	<i>NEUT5L::P<sub>ENO1</sub>-CC9-pFS108 gdh2-/- (Clone 1)</i>	This work
CFG278	<i>NEUT5L::P<sub>ENO1</sub>-CC9-pFS108 gdh2-/- (Clone 2)</i>	This work
CFG279	<i>NEUT5L::FRT gdh2-/- (from CFG277)</i>	This work
CFG281	<i>NEUT5L::FRT gdh2-/- (from CFG278)</i>	This work
CFG283	<i>NEUT5L:: P<sub>ENO1</sub>-CC9-pFS108</i>	This work
SCADH1G4A	<i>ADH1/adh1::P<sub>ADH1</sub>-GFP-caSAT1</i>	(47)

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*Amino acid-dependent alkalization is linked to mitochondrial function*

1056 **Table S2. Primers used in this study**

<b>p#</b>	<b>Primer Name</b>	<b>Sequence</b>	<b>Reference</b>
1	sgRNA-GDH2T	atttgTACATTGACTCCCCCTTTAGg	This study
2	sgRNA-GDH2B	aaaacCTAAAGGGGGAGTCAATGTAc	This study
3	RT-GDH2Top	GTTTAAACATTTACAGAACCACATCAAACAC TTCATCCCAAGTTAGTTTGAAACACGACTAA ctcgagTAAttTAGGG	This study
4	RT-GDH2Bot	CAGGGATAAAACCAGTGGAAATCCAAAACAT CCAAAACCTGATCAAATTGATCCTTTTTACC CTAaaTTActcgagTTAGTC	This study
5	GDH2-VerF	CACATAGAGTATGCATGCAC	This study
6	GDH2-VerR	GATTCAGCATCAACAGTGTC	This study
7	GDH2-GFPTop	GTTTACTCTAGAGGAATCGATTCTAATCCTG CTAAATTTTTGGAATTTATCAGTTCTATTAGA AAGGATTTTATTCAAAGGGATTGCTCAAGT ATGGTGCTGGCGCAGGTGCTTC	This study
8	GDH2-GFPBot	AAGCAAACCTTTAAATAAATAATTATAAATAGA ATTTTTGAAAATCAAGCATTTTCTCATAATTAT AGATAAATCTCTAAACGTATTTGAAACAACCT CTGATATCATCGATGAATTCGAG	This study
9	GDH2-GFPverrev	CTTCGGGCATGGCACTCTTG	This study
10	p91_FS95	GGCATAGCTGAAACTTCGGC	(15)
11	p112_5'ADH1test	ACAATATTTGATAGAGAC	(15)
12	p113_3'ADH1test	TTGAATCTACGAGACTC	(15)

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