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5 **Mitochondrial proline catabolism activates Ras1/cAMP/PKA-induced**
6 **filamentation in *Candida albicans***

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22 **Abstract**

23 Amino acids are among the earliest identified inducers of yeast-to-hyphal transitions in *Candida*
24 *albicans*, an opportunistic fungal pathogen of humans. Here, we show that the morphogenic
25 amino acids arginine, ornithine and proline are internalized and metabolized in mitochondria via
26 a *PUT1*- and *PUT2*-dependent pathway that results in enhanced ATP production. Elevated ATP
27 levels correlate with Ras1/cAMP/PKA pathway activation and Efg1-induced gene expression.
28 The magnitude of amino acid-induced filamentation is linked to glucose availability; high levels
29 of glucose repress mitochondrial function thereby dampening filamentation. Furthermore,
30 arginine-induced morphogenesis occurs more rapidly and independently of Dur1,2-catalyzed
31 urea degradation, indicating that mitochondrial-generated ATP, not CO₂, is the primary
32 morphogenic signal derived from arginine metabolism. The important role of the SPS-sensor of
33 extracellular amino acids in morphogenesis is the consequence of induced amino acid permease
34 gene expression, i.e., SPS-sensor activation enhances the capacity of cells to take up
35 morphogenic amino acids, a requisite for their catabolism. *C. albicans* cells engulfed by murine
36 macrophages filament, resulting in macrophage lysis. Phagocytosed *put1*^{-/-} and *put2*^{-/-} cells do
37 not filament and do not lyse macrophages, consistent with a critical role of mitochondrial proline
38 metabolism in virulence.

39

40 Introduction

41 *Candida albicans* is an opportunistic fungal pathogen that commonly exists as a benign member
42 of the human microbiome. Immunosuppression, or microbial dysbiosis, can predispose an
43 individual to infection, enabling this fungus to initiate and develop a spectrum of pathologies,
44 including superficial mucocutaneous or even life-threatening invasive infections [1, 2]. As a
45 human commensal, *C. albicans* can asymptotically colonize virtually all anatomical sites in
46 the host, each with a characteristic and unique microenvironment, with differing nutrient and
47 microbiome compositions, physical properties, and levels of innate immune defenses [3]. The
48 ability to colonize and infect discrete microenvironments is attributed to an array of virulence
49 characteristics, a major one being its morphological plasticity. As a pleomorphic organism, *C.*
50 *albicans* can assume at least three distinct morphologies: yeast-like, pseudohyphae, and true
51 hyphae, where the latter two are commonly referred to as filamentous morphologies (for review
52 see [4-7]). Strains that are genetically locked in either yeast or filamentous forms fail to mount
53 infections *in vitro* and *in vivo* infection models, supporting the concept that morphological
54 switching, rather than the specific morphology *per se*, is a requisite to virulence [4, 6, 8-10]. The
55 environmental signals known to trigger morphogenesis in *C. albicans* reflect the conditions
56 within the human host, such as temperature (37 °C) and CO₂, alkaline pH, the presence of serum,
57 N-acetylglucosamine, and a discrete set of amino acids.

58 Early studies examining amino acid-induced morphogenesis implicated metabolism as being
59 important for filamentation, and the inducing effects were shown to correlate to their specific
60 point-of-entry in metabolism [11-13]. The most potent inducers of filamentation are amino acids
61 that are catabolized to glutamate, such as arginine and proline, which enters the TCA cycle via
62 α -ketoglutarate. Importantly, arginine and proline can supply nitrogen and carbon for
63 intermediary metabolism and their catabolism provides energy to support diverse cellular
64 functions. Studies examining proline uptake and distribution during filamentous growth
65 suggested that proline catabolism results in an increase in the cellular reducing potential, i.e.,
66 enhanced levels of reduced flavoproteins were noted [11]. Several of the conclusions from these
67 earlier studies, in particular that filamentous growth of *C. albicans* is linked to repression of
68 mitochondrial activity [11-13], appear to conflict with more recent reports showing that

69 filamentation is accompanied by increased mitochondrial respiratory activity [14-16]. Clearly,
70 the underlying mechanisms through which amino acids induce filamentation remain to be
71 defined. In particular, the basis of arginine- and proline-induced morphogenesis needs to be
72 placed in context to the current mechanistic understanding of the signaling cascades implicated
73 in morphogenesis.

74 Among the central metabolic signaling pathways in *C. albicans* linked to morphogenesis, the
75 best characterized are the mitogen-activated protein kinase (MAPK) and the 3'-5'-cyclic
76 adenosine monophosphate/Protein Kinase A (cAMP/PKA) signaling systems, which activate the
77 transcription factors Cph1 and Efg1, respectively [8, 17, 18], reviewed in [4, 7, 19, 20]. Ras1 is
78 a small GTPase required for proper MAPK and cAMP/PKA signaling, and specifically for the
79 induction of filamentation by amino acids and serum [21, 22], reviewed in [20, 23]. Recently,
80 Grahl et al. have proposed that intracellular ATP levels and increased mitochondrial activity,
81 control the activation of Ras1/cAMP/PKA pathway [14]. In this intriguing model, the adenyl
82 cyclase (Cyr1/Cdc35) works cooperatively in a positive feedback loop with ATP as key input.
83 Accordingly, ATP promotes Cyr1 binding to the active GTP-bound form of Ras1 thereby
84 reducing the ability of Ira2 to stimulate the intrinsic GTPase activity of Ras1. As a consequence,
85 enhanced Cyr1 activity leads to elevated levels of cAMP and amplification of PKA-dependent
86 signaling, activating the effector transcription factor Efg1 and the expression of genes required
87 for filamentous growth [24-26], reviewed in [20, 23, 27, 28].

88 Some morphogenic signals appear to bypass the requirement for Ras1 (reviewed in [27, 28]).
89 By example, CO₂ is a well-characterized stimulus for morphological switching in *C. albicans*;
90 CO₂ binds directly and activates Cyr1 [29]. Ghosh et al. have proposed that arginine-induced
91 morphogenesis is the consequence of arginase (*CARI*) dependent metabolism to ornithine and
92 urea, and subsequent urea amidolyase (*DURI,2*) dependent generation of CO₂ from urea [30].
93 Also, the G protein-coupled receptor Gpr1, which has been implicated in amino acid-induced
94 morphogenesis, does not appear to require Ras1. Gpr1-initiated signals activate Cyr1 by
95 stimulating GTP-GDP exchange on the G α protein Gpa2; the active GTP-bound form of Gpa2
96 is thought to bind to the G α -binding domain within the N-terminal of Cyr1 leading to enhanced
97 cAMP production (reviewed in [20, 28]). It has been reported that Gpr1 senses the presence of

98 extracellular methionine [31] and glucose [32], however recently, lactate has been proposed to
99 be the primary activating ligand [33]. The role of Gpr1 in amino acid-induced morphogenesis
100 remains an open question.

101 *C. albicans* cells respond to the presence of extracellular amino acids using the plasma
102 membrane-localized SPS (Ssy1-Ptr3-Ssy5) sensor complex [34-36]. In response to amino acids,
103 the primary sensor Ssy1 (Csy1) is stabilized in a signaling conformation leading to Ssy5-
104 mediated proteolytic processing of two latently expressed transcription factors, Stp1 and Stp2
105 [34]. The processed factors efficiently target to the nucleus activating the expression of distinct
106 sets of genes required for assimilation of external nitrogen. Stp1 regulates the expression of
107 *SAP2*, encoding the major secreted aspartyl proteinase, and oligopeptide transporters (*OPT1* and
108 *OPT3*); whereas Stp2, derepresses the expression of a subset of amino acid permeases (AAP)
109 that facilitate amino acid uptake. *STP1* expression is controlled by nitrogen catabolite repression
110 (NCR), a supra-regulatory system that represses that utilization of non-preferred nitrogen sources
111 when preferred ones are available [37]. The endoplasmic reticulum (ER)-localized chaperone
112 Csh3, is required for the functional expression of both Ssy1 and AAPs, and thus acts as the most
113 upstream and downstream component of the SPS sensing pathway [35]. Strains lacking either
114 Ssy1 or Csh3 fail to efficiently respond to the presence of extracellular amino acids and serum
115 and exhibit impaired morphological switching [35, 36]. It has not previously been determined if
116 the SPS-sensor induces morphogenesis directly in response to extracellular amino acids, or
117 indirectly, the consequence of enhanced amino acid uptake and subsequent intracellular
118 signaling events.

119 In this report, we show how amino acid-induced and SPS-sensor-dependent signals are
120 integrated into the central signaling pathways that control yeast-to-hyphal morphological
121 transitions in *C. albicans*. Our results indicate that the augmented levels of intracellular ATP,
122 resulting from catabolism of proline in the mitochondria, correlate with activated
123 Ras1/cAMP/PKA and Efg1-dependent gene expression. The magnitude of the response is
124 sensitive to the levels of glucose in a manner consistent with glucose repression of mitochondrial
125 function. The SPS-sensor plays an indirect, but important, role in enhancing the uptake of the
126 inducing amino acids. Finally, we show that *C. albicans* cells express proline catabolic enzymes

127 when phagocytosed by murine macrophages, and that inactivation of proline catabolism
128 diminishes the capacity of *C. albicans* cells to induce hyphal growth and escape engulfing
129 macrophages.

130

131 **Results**

132 **Amino acid-induced morphogenesis is dependent on uptake**

133 We assessed the capacity of ornithine, citrulline, and the 20 amino acids commonly found in
134 proteins to induce filamentous growth in *C. albicans*. Wildtype (WT) cells were grown as
135 macrocolonies on MES-buffered (pH of 6.0) synthetic dextrose (2% glucose) medium containing
136 10 mM of each individual amino acid as sole nitrogen source. As shown in **Fig. 1A**, proline and
137 arginine strongly induced filamentous growth as evidenced by the formation of wrinkled
138 macrocolonies. Microscopic evaluation of cells from wrinkled colonies confirmed the presence
139 of extensive filamentous growth (mainly hyphae). Ornithine, a non-proteinogenic amino acid
140 and a catabolic intermediate in the degradation of arginine, induced pronounced filamentous
141 growth. Of the amino acids tested, aspartate consistently produced smooth macrocolonies
142 comprised of round cells, exclusively yeast-like in appearance. Consequently, aspartate was
143 chosen as a reference for subsequent studies.

144 Using quantitative RT-PCR (qRT-PCR) we analyzed the expression of known hyphae-
145 specific genes (HSG) *ECE1*, *EED1*, *HWPI*, *UME6*, *ALS3*, *HGC1*, *SAP4*, and *SAP5* [4] in cells
146 from colonies grown on media with arginine, proline, ornithine and aspartate. With the exception
147 of *EED1*, the expression of HSG were clearly induced in cells grown on media with morphogenic
148 amino acids, ≥ 7 -fold higher than in cells grown on aspartate (**Fig. S1**). *SAP4*, a known Efg1-
149 regulated gene [38], exhibited the highest level of induction, ≥ 80 -fold higher levels than in
150 aspartate grown cells. These experiments were repeated using liquid cultures, and the same
151 trends were observed (data not shown). These results confirm the appropriateness of using
152 macrocolonies to score amino acid-induced morphogenesis.

153 Next, we evaluated whether SPS-sensor activation was required for amino acid-induced
154 morphogenesis. This was accomplished by assessing SPS-sensor dependent Stp2 processing

155 [34]. A strain carrying a functional C-terminal HA tagged Stp2 (Stp2-HA; PMRCA44) was
156 grown in minimal ammonium-based synthetic dextrose (SD) medium, extracts were prepared 5
157 min after induction by the indicated amino acid. Arginine (R), asparagine (N), aspartate (D),
158 glutamine (Q), histidine (H), lysine (K), serine (S) and ornithine (Orn) efficiently activated the
159 SPS-sensor; extracts contained the shorter processed form of Stp2 (**Fig. 1B**, upper panel). Next,
160 we assessed Stp2-dependent promoter activation using an integrated P_{CAN1} -NanoLucTM-PEST
161 reporter construct; the expression of the luciferase signal is controlled by the *CAN1* promoter,
162 which is strictly dependent on the SPS-sensor and Stp2 (**Fig. S2A and S2B**). The inclusion of
163 the 41-amino acid PEST sequence confers a shorter NanoLucTM lifetime, which facilitates a
164 tighter coupling of transcription and translation [39]. Enhanced luminescence was observed only
165 in cells induced with the amino acids giving rise to Stp2 processing (**Fig. 1B**, lower panel).
166 Notably, proline, which induces robust filamentation, did not activate the SPS-sensor as no Stp2
167 processing or luminescence was detected. Conversely, aspartate, which does not induce
168 filamentous growth, robustly activated the SPS-sensor as determined by Stp2 processing and
169 enhanced luciferase activity. These results indicate that amino acid-induced morphogenesis is
170 not obligatorily coupled to SPS-sensor signaling.

171 The contribution of signals derived from the SPS sensing pathway on filamentation induced
172 by arginine, ornithine and proline was examined. Arginine, a potent inducer of the SPS-sensor
173 (**Fig. 1B**), induced filamentation in an SPS-sensor independent manner; filamentation was
174 observed in mutants lacking components of the SPS sensing pathway (**Fig. 1C**). By contrast,
175 ornithine, also a potent inducer of the SPS-sensor, induced filamentous growth in a strictly SPS-
176 sensor- and Stp2-dependent manner (**Fig. 1C**). Notably, Stp1, a transcription factor that induces
177 genes required for extracellular protein utilization, is not required for ornithine-induced
178 filamentation. Proline, which does not induce SPS-sensor signaling, promoted filamentous
179 growth in an SPS-sensor independent manner (**Fig. 1C**). Importantly, the filamentation was
180 greatly reduced in cells lacking *CSH3* (*csh3Δ/Δ*), a gene encoding a membrane-localized
181 chaperone required for the functional expression of Ssy1 and most amino acid permeases [35,
182 36] (**Fig. 1C**), clearly suggesting that amino acid uptake is required for amino acid-induced
183 morphogenesis.

184 The clear requirement of the SPS-sensor in facilitating ornithine-induced filamentation
185 provided the opportunity to rigorously test the notion that uptake is essential. Based on the
186 knowledge that amino acid permease-dependent uptake is dependent on Stp2 and not Stp1, we
187 used the CRISPR/Cas9 system to introduce *ssy1* null mutations in strains expressing
188 constitutively active Stp2 (*STP2**) or Stp1 (*STP1**) (**Fig. S3A**). The results clearly show that
189 *STP2**, but not *STP1** (**Fig. 1D**), bypasses the *ssy1* null mutation, indicating that the permease
190 responsible for ornithine uptake is indeed encoded by a SPS-sensor and Stp2 controlled gene.
191 Similarly, SPS-sensor dependence was observed for the inducing amino acids alanine,
192 glutamine, and serine (data not shown). Together, these results indicate that amino acid-induced
193 filamentous growth is dependent on the uptake of the inducing amino acid.

194

195 **Amino acid-induced morphogenesis is dependent on catabolism and Ras1/cAMP/PKA** 196 **signaling**

197 Two core signaling pathways, i.e., MAPK and cAMP/PKA, are known to transduce metabolic
198 signals that affect filamentous growth (**Fig. 2A**). We evaluated the capacity of amino acids to
199 induce filamentation in cells carrying null alleles of *RAS1* and the effector transcription factors,
200 *CPH1* and *EFG1* diagnostic for MAPK and cAMP/PKA signaling, respectively [8, 18, 40](**Fig.**
201 **2B**). Similar to wildtype, *cph1* Δ/Δ cells were wrinkled in appearance, indicating that amino acid
202 induced filamentation was independent of MAPK signaling. By contrast, the colonies derived
203 from *ras1* Δ/Δ and *efg1* Δ/Δ cells were smooth. As expected, the *efg1* Δ/Δ *cph1* Δ/Δ double mutant
204 strain also formed smooth colonies. These results indicate that the inducing signals are
205 transduced by the cAMP/PKA pathway. A clear dependence on Ras1/cAMP/PKA signaling was
206 also observed for other inducing amino acids, i.e., alanine, glutamine, and serine (data not
207 shown). Our results demonstrating that amino acid-induced morphogenesis is strictly dependent
208 on Ras1 is contrary to current models that postulate that amino acids-initiated signals are
209 transduced by Gpr1/Gpa2 (reviewed in [20, 28]. According to these models, amino acid signaling
210 should be Ras1 independent.

211 Our results regarding the clear Ras1-dependence suggested that amino acid-initiated signals

212 promote GTP-GDP exchange. To test this notion, we assessed the levels of Ras1-GTP in cells
213 after induction by amino acids (**Fig. 2C**). Our results clearly show that in contrast to cells induced
214 with aspartate, cells induced with arginine, proline and ornithine had increased levels of activated
215 Ras1 in its GTP bound form. We attempted to directly assess the requirement of adenylyl
216 cyclase, however, the previously characterized *cdc35Δ/Δ* (*cyr1*) strain [26] did not grow in the
217 synthetic media used here, even when the media was supplemented with 100 μg/ml uridine
218 and/or 10 mM dibutyryl cAMP (data not shown). The lack of growth of this strain, which was
219 contrary to our expectations, precluded a direct assessment of the role of Cyr1.

220 We tested whether amino acid catabolism was required to activate PKA-signaling by
221 examining the morphology of colonies from cells grown on medium containing arginine or
222 proline as sole nitrogen source and supplemented with enzyme specific inhibitors. In *C. albicans*,
223 arginine is primarily catabolized via the arginase (*CARI*) pathway commencing with the
224 hydrolysis of arginine to ornithine and urea. N^ω-hydroxy-nor-arginine (Nor-NOHA), a potent
225 competitive inhibitor of arginase [41], clearly inhibited arginine-induced filamentation in a dose-
226 dependent manner (**Fig. 2D**). Similarly, L-tetrahydrofuroic acid (L-THFA), a specific
227 competitive inhibitor of proline dehydrogenase (Put1)[42, 43], greatly impaired filamentation in
228 a dose-dependent manner (**Fig. 2E**). The data demonstrate that the arginine- and proline-inducing
229 signals are derived from their catabolism.

230 **Increased ATP resulting from mitochondrial metabolism of morphogenic amino acids** 231 **activate Ras1/cAMP/PKA signaling**

232 Intracellular levels of ATP are thought to provide a key input for Ras1/cAMP/PKA signaling
233 [14]. Consistent with this notion, in comparison to cells grown in the presence of non-inducing
234 nitrogen sources, such as aspartate and ammonium sulfate, cells grown in the presence of the
235 morphogenic amino acids arginine, ornithine or proline contained similar, and significantly
236 higher levels of ATP (**Fig. 3A**). Urea robustly induces filamentous growth, however, urea-
237 derived signals bypass Ras1; morphogenic induction is dependent on *DURI,2*-dependent
238 metabolism that generates CO₂ [30]. Interestingly, cells grown on media with urea, contained
239 significantly lower levels of ATP.

240 Arginine and ornithine are catabolized to proline in the cytoplasm, and proline is subsequently
241 metabolized to glutamate and then α -ketoglutarate in the mitochondria [12]. These metabolic
242 events generate the reduced electron donors, FADH₂ and NADH, which are oxidized by the
243 mitochondrial electron transfer chain leading to ATP synthesis (**Fig. 3A**). We posited that the
244 increased levels of ATP resulting from the catabolism of arginine, ornithine and proline is the
245 consequence of their shared metabolic pathway. To test this, we used methylene blue (MB),
246 which uncouples electron transport from the generation of a proton motive force across the inner
247 mitochondrial membrane. The inclusion of MB in media containing ornithine or proline
248 completely inhibited filamentous growth (**Fig. 3B**). Interestingly, the inhibitory effect of MB in
249 cells growing on arginine was not complete, and a higher concentration of MB was required to
250 noticeably inhibit filamentation. These latter findings suggest that an alternative arginine-
251 induced pathway that is independent of ATP-generating mitochondrial metabolism exists in *C.*
252 *albicans*.

253 We sought independent means to assess levels of reduced electron donors generated by the
254 metabolism of the morphogenic amino acids. The membrane-permeable redox indicator TTC
255 (2,3,5 triphenyltetrazolium chloride, colorless) is converted to TTF (1,3,5-triphenylformazan,
256 red) in the presence of NADH and has been used to monitor mitochondrial respiratory activity
257 of colonies [15, 44]. Colonies growing on proline, arginine, and ornithine exhibited a more
258 intense, deep red pigment than colonies growing on aspartate (**Fig. S4, top panel**). The redox-
259 sensitive dye resazurin can be used in liquid culture to monitor the reducing capacity of the
260 intracellular environment [45]; resazurin is non-fluorescent, but is readily reduced by NADH or
261 to a lesser extent by NADPH to highly red fluorescent resorufin (excitation 560 nm, emission
262 590 nm). Consistent with the results obtained using TTC, cells growing with proline, arginine,
263 or ornithine as sole nitrogen source exhibited 6 – 8-fold more resorufin fluorescence than
264 aspartate grown cells (**Fig. S4, bottom panel**). These results indicate that cells grown in the
265 presence of proline as the sole nitrogen source have a reducing intracellular environment, a
266 finding aligned with the previous report by Land et al. [11].

267

268 **Proline metabolism generates the primary signal for arginine-induced morphogenesis**

269 Arginine is degraded in a pathway that bifurcates after the initial reaction catalyzed by Car1,
270 which forms ornithine and urea (**Fig. 4A**). Ornithine is subsequently metabolized by ornithine
271 aminotransferase (*CAR2*) to form glutamate γ -semialdehyde, which spontaneously converts to
272 Δ^1 -pyrroline-5-carboxylate (P5C). P5C is converted to proline by the *PRO3* gene product.
273 Cytoplasmic proline is transported into the mitochondria where it is converted back to P5C by
274 proline oxidase (*PUT1*). Finally, the mitochondrial P5C is converted to glutamate by the *PUT2*
275 gene product (Marczak and Brandriss, 1989; Siddiqui and Brandriss, 1989), which is then
276 converted to α -ketoglutarate via Gdh2. Urea is further catabolized in the cytosol by urea
277 amidolyase (*DUR1,2*) forming NH_3 and CO_2 .

278 Based on our results demonstrating that the filament-inducing effect of ornithine and proline
279 requires mitochondrial respiration, we investigated if both branches of the bifurcated arginine
280 degradative pathway could independently trigger filamentous growth. To accomplish this, we
281 used a CRISPR/Cas9 strategy to construct PMRCA18-derived strains individually lacking *CAR1*
282 (**Fig. S3D**), *DUR1,2* (**Fig. S3E**), or *PUT1*, *PUT2* and *PUT3* (**Fig. S3F**), or both *PUT1* and
283 *DUR1,2* (**Fig. S3G**). Growth-based assays, on solid and in liquid media, confirmed that the *car1*-
284 *-* strain exhibited impaired growth on synthetic glucose medium (**SXD**) containing arginine as
285 a sole nitrogen source, whereas the strain grew like wildtype (WT) on **SXD** medium containing
286 either 10 mM ornithine, proline or urea (**Fig. 4B and Fig. S5**). As expected, and similar to
287 previous reports [30], the *dur1,2*-*-* strain exhibited severely impaired growth on medium
288 containing urea as sole nitrogen source, but grew well in media containing arginine, ornithine or
289 proline as sole nitrogen sources (**Fig. 4B and Fig. S5**). Cells lacking the proline oxidase (*put1*-
290 *-*) were able to grow in media containing arginine, but unable to grow when ornithine or proline
291 were the sole source of nitrogen (**Fig. 4B and Fig. S5**), indicating that ornithine utilization is
292 strictly dependent on the mitochondrial proline catabolic pathway (**Fig. 4B and Fig. S5**).

293 Interestingly, and quite surprisingly, the *put1*-*- dur1,2*-*-* double mutant strain retained the
294 ability to grow with arginine as sole nitrogen source, albeit slower, clearly suggesting that an
295 arginase-independent arginine utilization pathway exists in *C. albicans* (**Fig. 4B and Fig. S5**).
296 In subsequent growth-based assays the *car1*-*-* strain exhibited glucose-dependent growth
297 phenotypes. The *car1*-*-* strain did not grow when arginine was present as the sole nitrogen and

298 carbon source, and in the absence of glucose, the *car1*^{-/-} strain did not alkalinize the media (**Fig.**
299 **S6**). Thus, in the presence of high glucose, the arginase-independent pathway merely enables the
300 use of arginine as a nitrogen source.

301 Next we analyzed the expression of genes involved in arginine catabolism in cells after
302 shifting them to minimal medium containing 10 mM arginine (YNB+Arg) as sole nitrogen and
303 carbon source (**Fig. 4C**). One hour after the shift, the proline catabolic genes *PUT1* and *PUT2*
304 were significantly upregulated. The levels of *PUT3*, the proline activated transcription factor that
305 is constitutively bound to the promoter of *PUT1* and *PUT2*, did not change [46]. Strikingly,
306 *DUR1,2* gene expression remained constant. Contrary to the assumption that Dur1,2 is
307 responsible for alkalization of the medium, the consequence of the deamination of arginine-
308 derived urea [47], we observed that the *dur1,2*^{-/-} mutant still alkalinized the medium (**Fig. 4D**).
309 Notably, both *put1*^{-/-} and *put2*^{-/-} strains failed to grow in this medium (**Fig. 4D**), indicating that
310 the proline catabolic pathway branch of arginine utilization is essential for growth when arginine
311 is both carbon and nitrogen source. Accordingly, an increased flux through the proline branch of
312 the pathway and subsequent deamination of glutamate provides the likely explanation for the
313 alkalization of the medium (**Fig. 4A**).

314 Consistent with their ability to support growth, arginine, ornithine and proline induced the
315 expression of HA epitope-tagged Put2 (Put2-HA) (**Fig. 4E**). The induction was rapid, 1 h
316 following the shift from YPD to SXD (X = 10 mM Asp, Arg, Orn or Pro), Put2 expression was
317 derepressed in the presence of arginine and ornithine, almost to the levels observed by the
318 addition of proline. Aspartate did not induce Put2 expression. Together these results indicate that
319 arginine and ornithine are efficiently metabolized to proline, and metabolism associated with
320 proline branch is required for the use of these amino acids as energy sources for growth.

321 To test whether proline catabolism is required for arginine-induced morphogenesis, we tested
322 first whether morphogenesis in the presence of arginine can be reduced by Put1 inhibitor, L-
323 THFA (Zhu et al., 2002; Zhang et al., 2015). As expected, pharmacological inhibition of Put1
324 by L-THFA inhibited arginine-induced morphogenesis (**Fig. 4F**). We then carried out a genetic
325 analysis to dissect the pathway triggering filamentous growth in the presence of arginine.
326 Consistent with the existing model for arginine-induced morphogenesis [30], the *car1*^{-/-} strain

327 formed extensively wrinkled colonies in the presence of 10 mM urea comprised mainly of
328 filamentous cells (**Fig. 4G**). However, in comparison to wildtype colonies growing on arginine
329 media, wrinkling was delayed and was first noticeable after 48 h of incubation. On media with
330 an equimolar amount of arginine and urea (Arg + Urea) the *car1*^{-/-} strain developed wrinkled
331 colonies clearly visible after only 24 h. These findings suggest that arginine metabolism via the
332 proline branch induces filamentation more rapidly than the CO₂ (HCO₃⁻) generated by the
333 Dur1,2-dependent degradation of urea. Consistent with this notion, colonies formed by the *put1*^{-/-}
334 mutant remained relatively smooth even after 48 h of growth (**Fig. 4G**). In summary, our
335 results indicate that the metabolism associated with proline branch of the arginine degradation
336 pathway generates the primary and most rapid signal of arginine-induced morphogenesis.

337

338 **Proline utilization is sensitive to carbon source availability and independent of NCR** 339 **control**

340 The capacity of proline to stimulate filamentous growth is significantly affected by glucose
341 availability (**Fig. 5A**). In comparison to colonies formed on synthetic media with 10 mM proline
342 containing 2% glucose (SPD), colonies on media containing 0.2% glucose (SPD_{0.2%}) exhibited
343 larger feathery zones of hyphal cells emanating around their periphery. These findings are
344 reminiscent of reports that *C. albicans* cells grown on media with methionine as nitrogen source
345 and low glucose exhibit robust filamentation [31]. We considered the possibility that glucose
346 repression of mitochondrial function, known to occur in *Saccharomyces cerevisiae* [48, 49], may
347 underlie the difference. Consistent with this notion, macrocolonies formed on SPD were deeper
348 red in color when overlaid with TTC than macrocolonies formed on SPD_{0.2%} or on media with
349 1% glycerol (SPG). The lighter red color of macrocolonies on low glucose, or on glycerol,
350 confirm that cells have lower intracellular levels of NADH, i.e., under derepressing conditions
351 when mitochondria can efficiently oxidize NADH (**Fig. 5A**).

352 We tested the notion that at low glucose concentrations, i.e., non-repressing conditions, cells
353 use proline as a carbon source. Proline utilization was assayed directly by measuring the amount
354 of residual proline in culture supernatants after a 2 h incubation period. In media containing 2%

355 glucose, cells took up < 2% of the proline. By contrast, cells growing in low glucose (0.2%) or
356 1% glycerol used 12 – 15% of the available proline (**Fig. 5B**). The expression of Put2 was
357 independent of glucose, as the levels of Put2 were similar (**Fig. 5B, insert**). These results indicate
358 that proline is taken up and metabolized more efficiently in cells under non-repressing
359 conditions, suggesting that mitochondrial activity is subject to glucose repression.

360 To critically test this, we assessed the effect of varying the glucose concentration from 0.05 -
361 4%. Cells were grown for 16 h in media containing the pH indicator bromcresol purple. At high
362 glucose concentrations (0.5 – 4%) the media remained acidic, indicating cells were growing
363 fermentatively using proline merely as a nitrogen source (**Fig. 5C**). By contrast, at glucose
364 concentrations $\leq 0.2\%$, the media became alkaline, indicating that cells were respiring and using
365 proline as the primary energy source. The increased flux through the proline pathway is expected
366 to yield elevated NH_3 generated by the mitochondrial glutamate dehydrogenase (*GDH2*)
367 catalyzed deamination of glutamate. To directly assess mitochondrial activity under these
368 conditions, we carried out extracellular oxygen consumption analysis in a high-throughput
369 microplate format (**Fig. S7**). Cells grown in repressing SPD had the lowest oxygen consumption
370 whereas those grown at $\text{SPD}_{0.2\%}$ had the highest, higher than cells grown in SPG . As previously
371 pointed out, Put2 levels were similar across all conditions (**Fig. 5B**). Together, these results
372 indicate that proline is taken up and then metabolized more efficiently in cells growing under
373 low glucose concentrations. Consistent with this finding, Put2 expression was derepressed in
374 rich media containing yeast extract and peptone when non-repressing, non-fermentative carbon
375 sources replaced glucose; i.e., glycerol or lactate (**Fig. 5D**). Similarly, cells express elevated
376 levels of Put2 when grown in hyphal inducing Spider medium, a medium rich in amino acids
377 and mannitol as a primary carbon source.

378 Nitrogen regulation of transcription in fungi is a suprapathway response that is commonly
379 referred to as nitrogen catabolite repression (NCR), which functions to ensure that cells
380 selectively use preferred nitrogen sources when available. Briefly, NCR regulates the activity of
381 GATA transcription factors Gln3 and Gat1; in the presence of preferred nitrogen sources, these
382 factors do not gain access to the promoters of NCR-regulated genes (reviewed in[50]). Previous
383 studies have shown that certain amino acids, traditionally classified as poor (e.g., proline) in *S.*

384 *cerevisiae*, were readily utilized by *C. albicans* mutants lacking Gln3 and Gat1 [51]; the
385 introduction of null alleles of both *GLN3* and *GAT1* in *C. albicans* did not impair growth using
386 proline as sole nitrogen source, whereas growth on urea was severely affected. Consistent with
387 these findings, we found that Put2-HA was constitutively expressed in *gln3Δ/Δ gat1Δ/Δ* mutant
388 grown in medium containing high levels of the preferred nitrogen source ammonium sulfate
389 (**Fig. 5E**). Our data indicate that in *C. albicans* proline utilization is not subject to NCR, a
390 conclusion aligned with recently published transcriptome analyses[46].

391

392 **Proline induces hyphal growth within phagosomes and enables *C. albicans* to escape from** 393 **engulfing macrophages**

394 We sought to place our novel insights regarding the critical role of proline metabolism in the
395 induction of hyphal growth in a broader biological context and tested whether proline catabolism
396 affects the capacity of *C. albicans* cells to form hyphae within macrophages and escape killing.
397 First, using indirect immunofluorescence microscopy we examined whether Put2-HA is
398 expressed in *C. albicans* cells engulfed by murine RAW264.7 macrophages (**Fig. 6A**). *C.*
399 *albicans* CFG185 (*PUT2/PUT2-HA*) cells were co-cultured with macrophages (MOI of 5:1;
400 C:M) for 90 min. Strain CFG185 exhibits activation of proline catabolism in the presence of
401 arginine, ornithine, and proline (**Fig. 4E**). The macrophages were imaged using antibodies
402 against the HA tag (1^o, rat anti-HA; 2^o, goat anti-rat antibody conjugated to Alexa Fluor 555)
403 and LAMP-1, a lysosomal marker that is enriched in phagosomes. Confocal images clearly
404 showed that *C. albicans* cells engulfed by macrophages express Put2, and that the Put2
405 expressing fungal cells localized to Lamp1 compartments (see the orthogonal view of merged
406 channels, lower left panel). The results indicate that *C. albicans* cells within macrophage
407 phagosomes express Put2.

408 Next, we assessed the importance of the proline catabolic pathway components in the capacity
409 to escape macrophage. To facilitate comparisons with results obtained in other laboratories, we
410 repeated the construction of the proline catabolic pathway mutations in the SC5314 strain
411 background; strains lacking *PUT1*, *PUT2*, *PUT3* or both *PUT1* and *PUT2* were constructed using

412 CRISPR/Cas9. The full genome of each mutant strain was sequenced; the sequence coverage
413 varied from 42 – 65X and after assembly the contig coverage accounted for ≥ 98 of the reference
414 SC5314 genome (Assembly 22, version s06-m01-r01; [52]). Each strain was found to carry the
415 intended null mutation in the correct chromosomal locus and no large scale dissimilarities to the
416 reference genome or off-target mutations were evident. Furthermore, no phenotypic differences
417 were detected in comparison to the PMRCA18-derived strains (data not shown).

418 As expected, SC5314 (WT) and CRISPR/Cas9 control strains (pV1093 and pV1524), lacking
419 guide sequences to target Cas9, exhibited robust hyphal growth when co-cultured with
420 RAW264.7 macrophages (**Fig. 6B**). By contrast, and similar to heat killed SC5314, the strains
421 carrying *put1*^{-/-}, *put2*^{-/-}, *put3*^{-/-} and *put1*^{-/-} *put2*^{-/-} mutations were unable to efficiently form
422 filaments from within engulfing macrophages (**Fig. 6B**). As hyphal formation enables *C.*
423 *albicans* cells to escape macrophages and thereby facilitates survival, we analyzed the
424 candidacidal activity of macrophages by assessing fungal cell viability by assessing colony
425 forming units (CFU). Consistent with our microscopic analysis, in comparison to wildtype cells,
426 the proline mutants were killed more efficiently (**Fig. 6C**). Together, these results indicate that
427 *C. albicans* cells rely on proline catabolism to induce hyphal growth in phagosomes, a response
428 that facilitates escape from killing by macrophages.

429

430

431 **Discussion**

432 In this study we have found that ATP generating mitochondrial proline catabolism is required to
433 induce hyphal development of *C. albicans* cells in phagosomes of engulfing macrophages. The
434 finding that proline catabolism, also required for the utilization of arginine and ornithine, is
435 required to sustain the energy demands of hyphal growth provides the basis to understand the
436 central role of mitochondria in fungal virulence. The energy status of the fungal cell is clearly a
437 key signal that engages the genetic programs underlying yeast-to-hyphal transitions. The
438 dependence on the energy producing proline catabolic pathway to induce *C. albicans* cells to
439 switch morphologies is instrumental in their ability to escape from macrophages. Our results are
440 consistent with a recent model postulating that elevated cellular levels of ATP induces hyphal

441 morphogenesis [14] and with early reports that amino acid catabolism promotes filamentous
442 growth [12, 13, 53]. Our experimental findings are schematically summarized in Fig. 7.

443 Our work provides a framework to integrate several fragmentary observations regarding
444 amino acid-induced morphogenesis. For example, Land et al. [11, 12] observed that the most
445 potent morphogenic amino acids arginine and proline are those metabolized to glutamate. Our
446 results show that this occurs strictly via the mitochondrial localized proline utilization pathway
447 essentially as described in *S. cerevisiae* [54-56] with the exception that proline metabolism is
448 not under nitrogen regulation (**Fig. 5E**, [46]). Consistently, ornithine, an intermediate in arginine
449 catabolism, also acts as a potent inducer of morphogenesis (**Fig. 1A**; [12, 53]). Glutamate is
450 further converted to α -ketoglutarate, an intermediate in the TCA cycle. These metabolic
451 reactions are coupled to the generation of reduced electron carriers FADH₂ and NADH, which
452 are oxidized in the mitochondria powering ATP synthesis. Amino acid induction of hyphal
453 growth exhibits a strict requirement for Ras1 (**Fig. 2B**) and cells grown in the presence of these
454 inducing amino acids have high levels of active Ras1 (**Fig. 2C**) and elevated levels of
455 intracellular ATP (**Fig. 3A**). The metabolic inhibitors nor-NOHA (Car1) and L-THFA (Put1)
456 and the mitochondrial uncoupler methylene blue (MB) block the induction of filamentation (**Fig.**
457 **2D, E** and **Fig. 3B**). Our analysis demonstrates that arginine and proline induce morphogenesis
458 by virtue of a shared metabolic pathway (**Fig. 4C-F**).

459 Together, our findings are well aligned to the recent model proposed by Grahl et al. [14],
460 where mitochondrial ATP synthesis facilitates Ras1 activation in cooperation with the adenylyl
461 cyclase (Cyr1) leading to increased cAMP production and to activation of the Efg1 transcription
462 factor. The finding that arginine-induced hyphal growth occurs rapidly (**Fig. 4G**), suggests that
463 a brief exposure to arginine may suffice to trigger filamentous growth. According to Grahl et al.
464 (2015), Ras1 activation by ATP appears to be independent of the AMP kinase, a key regulator
465 of cellular energy homeostasis. The ATP-binding pocket within the active site of mammalian
466 adenylyl cyclase has been shown to act as an ATP sensor [57]. Although it has been proposed
467 that Cyr1 may function similarly as an ATP sensor this has yet to be confirmed in *C. albicans*.
468 Regardless of the mechanism, exceeding a critical threshold of ATP is likely required to induce
469 cAMP synthesis. It is known that the cAMP produced by Cyr1 does not necessarily correlate to

470 the strength of the inducer and that transient short-lived spikes in cAMP are sufficient to trigger
471 phosphorylation and eventually activation of Efg1 [27]. Consequently, spikes of ATP transiently
472 generated by proline catabolism may efficiently induce hyphal specific genes (*HSG*).

473 We have clearly shown that arginine-, ornithine- and proline-induced hyphal growth is
474 dependent on Ras1, which is not accounted for by other models of amino acid-induced
475 morphogenesis (reviewed in [4, 7], despite the fact that Ras1 is known to be important in
476 induction of filamentous growth in the presence of amino acid-rich serum [22]. Both the
477 presumed amino acid sensitive Gpr1-Gpa2 pathway [58, 59] and the Dur1,2-dependent CO₂
478 model for arginine-induced morphogenesis [30] are thought to bypass Ras1 and involve direct
479 interactions with adenylyl cyclase (Cyr1). Also, contrary to the previous report [30], CO₂
480 generated by the Dur1,2-dependent catabolism of urea is not the primary morphogenic signal.
481 Specifically, induction of filamentous growth in the presence of arginine or proline as sole
482 nitrogen source proceeds more quickly than that observed by the metabolism of urea (**Fig. 4G**).
483 In addition, *DURI,2* expression is tightly regulated by NCR, i.e., in the presence of ammonia,
484 urea metabolism is repressed [51]. By contrast, the conversion of arginine to proline is not under
485 NCR control (**Fig. 5E**, [46]). Finally, when cells were shifted from YPD to medium containing
486 arginine as sole carbon and nitrogen source, proline catabolic genes (*PUT1* and *PUT2*) were
487 derepressed much faster than *DURI,2* (**Fig. 4C**), indicating that arginine is rapidly converted to
488 proline. We have noted that the constitutive expression of arginase represents a common and
489 undesired technical problem in proteomic analyses using SILAC (Stable Isotope Labeling
490 by/with Amino acids in Cell culture) due to the rapid conversion of arginine to proline in
491 eukaryotes [60-63]. In *Schizosaccharomyces pombe*, the deletion of two arginase genes (one a
492 *CAR1* homologue) and the single ornithine transaminase (*CAR2* homologue) rectified this
493 problem [60]. We predict, that similar deletions would be helpful in the quantitative analysis of
494 the *C. albicans* proteome.

495 Earlier reports by Nickerson and Edwards [64] and Land et al. [11] suggested that
496 mitochondrial activity is repressed during filamentous growth. By contrast, other more recent
497 work has shown that hyphal formation occurs predominantly under aerobic conditions [16] and
498 is associated with increased respiratory activity [14, 15]. Based on our findings (**Fig. 5C**), the

499 seemingly conflicting observations could be explained if, as in *S. cerevisiae*, the synthesis of
500 mitochondrial respiratory enzymes are subject to glucose repression [65, 66]. There is
501 surprisingly little information available regarding glucose repression of mitochondrial function
502 in *C. albicans*, and whether the regulatory circuits are wired similar to those in *S. cerevisiae*.
503 However, we note that Land et al. [11] used growth conditions with high glucose (~1.8%; 100
504 mM), whereas studies by [14, 15] were carried out using low glucose (10 mM, i.e., ≈ 0.2%).

505 In striking contrast to the current view that *C. albicans* mitochondrial function is insensitive
506 to glucose repression [67-69], our results clearly demonstrate that glucose represses respiration
507 in the presence of proline (**Fig. S7**). Cells grown aerobically in high glucose exhibit fermentative
508 metabolism (**Fig. 5C**), i.e., the well-characterized Crabtree effect [70]. In glycolysis, conversion
509 of glucose to pyruvate is coupled to reduction of NAD⁺ and to the generation of ATP. Only small
510 amounts of the cofactor is available in the cytosol. Consequently, when mitochondrial functions
511 are glucose repressed, cells use fermentation to oxidize NADH and regenerate NAD⁺, thereby
512 enabling cytoplasmic ATP synthesis to continue. Under conditions when proline is the sole
513 nitrogen source and high glucose is present, cells use glucose for energy and as carbon-source,
514 whereas proline catabolism merely supplies cells with nitrogen, i.e., proline utilization is low
515 (**Fig. 5B**). However, when glucose becomes limiting (<0.2%), the respiratory capacity of
516 mitochondria increases (**Fig. S7**), enabling cells to efficiently oxidize NADH and generate ATP
517 by oxidative phosphorylation; under these conditions cells use proline for energy and as the
518 carbon- and nitrogen-source, i.e., proline utilization is high (**Fig. 5B**). Together our results show
519 that proline metabolism is a sensitive indicator of mitochondrial function in *C. albicans*.

520 Our observation that high glucose represses mitochondrial function, provides a mechanistic
521 understanding of how high glucose inhibits hyphal morphogenesis [13, 31]. Cells grown on 2%
522 glucose have elevated levels of reduced cofactors, such as NADH (**Fig. 5A**), suggesting that the
523 capacity of mitochondria to oxidize NADH is suboptimal, i.e., the cellular capacity to regenerate
524 NAD⁺ is rate limiting, a phenomenon termed over-flow metabolism [48]. It is important to note
525 that, based on the *S. cerevisiae* paradigm, the pyruvate formed in glycolysis needs to be
526 converted to acetyl-CoA to prime the TCA cycle. The mitochondrial-localized pyruvate

527 dehydrogenase complex is predominantly responsible for the conversion of pyruvate to acetyl-
528 CoA during glucose-limited, respiratory growth [65, 66]. Indeed, pharmacological inhibition of
529 glycolysis has been shown to arrest filamentous growth of *C. albicans* even in the presence of
530 proline [11]. Alternatively, β -oxidation of lipids may contribute the necessary acetyl-CoA [9].

531 We have placed the SPS sensing pathway, the primary sensing system of extracellular amino
532 acids, in context to the major intracellular signaling pathways governing in nutrient regulated
533 morphogenesis. SPS-sensor initiated signals do not directly induce hyphal growth, but rather
534 facilitate morphogenesis by up-regulating the capacity of cells to take up inducing amino acids
535 (Fig. 7). Experimental support for this conclusion includes the following observations. First,
536 amino acid-induced activation of SPS-sensor signaling does not strictly correlate with the
537 induction of filamentous growth (Fig. 1A). Second, the inability of a *ssy1* null mutant to undergo
538 morphogenesis can be rescued by expressing a constitutively active form of Stp2 (*STP2**) but
539 not Stp1 (*STP1**). Stp2 is the effector transcription factor that controls amino acid permease gene
540 expression, whereas Stp1 activates the expression of secreted aspartyl proteases and oligopeptide
541 transporters [34]. Consistently, and similar to Kraidlova et al. [71], we found that the expression
542 of six *C. albicans* orthologues (*GAP1-GAP6*) of the *S. cerevisiae* general amino acid permease
543 (*GAP1*) are regulated by the SPS sensing system, perhaps with the exception of *GAP4*
544 expression, which is comparatively expressed at very low levels (data not shown). Third,
545 filamentous growth is dependent on amino acid catabolism. The weak filamentation observed in
546 the *csH3* Δ/Δ mutant grown in 10 mM proline can be attributed to the residual uptake of proline
547 as previously described [35]; apparently, the residual systems are expressed and function at high
548 extracellular concentrations of proline [53, 72]. Thus, the filamentous growth defect observed in
549 cells lacking a functional SPS sensing pathway, i.e., *SSY1* or *CSH3* null mutants, is due to the
550 inability to efficiently take up inducing amino acids from the extracellular environment, a
551 requisite for their metabolism [35, 36].

552 Together our findings have important implications on understanding how *C. albicans* cells
553 interact with host immune cells. Transcriptomic studies examining macrophage-*C. albicans*
554 interactions by Lorenz et al. [9] showed that arginine biosynthesis genes are peculiarly
555 upregulated in phagocytosed cells. Furthermore, the results suggest that the phagosome is likely

556 a glucose-poor environment as an increased expression of genes that favor gluconeogenesis and
557 mitochondrial function was also noted [9]. Interestingly, arginine utilization appears to proceed
558 concomitant with arginine biosynthesis as deduced from the increased arginase transcripts in
559 phagocytosed cells [9, 30]. In a follow-up study, the apparent upregulation of arginine
560 biosynthesis was suggested to be a response to the macrophage oxidative burst [73].
561 Interestingly, the expression of *DUR1,2* in phagocytosed cells was not significantly altered. Our
562 finding that the enzymes responsible for proline utilization are upregulated indicates that proline
563 is either made available by the host or is the result of arginine catabolism.

564 In the light of these results, the challenging question is where the hyphae inducing amino
565 acids come from, from the macrophage or from nutrients stored within *C. albicans* cells prior to
566 their being phagocytosed. In *S. cerevisiae*, > 90% of free arginine is sequestered in the vacuole
567 and the non-compartmentalized and cytosolic arginine is catabolized by arginase [74]. Given
568 that arginine is catabolized to proline via the arginase pathway with ornithine acting as a
569 transitory intermediate, it is possible that vacuolar stores of arginine are activated in the
570 phagosome to support the demand for cellular energy. When glucose becomes limiting, *C.*
571 *albicans* may rely on the catabolism of amino acids, particularly proline, as primary energy. This
572 is reminiscent of the requirement of proline catabolism for Trypanosome survival in the Tsetse
573 fly vector [75-78].

574 Proline-induced morphogenesis is repressed under acidic conditions [13, 53], presumably a
575 condition confronting newly phagocytized *C. albicans* cells. This raises the interesting
576 conundrum as to how *C. albicans* cells deal with this environmental challenge and filament. It
577 is possible that Stp2-mediated alkalization of the phagosome reported by Vylkova and Lorentz
578 [79] is a key predisposing event that facilitates proline-induced morphogenesis. We found that
579 alkalization is not Dur1,2-dependent (**Fig. 4D**), indicating that an alternative mechanism triggers
580 alkalization. Accordingly, the Stp2-dependent induction of arginine uptake and its subsequent
581 Put1- and Put2-dependent metabolism generates glutamate, which is deaminated to α -
582 ketoglutarate by glutamate dehydrogenase (Gdh2) (**Fig. 7**). The resulting NH₃ may provide the
583 explanation for the observed alkalization. As already pointed out, the source of amino acids in
584 the macrophage phagosome remains a very interesting question. Numerous metabolic signatures

585 appear to reflect a microenvironment with a poor nitrogen content. For example, based on the
586 transcriptional analysis of the *C. albicans*-macrophage interaction, *OPT1*, encoding an
587 oligopeptide transporter, is upregulated in phagocytosed cells [9]. *OPT1* expression is controlled
588 by the SPS-sensor signaling and the downstream transcription factor Stp1 [34, 37]. *STP1*
589 expression is itself under tight NCR control [37]. Thus, the upregulated expression of *OPT1*
590 strongly suggests that NCR is relieved in phagocytosed cells and that sufficient levels of amino
591 acids are present to induce the SPS-sensor. As to the origin of amino acids in the phagosome, *C.*
592 *albicans* may excrete amino acids liberated from storage compartments, loaded during growth
593 in rich media. In *S. cerevisiae*, under defined conditions, amino acids are known to be excreted
594 at detectable levels [80] and under certain circumstances activate SPS-sensor signaling [81].
595 Thus, amino acids may provide an autocrine function to induce filamentous growth of
596 phagocytosed *C. albicans* cells.

597 The results presented here provide a clear example of how *C. albicans* cells sense and respond
598 to nutrients present in the host to ensure proper nutrient uptake and continued survival. The
599 molecular components underlying nutrient uptake are often referred to as virulence factors.
600 When afforded the opportunity, *C. albicans* will alter developmental programs to optimize
601 nutrient uptake systems that enable the better exploit host environments and to evade the primary
602 immune response [3, 82, 83]. The identification and understanding of fungal virulence factors is
603 necessary to therapeutically disturb their function upon infectious growth and thereby facilitate
604 the ability of host immune systems to re-establish and maintain the integrity of the host. We are
605 excited by the prospect of exploiting mitochondrial proline metabolism to probe the nutrient
606 environment of the macrophage phagosome, a currently poorly characterized environment.

607

608

609 **Materials and methods**

610 **Strains, media and chemicals**

611 *C. albicans* strains are listed in Supplementary Information Table S1 and all primers used are
612 listed in Table S2. All strains were cultivated in YPD medium (1% yeast extract, 2% peptone,

613 2% glucose) at 30 °C. Minimal synthetic dextrose (SD) medium containing 0.17% YNB (Yeast
614 Nitrogen Base without amino acids and without ammonium sulfate; Difco™), 2% glucose, and
615 5 g/l ammonium sulfate (≈ 38 mM) was used as indicated. Media were made solid by 2% (w/v)
616 Bacto agar. Where appropriate, 100 or 200 μ g/ml nourseothricin (Nou; Jena Biosciences, Jena,
617 Germany) was added to the medium. The ability of amino acids to induce filamentous growth
618 was determined on buffered solid synthetic (SXD) media containing 0.17% YNB, 2% glucose,
619 and 10 mM of the indicated amino acid (X) as sole nitrogen source, or at concentrations as
620 described in the figure legends. Fifty mM 2-(N-morpholino) ethanesulfonic acid (MES) was
621 included in media and the pH was adjusted to 6.0 using NaOH. To minimize residual nitrogen,
622 the SXD media were made solid using 2% (w/v) highly purified agar (Biolife, Milano, Italy).
623 Where indicated 0.2% glucose, 1% lactate or 1% glycerol replaced 2% glucose as carbon source.
624 The following media were used to screen CRISPR/Cas9-derived knockout phenotypes: YPD-
625 MM; SUD; SPD; and YNB+Arg+BCP. YPD-MM is standard YPD supplemented with 1.5
626 mg/ml MM (2-((((4-methoxy-6-methyl)-1,3,5-triazin-2-yl]-amino)carbonyl)amino]-sulfonyl)-
627 benzoic acid; Dupont™ Ally); SUD and SPD were prepared as SXD containing urea (U), or
628 proline (P) as sole nitrogen source; YNB+Arg+BCP contains 0.17% YNB, 10 mM arginine
629 (Arg) as sole nitrogen and carbon source, and 0.03 μ g/mL bromocresol purple (BCP; Sigma) as
630 indicator, with the pH adjusted to 4.0 using 1 M HCl. Growth in the presence of specific
631 metabolic inhibitors was assessed on media containing nor-NOHA (N-hydroxy-nor-L-arginine;
632 BioNordika AB, Sweden) prepared in 100% dimethyl sulfoxide (DMSO) as 56 mM concentrated
633 stock; a 26 mM working stock was prepared freshly diluting in ddH₂O. L-tetrahydro-2-furoic
634 acid (L-THFA; Sigma) and methylene blue (MB; Sigma), were freshly prepared in ddH₂O as 1
635 M and 3 mM stocks, respectively. *Escherichia coli* strain DH10B™ was used for the
636 construction of plasmids; LB medium supplemented where required with carbenicillin (Cb, 50
637 μ g/ml), Nou (50 μ g/ml), and/or chloramphenicol (Cm, 30 μ g/ml). LB was made solid by 1.5%
638 Bacto agar. Liquid cultures were grown with agitation at 150-200 rpm. The density of yeast
639 suspensions was determined and adjusted ($1 \text{ OD}_{600} = 3 \times 10^7 \text{ CFU/ml}$) [84]. Sterile Milli-Q™
640 ddH₂O was used in all experiments.

641

642 CRISPR/Cas9 mediated gene inactivation

643 The CRISPR/Cas9 gene editing was used to inactivate both alleles of *SSY1* (C2_04060C), *CSH3*
644 (C4_03390W), *CARI* (C5_04490C), *PUT1* (C5_02600W), *DURI,2* (C1_04660W), *IRA2*
645 (C1_12450C), *PUT1* (C5_02600W), *PUT2* (C5_04880C) or *PUT3* (C1_07020C). Sequences of
646 synthetic guide RNAs (sgRNAs), repair templates, and verification primers are listed in Table
647 S2. The solo system plasmids pV1093 or pV1524 were used [85, 86]. These plasmids contain a
648 cassette comprised of the *Candida/Saccharomyces* codon-optimized *CAS9* endonuclease gene,
649 *NAT* gene (recyclable in pV1524), sgRNA cloning site, and flanking sequences for genomic
650 integration. For pV1093 and its derivative plasmids, the cassettes were integrated in one of the
651 *ENO1* loci, whereas pV1524 and its derivatives were integrated in one of the *NEUT5* loci. The
652 sgRNAs were designed as described [87] and were inserted in pV1093 or pV1524 by linker
653 ligation. To summarize, oligo pairs p43/p44 (*SSY1*), p49/p50 (*CSH3*), p55/p56 (*CARI*), p61/p62
654 (*DURI,2*), p67/p68 (*IRA2*), p73/p74 (*PUT1*), p79/p80 (*PUT2*), and p85/p86 (*PUT3*), were
655 separately phosphorylated and annealed prior to ligating them to dephosphorylated *Esp31*
656 (*BsmBI*)-digested pV1093 or pV1524. Ligation reactions were purified and introduced into *E.*
657 *coli* by electroporation. Transformants were selected on solid LB+Cb (or +Nou for pV1524
658 cloning) incubated overnight at 37 °C. Plasmids were sequenced using primer p91 (FS95).
659 Plasmids (3 to 6 µg) containing the 20-bp sgRNA for *SSY1* (pFS013), *CSH3* (pFS017), *CARI*
660 (pFS024), *DURI,2* (pFS039), *IRA2* (pFS028), *PUT1* (pFS080, pV1093 derivative), *PUT1*
661 (pFS088, pV1524 derivative), *PUT2* (pFS083) and *PUT3* (pFS084) were digested with *KpnI* and
662 *SacI* to release the cassette. Repair templates (RT) containing stop codon and specific restriction
663 site were produced by template-less PCR using oligo pairs p45/p46 (*SSY1*), p51/p52 (*CSH3*),
664 p57/p58 (*CARI*), p63/p64 (*DURI,2*), p69/p70 (*IRA2*), p75/p76 (*PUT1*), p81/p82 (*PUT2*), and
665 p87/p88 (*PUT3*). PCR-purified digested plasmid and repair templates were co-transformed into
666 *C. albicans* cells at a 1:3 ratio (w/w, plasmid:repair template). The hybrid lithium acetate/DTT-
667 electroporation method, with minor modifications, was used for transforming *C. albicans* [88].
668 After applying 1.5 kV of electric pulse, cells were recovered in YPD medium supplemented with
669 1 M sorbitol for at least 4 h and then plated on YPD+Nou plates; Nou^R colonies were selected 2
670 days after plating. Nou^R transformants were pre-screened according to the expected phenotype

671 prior to PCR and restriction analysis using primers and restriction enzymes indicated in Table
672 S2 (**Fig. S3**).

673

674 **Full genome shotgun sequencing**

675 Genomic DNA was isolated from *put1*^{-/-} (CFG139), *put2*^{-/-} (CFG207), *put3*^{-/-} (CFG146), *put1*^{-/-}
676 *put2*^{-/-} (CFG159) and CRISPR/Cas9 control strains CFG181 (pV1093) and CFG182 (pV1524)
677 and sequenced. Prior to library construction, extracted DNA was purified with Agencourt
678 AMPure[®] XP beads (Beckman Coulter, USA) in order to remove short sequences (<100 bp).
679 Aliquots (25 μ l) of DNA were mixed with 45 μ l of AMPure beads with a ratio of 1:1.8 and
680 incubated 15 min. Initial DNA concentrations following purification were evaluated using
681 Quant-iT PicoGreen dsDNA Assay kit (ThermoFisher, USA) as described (Logares & Feng,
682 2010). Absorbance was measured at 530 nm, using a Tecan Ultra 384 SpectroFluorometer
683 (PerkinElmer, USA).

684 Library construction was carried out with the QIAGEN-FX kit (Qiagen, Germany) with a
685 DNA input of 100 ng DNA per sample and a digestion time of 13 min without enhancer.
686 Following fragmentation, adapter sequences were ligated, and ligated DNA fragments were
687 amplified by 9 cycles of PCR and DNA was purified with AMPure[®] XP beads. The quality of
688 the library samples were evaluated with an Agilent Bioanalyzer using DNA1000 cartridges. The
689 average length of the fragments excluding adapter sequences was 455 bp.

690 Prior to sequencing, the samples were denatured with 0.2 N NaOH. A final volume of 570
691 μ l of pooled library was mixed with denatured Phix control (30 μ l) and loaded on an Illumina
692 Mi-Seq 2x300 flow-cell and reagent cartridge. De-multiplexing and removal of indexes and
693 primers were done with the Illumina software v. 2.6.2.1 on the instrument according to the
694 standard Illumina protocol. Initial de novo assembly of quality controlled reads was done with
695 SPADES v. 3.11.1 and standard settings [89]. Mapping of assembled contigs was done with
696 Ragout v 2.0 [90] using Sibelia for synteny detection[91]. Visualization of results and generation
697 of reports on the assembly quality and other factors were done with QUAST v. 4.6.1 [92].

698

699 **NanoLuc transcription-translation reporter of SPS-sensor activation**

700 The NanoLuc-PEST (Nlucp) construct was used to create the reporter of SPS-sensor dependent
701 transcription (**Fig. S2**). The presence of PEST sequences ensures rapid degradation of NanoLuc
702 luciferase, thereby enhancing sensitivity [39]. Up- and downstream regions of the *CAN1* ORF
703 were amplified using genomic DNA from PMRCA18 as template and primers p92/p93 (0.9 kB
704 upstream) and p96/p97 (0.98 kB downstream) (**Table S2**). An approximately 0.7 kB Nlucp gene
705 sequence was amplified from plasmid pCA873 [93] using primers p94/p95. These amplicons
706 were digested with appropriate FastDigest enzymes (Thermo Scientific) and purified; i.e., the
707 *CAN1* upstream amplicon was digested with *KpnI/XhoI*, the *CAN1* downstream with *XbaI/NotI*,
708 and Nlucp DNA fragment with *XhoI/BamHI*. Using T4 DNA Ligase (Thermo Scientific), the
709 upstream fragment was first ligated to *KpnI/XhoI*-digested pSFS2a vector [88] creating pFS006.
710 The purified Nlucp DNA was then ligated into *XhoI/BamHI* restricted pFS006 creating pFS007.
711 Finally, the downstream fragment was ligated into *XbaI/NotI* restricted pFS007 creating pFS010.
712 The plasmids were introduced into *E. coli* and transformants selected on LB+Cm+Nou plates
713 incubated at 30 °C. The desired reporter construct, purified from *KpnI/NotI* restricted pFS010,
714 was introduced into *C. albicans* wildtype (PMRCA18) and SPS-deficient mutant strains
715 (*ssy1Δ/Δ*, *ssy5Δ/Δ*, and *stp2Δ/Δ*) by electroporation. Selection was carried out on YPD+Nou and
716 NAT^R clones carrying the integrated Nlucp construct were identified by PCR.

717 For analysis of amino acid-induced SPS-sensor activation, Nano-Glo[®] Luciferase Assay
718 System (Promega GmbH, Germany) was used following the manufacturer's protocol. Briefly,
719 log phase SD cultures were first standardized to OD \approx 0.8 before adding 50 μ l of the cell
720 suspension into each well of Nunc 96 well microplate (white). Then, cells were induced with 50
721 μ M of the indicated amino acids for 2 h at 30 °C. Fifty microliters (50 μ l) of Nano-Glo substrate
722 diluted 1:50 in the supplied lysis buffer was added into each well of the microplate. After 3 min,
723 bioluminescence was captured using microplate luminometer (Orion II, Berthold Technologies
724 GmbH & Co. KG, Germany). Luminescence reading from treated wells were deducted from
725 wells spiked with ddH₂O serving as uninduced control.

726

727 **Filamentation assay**

728 Solid filamentation assay was performed as described [14]. Briefly, cells from overnight YPD
729 liquid cultures were harvested, washed once, and resuspended in sterile ddH₂O. The cell density
730 of cell suspensions was adjusted to OD₆₀₀ ≈ 8 before spotting 10 μl onto solid media. Plates were
731 allowed to dry at room temperature before incubating at 37 °C as indicated to allow
732 macrocolonies to form. Filamentation assays in the presence of metabolic inhibitors, nor-NOHA
733 or L-THFA, were performed in a 6-well microplate format (~5 ml/well); otherwise, all assays
734 were carried out using standard Petri plates (~35 ml/plate). For filamentation assays in liquid
735 cultures, cells were washed and then adjusted to OD₆₀₀ ≈ 25. Cells were diluted in pre-warmed
736 liquid medium at OD₆₀₀ ≈ 0.5 and then incubated at 37 °C with vigorous agitation for the
737 specified time. Cell morphologies were assessed under epifluorescence microscopy using
738 calcofluor white stain (CFW, Fluorescent Brightener 28, 1 mg/ml; Sigma).

739

740 **qRT-PCR**

741 Hyphal specific gene (HSG) expression in 24 h old macrocolonies was analyzed as follows:
742 using a sterile glass slide, three to four macrocolonies of wildtype strain (PMRCA18) were
743 collected by scraping and suspended in 1 ml of ice-cold PBS. Cells were harvested by
744 centrifugation at 10,000 x g for 3 min (4 °C), snap frozen in liquid nitrogen and then stored at -
745 80 °C until processed for RNA extraction. Gene expression in liquid grown cells was analyzed
746 as follows: cells from overnight YPD cultures were harvested by centrifugation, washed and
747 resuspended at an OD₆₀₀ ≈ 25 in pre-warmed liquid medium and incubated at 37 °C for 2- and
748 4- h before harvesting the cells by centrifugation; the cell pellets were snap frozen in liquid
749 nitrogen. For arginine catabolic gene expression analysis, SC5314 was used as wildtype strain.
750 Briefly, cells from log phase YPD culture growing at 30 °C were harvested, washed 3X with
751 PBS, diluted in pre-warmed YNB+Arg medium (pH = 6.0, without BCP) at an OD₆₀₀ ≈ 0.5, and
752 then incubated for 1 h at 37 °C under aeration. A portion of the washed cells were snap-frozen
753 in liquid nitrogen to serve as reference (t = 0). Following 1 h incubation, cells in YNB+Arg were
754 immediately harvested and then snap-frozen in liquid nitrogen for RNA extraction. To analyze

755 the dependence of *GAP* genes expression to SPS pathway (i.e., *Ssy1*), wildtype (PMRCA18) and
756 *ssy1Δ/Δ* (YJA64) cells were grown to log phase in SD medium at 30 °C before spiking with 1
757 mM of glutamine or ddH₂O for 30 min. Cells were collected from induced (glutamine) and non-
758 induced (ddH₂O) cultures and snap-frozen in liquid nitrogen.

759 Total RNA was extracted from frozen cell pellets using RiboPure-Yeast Kit (Ambion®, Life
760 Technologies) essentially following the instructions of the supplier with the exception that cells
761 were subjected to extra bead-beating step (Bio-Spec; 1 × 60 sec, 4 M/s). DNase-treated RNA
762 extracts were reverse-transcribed using SuperScript III and Random Primers (Invitrogen, Life
763 Technologies). cDNA preparations were diluted 1/40 in ddH₂O and 5 μl were used as template
764 for qPCR using KAPA SYBR Green (Kapa Biosystems). Gene specific primers (500 nM) were
765 added and reactions were performed in a Rotor-Gene 6000 (software version 1.7). The $\Delta\Delta C_t$
766 method ($2^{-\Delta\Delta C_t}$) was used to quantitate the relative levels of gene expression.

767

768 **ATP quantification**

769 A bioluminescence-based ATP detection kit (Molecular Probes, Invitrogen) was used to quantify
770 ATP levels in macrocolonies grown on SXD medium as indicated. ATP was extracted from
771 eight, 24 h-old macrocolonies harvested using a sterile glass slide and then suspended in 1 ml
772 sterile ice-cold Tris Buffered Saline (TBS; 50 mM Tris-Cl, pH 7.5, 150 mM NaCl). Cells were
773 harvested at 10,000 x g for 3 min (4°C) before re-suspending the entire pellet in TCA buffer
774 containing 100 mM Tris-HCl (pH = 8.0), 10% trichloroacetic acid (TCA), 25 mM ammonium
775 acetate, and 4 mM EDTA. Cell suspension was transferred to pre-chilled tubes containing glass
776 beads and then subjected to bead beating (Bio-Spec; 5 × 1 min, 4 M/s with 2 min on ice between
777 pulses). Cell lysates were collected and a portion of the supernatant was analyzed for ATP
778 following the instruction of the manufacturer. Luminescence was analyzed using microplate
779 reader (Berthold) using 1 sec integration time. A portion of the same lysate was used to
780 determine total protein concentration using the bicinchoninic acid (BCA; Sigma) assay. Results
781 presented are average of ATP normalized to total protein concentration analyzed from 3
782 biological replicates; each replicate is an average of 2-3 technical replicates.

783

784 **Immunoblotting**

785 For Stp2 cleavage analysis, cells expressing Stp2-HA (PMRCA48) were grown to saturation in
786 SD liquid medium overnight at 30 °C and then refreshed the following morning in 25 ml of fresh
787 SD medium at a starting OD₆₀₀ ≈ 0.3. Cells were grown in a 30 °C-shaker to an OD₆₀₀ of 1.5-
788 2.2. For induction experiments, a 500-μl aliquot of log phase culture were separately added to
789 tubes containing the indicated amount and type of amino acids or an equal volume of water for
790 control, and then incubated for 5 min at 30 °C in a thermoblock shaking at 700 rpm. For Put2-
791 HA expression analysis, cells from overnight YPD cultures were harvested, washed and then
792 grown as indicated. Whole cell lysates were prepared using NaOH/TCA method as described
793 previously with minor modifications [94]. Cells were lysed on ice with 280 μl of ice-cold 1.85
794 M NaOH with 7% β-Mercaptoethanol for 15 min; proteins were precipitated ON at 4 °C by
795 adding the same volume of cold 50% TCA. Protein pellets were quickly washed with ice-cold 1
796 M Tris base (pH = 11) and then resuspended in equal volume of Tris-HCl (pH = 8.0). In some
797 instances, as indicated, due to highly variability in protein recovered from certain types of cells
798 (i.e., yeast and filamentous forms) sample loading was normalized based on protein content.
799 Samples were denatured in 2X SDS sample buffer at 95-100 °C for 5 min, the proteins were
800 resolved in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 4-
801 12% pre-cast gels (Invitrogen) and analyzed by immunoblotting on nitrocellulose membrane
802 according to standard procedure. For Stp2-HA and Put-HA detection, HRP-conjugated anti-HA
803 antibody (Pierce) was used at 1:2,500 dilution. For loading control, HRP-conjugated rat
804 monoclonal α-tubulin antibody [YOL1/34] (Abcam) was used at 1:10,000 dilution. Membranes
805 were blocked using TBST (TBS + 0.1% Tween) containing 10% skimmed milk; antibodies were
806 diluted in TBST containing 5% skimmed milk. Immunoreactive bands were visualized by
807 enhanced chemiluminescent detection system (SuperSignal Dura West Extended Duration
808 Substrate; Pierce) using ChemiDoc MP system (BioRad). Densitometric analyses were
809 performed using ImageJ.

810

811 **Active Ras1-Pull Down Assay**

812 Active Ras1 (Ras1-GTP) was analyzed in macrocolonies using Pierce Active Ras Pull-Down Kit
813 (Thermo Scientific) following the manufacturer's instructions, but with an extra bead-beating
814 step to ensure optimal disruption of cells. Five 24 h-old macrocolonies were scraped, pooled,
815 and suspended in 1 ml ice-cold TBS in 2-ml microcentrifuge tubes (with caps). Cells were
816 collected by centrifugation at 10,000 x g for 3 min (4 °C) and then resuspended in 400 µl of
817 Lysis/Binding/Washing buffer (1X, Pierce kit) supplemented with protease cocktail
818 (cOmplete™ mini, EDTA-free; Roche) and 1 mM PMSF. Pre-chilled glass beads were added,
819 cell suspensions were subjected to multiple cycles of bead beating (6 × 40 sec, 4 M/s, 2 min on
820 ice between pulses). After an initial clarification step at 1,000 rpm for 5 min, supernatants were
821 collected and total protein was determined using the BCA assay. The concentration of protein in
822 lysates was adjusted to 2 mg/ml using the lysis buffer as diluent and then 500 µg of protein was
823 used for the immunoprecipitation. We used 12.5 µg protein for input and eluted bound protein
824 in 25 µl. Proteins were resolved by SDS-PAGE and analyzed by immunoblotting. Total Ras and
825 active Ras-GTP were probed with primary monoclonal anti-Ras clone X (1:300) included in the
826 kit, and secondary goat anti-mouse antibody (1:10,000; Pierce). For loading control, α-tubulin
827 conjugated to HRP (1:10,000) was used. Membranes were blocked and the primary antibody
828 diluted in TBST containing 3% BSA; the secondary and loading control antibodies were diluted
829 in TBST containing 5% skimmed milk. Results presented are representative of at least 3
830 independent experiments.

831

832 **Growth Assays**

833 For drop plates, cells from log phase YPD cultures grown at 30 °C were harvested, washed, and
834 then adjusted to OD₆₀₀ ≈ 1. Five microliters of 10-fold serially diluted cell suspension were
835 spotted onto the surface of the indicated SXD media and incubated at 30 °C for 2-3 days and
836 photographed. For liquid assays, washed cells from log phase YPD cultures were diluted in the
837 indicated SXD liquid medium to a starting OD₆₀₀ ≈ 0.05, and 300 µl were transferred into each
838 well of a 10 x 10-well microplate and grown continuously for > 20 h at 30 °C with constant

839 agitation. OD₆₀₀ readouts were captured using BioScreen C MBR analyzer (Oy Growth Curves
840 Ab Ltd, Helsinki, Finland).

841

842 **Resazurin Reduction Assay**

843 The membrane permeant, non-destructive redox indicator, Resazurin (Sigma), was used to
844 measure the metabolic activity of intact cells growing in SXD. Briefly, cells from overnight YPD
845 cultures were harvested, washed once with sterile ddH₂O, and adjusted to OD₆₀₀ ≈ 0.01 (~3 x
846 10⁵ CFU/ml) using the YNB-glucose base medium (~1.05x strength, pH = 6.0). Using a multi-
847 channel pipette, 95 µl of this cell suspension were added to the well of a 96-well microplate
848 followed by addition of 5 µl of 200 mM amino acid stock (10 mM final concentration). Plates
849 were incubated at 37°C for 2 h with agitation protected from light. After 2 h, 20 µl of filtered
850 Resazurin dye (0.15 mg/ml) was added to each well and incubated for 2 h at 37 °C before
851 measuring the fluorescence (560 nm excitation/590 nm emission) using EnSpire microplate
852 reader (PerkinElmer).

853

854 **TTC Overlay Assay**

855 Macrocolonies grown on the indicated plates for 24 h were overlaid with 2 ml of molten TTC-
856 agar solution (50-55 °C) containing 0.1% TTC (2,3,5 triphenyltetrazolium chloride; Sigma)
857 dissolved in 6.7 mM potassium phosphate buffer (PPB, pH = 7.0) with 1% agar [15]. Plates were
858 photographed 30 min after the overlaid solution became solid.

859

860 **Extracellular oxygen consumption assay**

861 Oxygen consumption assay was performed in *C. albicans* grown in synthetic proline medium
862 containing the indicated carbon source (i.e., 2% glucose (SPD), 0.2% (SPD_{0.2%}), or 1% glycerol
863 (SPG) using the Extracellular Oxygen Consumption Assay (Abcam, ab197243) following
864 manufacturer's protocol. Briefly, cells from log phase YPD culture were harvested, washed 3X
865 with PBS, and then diluted in the indicated media at OD₆₀₀ ≈ 0.3. A 150 µl cell suspension was

866 added into each well of a 96-well microplate with black walls and clear bottom. Ten microliters
867 (10 μ l) of Extracellular Oxygen Consumption Reagent or medium were then added into each
868 well, mixed gently by moving the plate on a circular motion, and then spiked with either medium
869 or control. FCCP (final conc. 10 μ M) and antimycin (final conc. 10 μ g/ml) were used as positive
870 and negative controls, respectively. Plates were analyzed using Enspire microplate reader using
871 Time Resolved Fluorescence (TRF). Signals were read every 90 sec for 120 repeats with optimal
872 delay time of 30 μ s and gate (integration) time of 100 μ s. Signal from wells without cells were
873 used as background signal.

874

875 **Quantification of proline**

876 The concentration of proline in media and in cell extracts was analyzed using the quantitative
877 ninhydrin method [95]. Proline utilization was assessed as follows: cells grown overnight in
878 YPD were washed and resuspended to an $OD_{600} \approx 0.5$ in pre-warmed synthetic proline media
879 containing 10 mM of proline and the indicated carbon source. The cultures were incubated under
880 constant aeration for 2 h at 37 °C, and the amount of proline in culture supernatants was analyzed.
881 Proline utilization was defined by comparison to non-inoculated media.

882

883 ***C. albicans* co-culture with murine macrophages**

884 The murine macrophage cell line RAW264.7 (ATCC) was cultured and passaged in Dulbecco's
885 modified Eagle's medium/high glucose (HyClone, GE Healthcare Life Sciences, Amersham,
886 UK) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μ g/ml
887 streptomycin (hereafter referred as D10) at 37°C with 5% CO₂. Prior to co-culture with *C.*
888 *albicans*, RAW264.7 cells (1×10^6) in D10 medium were seeded on a 24-well microplate
889 containing sterile cover slips and were allowed to adhere overnight in a humidified chamber at
890 37°C and 5% CO₂. Fungal cells (3×10^8) were harvested from overnight YPD cultures and
891 stained with 1 mg/ml FITC solution in 0.1 M NaHCO₃ buffer (pH = 9.0) in the dark for 15 min
892 at 30 °C. Cells were washed 3X with PBS before resuspending in equal volume of PBS. Fungal
893 cells were added to macrophage at MOI of 3:1 (*Candida*:Macrophage, C:M) and were then

894 allowed to interact for 30 min. Non-phagocytosed cells were removed by washing the cells at
895 least 5X with pre-warmed Hank's Balanced Salt Solution (HBSS) and 1X with D10 medium.
896 Cells were allowed to interact for an additional 4 h in fresh D10 medium before fixing with 3.7%
897 formaldehyde-PBS for 15 min in the dark at room temperature. Fixed cells were then washed
898 3X with PBS before staining with calcofluor white (10 µg/ml) for 1 min. After 2X PBS washing,
899 coverslips were mounted on glass slides using ProLong™ Gold antifade reagent (Invitrogen).
900 Images were obtained using LSM 800, 63x/1.2 oil.

901

902 ***C. albicans* killing by murine macrophages**

903 The survival of *C. albicans* co-cultured with macrophages was assessed by colony forming units
904 (CFU) analysis. Briefly, RAW264.7 cells in D10 were seeded into a 96-well microplate at a
905 density of 1×10^5 per 200 µl and allowed to adhere overnight. *C. albicans* cells from overnight
906 YPD cultures were processed without staining and added at a MOI of 3:1 (C:M). The co-cultures
907 were incubated for 3 h prior to assessing fungal cell viability by CFU; each well was treated to
908 final concentration of 0.1 % Triton X-100 for 2 min to lyse macrophage and serial dilutions were
909 prepared and plated onto YPD. CFUs were counted 2 days after incubation at 30 °C. The ability
910 of macrophages to kill *C. albicans* (% killing) was determined by comparison of fungal CFU
911 recovered in the absence of macrophages.

912

913 **Indirect immunofluorescence microscopy of phagocytosed *C. albicans***

914 RAW264.7 cells were co-cultured with *C. albicans* cells, CFG185 (*PUT2/PUT2-HA*), for 90 min
915 on glass coverslips at a MOI of 5:1 (C:M). Cells were fixed in 3.7% formaldehyde-PBS for 15
916 min, and permeabilized in 0.25% Tween-20 for 15 min, both incubations were at room
917 temperature. The fixed and permeabilized cells were incubated in zymolyase buffer (2U
918 zymolyase 100T (Zymo Research, Irvine, CA, USA), 10 mM DTT in PBS) for 1 h at 30 °C.
919 After washing, cells were incubated at room temperature in 0.25% Tween-20 for 10 min and
920 blocked in 5% FBS for 30 min. Cells were incubated overnight at 4 °C with rat anti-HA (Roche,
921 Germany, #1867423) and rabbit anti-Lamp1 (Abcam, UK, #ab24170) primary antibodies diluted

922 1:500 in 0.25% Tween-20. Cells were washed with PBS and incubated 2 h with Alexa flour 488
923 goat anti-rabbit (Invitrogen, Eugene, OR, USA #A11034) and Alexa flour 555 goat anti-rat
924 (Invitrogen, Eugene, OR, USA #A11034) secondary antibodies diluted 1:500 in 0.25% Tween-
925 20. Images were captured on a Zeiss 510 Meta confocal microscope, 63x/1.4 oil. Orthogonal
926 views were constructed in FIJI imaging software.

927

928

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941

942

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- 1223

1224 **Supporting Information**

1225 **Fig S1. Hyphae-specific gene (*HSG*) expression in *C. albicans* grown in the presence of inducing**
1226 **amino acids.**

1227 **Fig S2. NanoLuc™ luciferase assay for analysis of Stp2 target gene expression.**

1228 **Fig S3. CRISPR/Cas9-mediated gene inactivation in *C. albicans*.**

1229 **Fig S4. Metabolic activity of *C. albicans* grown in the presence of inducing and non-inducing**
1230 **amino acids.**

1231 **Fig S5. Growth curves of arginase-pathway mutants in different nitrogen sources.**

1232 **Fig S6. Neutralization of medium containing amino acid as sole carbon and nitrogen source**
1233 **remains intact in mutant lacking *DURI,2*.**

1234 **Fig S7. Carbon source and mitochondria-dependent oxygen consumption of *C. albicans*.**

1235 **Table S1. Strains used in this study.**

1236 **Table S2. Primers used in this study**

1237

1238

1239 **Figures**

1240 **Fig 1. Amino acid-induced morphogenesis is dependent on amino acid uptake. A.**

1241 Macrocolonies of wildtype *C. albicans* (PMRCA18) grown on SXD medium containing 10 mM
1242 of the indicated amino acids (X = Pro, Arg, Orn or Asp) and 2% glucose after 48 h of growth at
1243 37 °C (upper panels). Cells scraped from macrocolonies stained with calcofluor white (lower
1244 panels); scale bars = 30 μ. **B.** Amino acid-induced SPS-sensor signaling. Cells expressing Stp2-
1245 6XHA (PMRCA48) were grown to log phase in SD medium and induced with 50 μM or 5 mM
1246 of the indicated amino acids for 5 min at 30 °C. The levels of latent and processed Stp2 in extracts
1247 were analyzed by immunoblotting (upper panels). Similarly, reporter strain (CFG001) carrying
1248 an integrated P_{CANI}-NanoLuc™-PEST construct was grown to log phase in SD medium and
1249 induced with 50 μM of the indicated amino acids for 2 h at 30 °C. The average luciferase signal

1250 (ave. \pm CI, 95% CL) are plotted; threshold for significance $\geq 1.5X$ fold change). **C.**
1251 Macrocolonies of wildtype (WT; PMRCA18) and strains carrying mutations inactivating SPS-
1252 sensing pathway components *ssyl* Δ/Δ (YJA64), *ssy5* Δ/Δ (YJA53), *stp1* Δ/Δ (PMRCA59),
1253 *stp2* Δ/Δ (PMRCA57), *stp1* Δ/Δ *stp2* Δ/Δ (PMRCA94) and *csh3* Δ/Δ (PMRCA12) grown on the
1254 indicated SXD media. **D.** Constitutively active Stp2* but not Stp1* bypasses the filamentous
1255 growth defect of a *ssyl* null mutant in the presence of ornithine. Macrocolonies of WT
1256 (PMRCA18), *STP1** (PMRCA23), *STP2** (PMRCA44), *ssyl*^{-/-} *STP1** (CFG078), and *ssyl*^{-/-}
1257 *STP2** (CFG073) grown on SQD with ornithine (O) as sole nitrogen source. Images in C and D
1258 were obtained after 24 h of incubation 37 °C.

1259
1260 **Fig 2. Amino acid-induced morphogenesis is dependent on catabolism and Ras1 activated**
1261 **Efg1-dependent transcription.** **A.** Scheme of possible signaling pathways controlling amino
1262 acid-induced morphogenesis. **B.** Amino acid-induced morphogenesis requires a functional
1263 Ras1/cAMP/PKA pathway (Efg1-dependent) but not on the MAPK signaling pathway (Cph1-
1264 dependent). Wildtype (WT; PMRCA18) and strains lacking Ras1 (CDH107), Cph1 (JKC19),
1265 Efg1 (HLC52), and both Cph1 and Efg1 (HLC54) were spotted onto the indicated SXD media
1266 (X = Pro, Arg, Orn or Asp) and incubated at 37 °C for 48 h. **C.** Levels of active GTP bound form
1267 of Ras1 (Ras1-GTP) increase upon amino acid induction. Extracts were prepared from pooled
1268 WT (PMRCA18) macrocolonies grown for 24 h at 37 °C on the specified SXD medium. The
1269 levels of total Ras1 and the activated forms (Ras1-GTP) were determined by
1270 immunoprecipitation. **D.** Arginine catabolism is required for arginine-induced morphogenesis.
1271 Cells were spotted on SRD (Arg) supplemented with nor-NOHA, a competitive inhibitor of
1272 arginase. **E.** Proline catabolism is required for proline-induced morphogenesis. Cells were
1273 spotted on SPD (Pro) supplemented with L-THFA, a competitive inhibitor of proline
1274 dehydrogenase. For **D** and **E**, macrocolonies (PMRCA18) were grown at 37 °C and
1275 photographed after 72 h. Lower images are magnified 2X in comparison to upper images.

1276
1277 **Fig. 3. Amino acid-induced morphogenesis requires mitochondrial oxidative**

1278 **phosphorylation. A.** ATP levels in macrocolonies (PMRCA18) formed 24 h after spotting cells
1279 on the indicated SXD medium (X = Asp, Arg, Orn, Pro, Am (ammonium sulfate) or Urea)
1280 incubated at 37 °C. The levels of ATP in three biological replicates normalized to total protein
1281 are plotted. The values from each biological replicate is the average of 2-3 technical replicates.
1282 Statistically significant changes in ATP levels, as compared to cells grown on Asp, are indicated
1283 (ave. ± CI; **, p value < 0.01; *, p value < 0.05). **B.** Uncoupling of mitochondria reduces amino
1284 acid-induced filamentation. Cells (PMRCA18) were spotted on SXD media (X = Arg, Orn or
1285 Pro) supplemented with indicated amount of methylene blue (MB); macrocolonies were grown
1286 at 37 °C and photographed after 24 h.

1287
1288 **Fig. 4. A bifurcated pathway for arginine-induced morphogenesis. A.** Scheme of arginine
1289 catabolic pathway. **B.** Growth-based assays. Five microliters (5 µl) of serially diluted cells were
1290 spotted onto the surface of SXD (X = Am (Ammonium sulfate), Arg, Orn, Pro or Urea) and then
1291 grown for 48 h at 30°C. Strains used: wildtype (WT; PMRCA18), *car1*^{-/-} (CFG077), *dur1,2*^{-/-}
1292 (CFG091), *put1*^{-/-} (A) (CFG122), *put1*^{-/-} (B) (CFG155), and *put1*^{-/-} *dur1,2*^{-/-} (CFG158). **C.**
1293 Arginine rapidly derepresses proline catabolic genes. *PUT1*, *PUT2*, *PUT3* and *DUR1,2*
1294 expression in wildtype (SC5314) cells 1 h at 37 °C after shifting from YPD (t = 0) to YNB+Arg
1295 (pH = 6.0). Gene expression was determined by qRT-PCR using the levels of *RIP1* to normalize
1296 expression [96]. **D.** Proline catabolic pathway is required for growth in arginine as a sole nitrogen
1297 and carbon source. Cells with the indicated genotypes were harvested from log phase YPD
1298 cultures and diluted in YNB+Arg+BCP medium (pH = 4.0) to OD₆₀₀ ≈ 0.01, cultures were
1299 incubated for 16 h at 37 °C. Alkalinization (shift to purple) correlates with growth. Strains used:
1300 WT (SC5314), *dur1,2*^{-/-} (CFG246), *put1*^{-/-} (CFG149), and *put2*^{-/-} (CFG143). Identical results
1301 were obtained using PMRCA18-derived mutants. **E.** Rapid activation of proline catabolism in
1302 the presence of arginine, ornithine, and proline. Immunoblot analysis of Put2-HA in whole cell
1303 lysates prepared from CFG185 (*PUT2/PUT2-HA*) cells grown at 37 °C in the specified SXD
1304 media for the indicated times. Cells were pre-grown in YPD and inoculated at an OD₆₀₀ of 0.5.
1305 Levels of α-tubulin were used to control loading. **F.** Pharmacological inhibition of proline
1306 dehydrogenase (Put1) reduced filamentous growth of *C. albicans* in the presence of arginine.

1307 Macrocolonies of wildtype (WT; PMRCA18) grown at 37 °C for 72 h on SRD medium
1308 supplemented with L-THFA as indicated. **G.** Filamentous growth of strains on SXD media (X =
1309 Arg, Urea, or Arg + Urea as sole nitrogen sources). Macrocolonies were grown at 37 °C and
1310 photographed at 24 and 48 h. Strains used: wildtype (WT; PMRCA18), *car1*^{-/-} (CFG077),
1311 *dur1,2*^{-/-} (CFG091), and *put1*^{-/-} (CFG122).

1312

1313 **Fig 5. Proline utilization is influenced by carbon source but not by NCR.** **A.** Filamentous
1314 growth is more robust at lower glucose level. Wildtype cells (PMRCA18) from overnight YPD
1315 liquid cultures were washed and then adjusted to OD₆₀₀ of 8, 10 µl aliquots were spotted on
1316 media containing 10 mM of proline and the indicated levels of carbon source. Plates were
1317 incubated at 37 °C and photographed at 24, 48 and 72 h. TTC overlay assay was performed on a
1318 replica plate containing 24 h-old macrocolonies (shown in 24 h panel). **B.** Proline utilization is
1319 higher under glucose limited conditions. The levels of proline remaining in culture supernatants
1320 of WT (PMRCA18) after 2 h of growth at 37 °C in the presence of different carbon source, as
1321 indicated. Results shown are from 5 biological replicates (Ave. ± CI, 95% CL; ***, p value <
1322 0.001). Immunoblot analysis of Put2-HA and α-tubulin (loading control) in cell extracts prepared
1323 from CFG185 (*PUT2/PUT2-HA*) grown under identical conditions (inset). **C.** Respiratory
1324 growth predominates as glucose level decreases. WT cells were diluted to OD₆₀₀ of 0.5 in pre-
1325 warmed synthetic proline media containing 10 mM proline (YNB+Pro+BCP) and the indicated
1326 levels of glucose with the initial pH adjusted to 6.0. Cultures were grown for 16 h under vigorous
1327 agitation at 37 °C prior to photographing the culture tubes. Glycerol was used as respiratory
1328 growth control. **D.** Put2 is highly expressed in cells grown in the presence of non-glucose carbon
1329 sources. Immunoblot analysis of cell extracts prepared from CFG185 (*PUT2/PUT2-HA*) cells
1330 grown in YPGlu (YP+2% glucose = YPD), YPGly (YP + 1% glycerol), YPLac (YP + 1%
1331 lactate), or Spider medium (with 1% mannitol) at 37 °C for the timepoints indicated. Cells were
1332 pre-grown in YPD and inoculated at an OD₆₀₀ of 0.5. **E.** Proline utilization is insensitive to NCR.
1333 Immunoblot analysis of cell extracts prepared from CFG184 (*gln3Δ/Δ gat1Δ/Δ PUT2/PUT2-*
1334 *HA*) grown at 30 °C in SD_{0.2%} medium, which contains 10 mM ammonium sulfate (Am) and
1335 0.2% glucose, supplemented with 10 mM of the nitrogen sources and harvested at the timepoints

1336 as indicated. Cells were pre-grown in SD and then subcultured to log phase in SD_{0.2%}.

1337

1338 **Fig 6. Mitochondrial proline catabolism is required by *C. albicans* cells to escape murine**
1339 **macrophages. A.** Confocal immunofluorescence microscopy of *C. albicans* cells expressing
1340 Put2-HA (CFG185) in the phagosomes of RAW264.7 macrophages. Primary antibodies (rat anti-
1341 HA and rabbit anti-Lamp1) and secondary antibodies (Alexa Fluor 555 conjugated goat anti-rat
1342 antibody and Alexa Fluor 488 conjugated goat anti-rabbit) were used to visualize Put2 and the
1343 lysosomal compartment, respectively. Orthogonal view of merged channels is shown in the lower
1344 right panel. Scale bar = 10 μ . **B.** Proline catabolism is required for hyphal growth of *C. albicans*
1345 in macrophages. Wildtype (WT; SC5314), heat killed WT, *put1*^{-/-} (CFG139), *put2*^{-/-} (CFG207),
1346 *put3*^{-/-} (CFG146), *put1*^{-/-} *put2*^{-/-} (CFG159) and CRISPR/Cas9 control strains CFG181
1347 (pV1093) and CFG182 (pV1524) pre-grown in YPD and stained with FITC were co-cultured
1348 with RAW264.7 macrophages at a MOI of 3:1 (C:M). After 30 min, external non-phagocytosed
1349 cells were removed by washing, and the co-cultures were incubated an additional 4 h. External
1350 (escaping) hyphal cells were stained with calcofluor white (CFW). Scale bar = 10 μ . **C.**
1351 Macrophage killing of *C. albicans*. Strains as in **B** were co-cultured with RAW264.7 at a MOI
1352 of 3:1 (C:M) for 3 h. After lysing macrophages, viability of *C. albicans* was assessed by
1353 quantitating the number of CFU. The percent killing was determined by comparison to the
1354 viability of cells grown in the absence of macrophages.

1355

1356 **Fig 7. Arginine induces morphogenesis in *C. albicans* through mitochondria-dependent**
1357 **activation of Ras1/cAMP/PKA pathway.** The presence of extracellular arginine enhances
1358 arginine uptake by binding to the SPS-sensor, leading to the endoproteolytic activation of
1359 transcription factor Stp2. The active form of Stp2 efficiently targets to the nucleus and binds the
1360 UAS_{aa} in the promoter of genes encoding amino acid permeases (*AAP*). Amino acid permeases
1361 are cotranslationally inserted into the membrane of the ER, and transported to the plasma
1362 membrane (PM, arrow) via the secretory pathway, a process that requires the ER membrane-
1363 localized chaperone Csh3. The increased functional expression of amino acid permeases (Aap)

1364 lead to an enhanced capacity to take up arginine. Intracellular arginine is catabolized by arginase
1365 (Car1) yielding ornithine and urea. Urea is further degraded by the urea amidolyase (Dur1,2)
1366 generating CO₂ and NH₃. Ornithine is further catabolized to proline in the cytoplasm in a series
1367 of enzymatic reactions starting with the ornithine aminotransferase (Car2). Proline is transported
1368 into the mitochondria where it is catabolized by Put1 and Put2 forming glutamate. These
1369 reactions generate the reduced electron carriers FADH₂ and NADH,H⁺, respectively. Glutamate
1370 is oxidized by glutamate dehydrogenase (Gdh2) forming α -ketoglutarate in a reaction that
1371 liberates NH₃ and generates NADH,H⁺. α -ketoglutarate feeds directly into the mitochondria-
1372 localized TCA cycle. The reduced electron carriers generated by proline, glutamate and TCA
1373 cycle metabolic events are oxidized in reactions coupled to the generation ATP by mitochondrial
1374 oxidative phosphorylation. The elevated levels of ATP in the cytoplasm activate the adenyl
1375 cyclase (Cyr1) in a Ras1-dependent manner, which activates the downstream protein kinase A
1376 (PKA) signaling pathway and the effector transcription factor Efg1. The active phosphorylated
1377 form of Efg1 binds the UAS_{Efg1} in the promoter of hyphal specific genes (*HSG*), thereby inducing
1378 yeast-to-hyphal morphogenesis. The catabolism of arginine via the proline pathway induces
1379 hyphal growth more rapidly (FAST) than the Dur1,2 generated CO₂ (slow). Mitochondrial
1380 activity is repressed in the presence of high glucose.

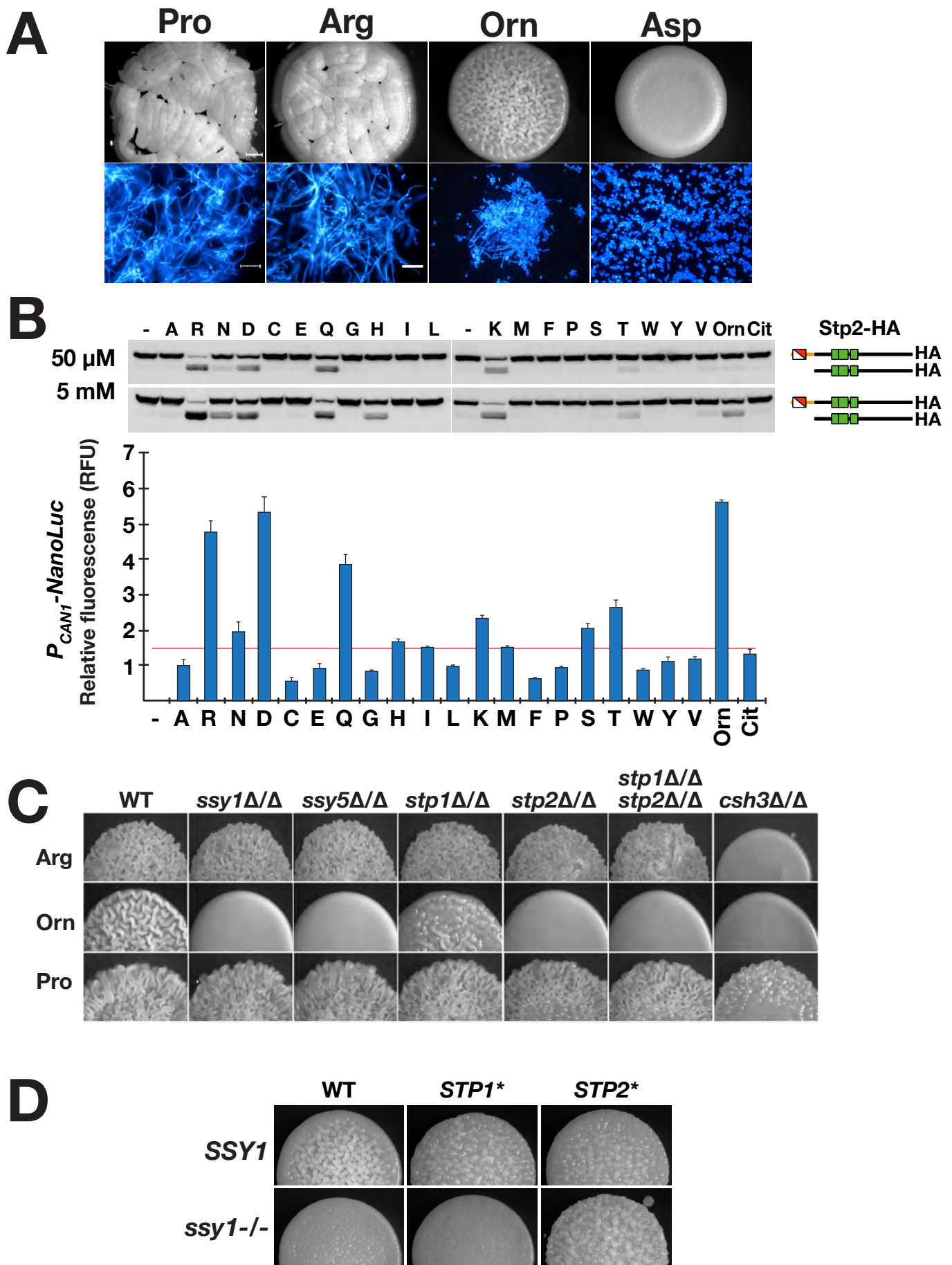


Fig. 1 Silao et al.

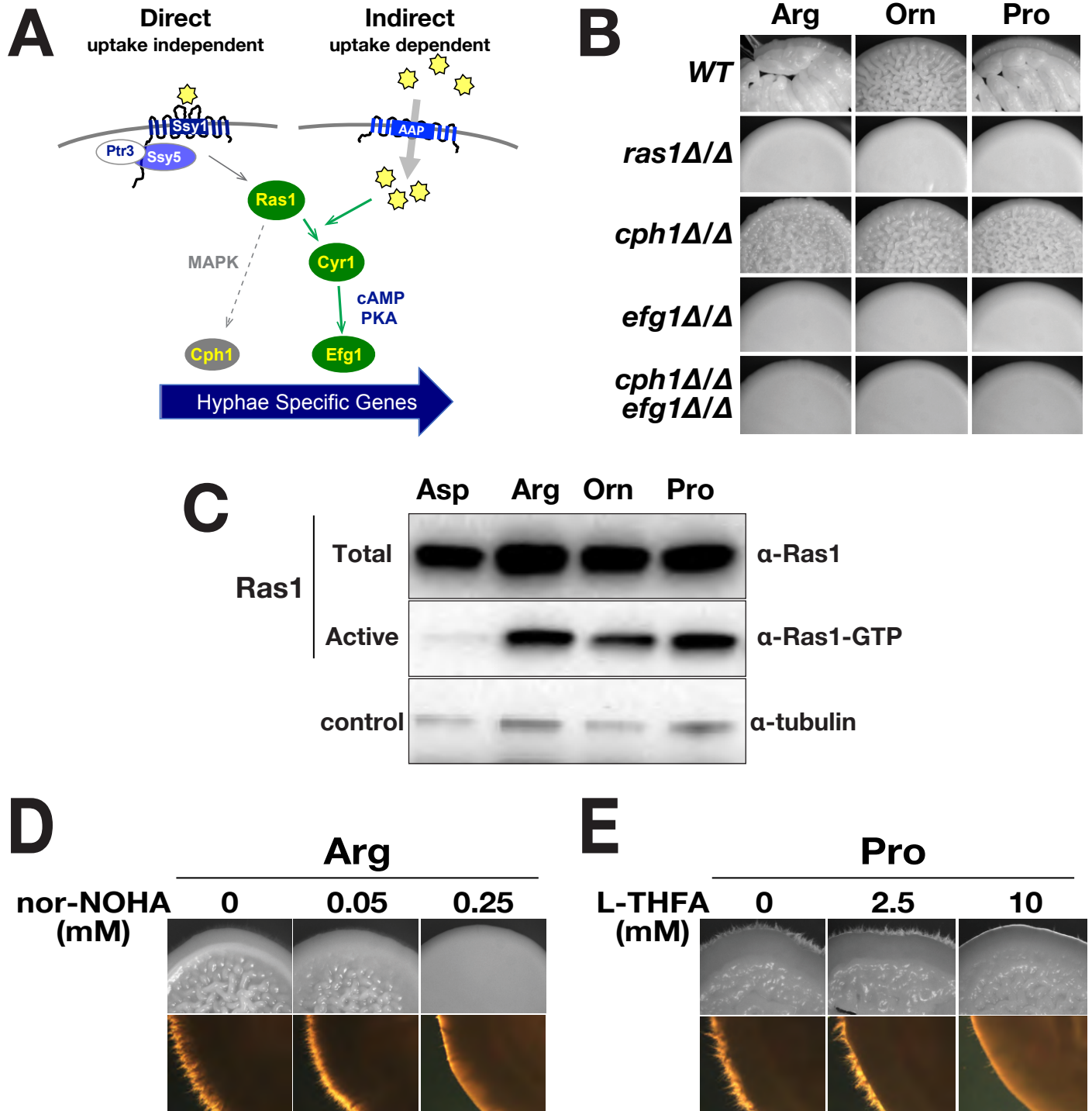


Fig. 2 Silao et al.

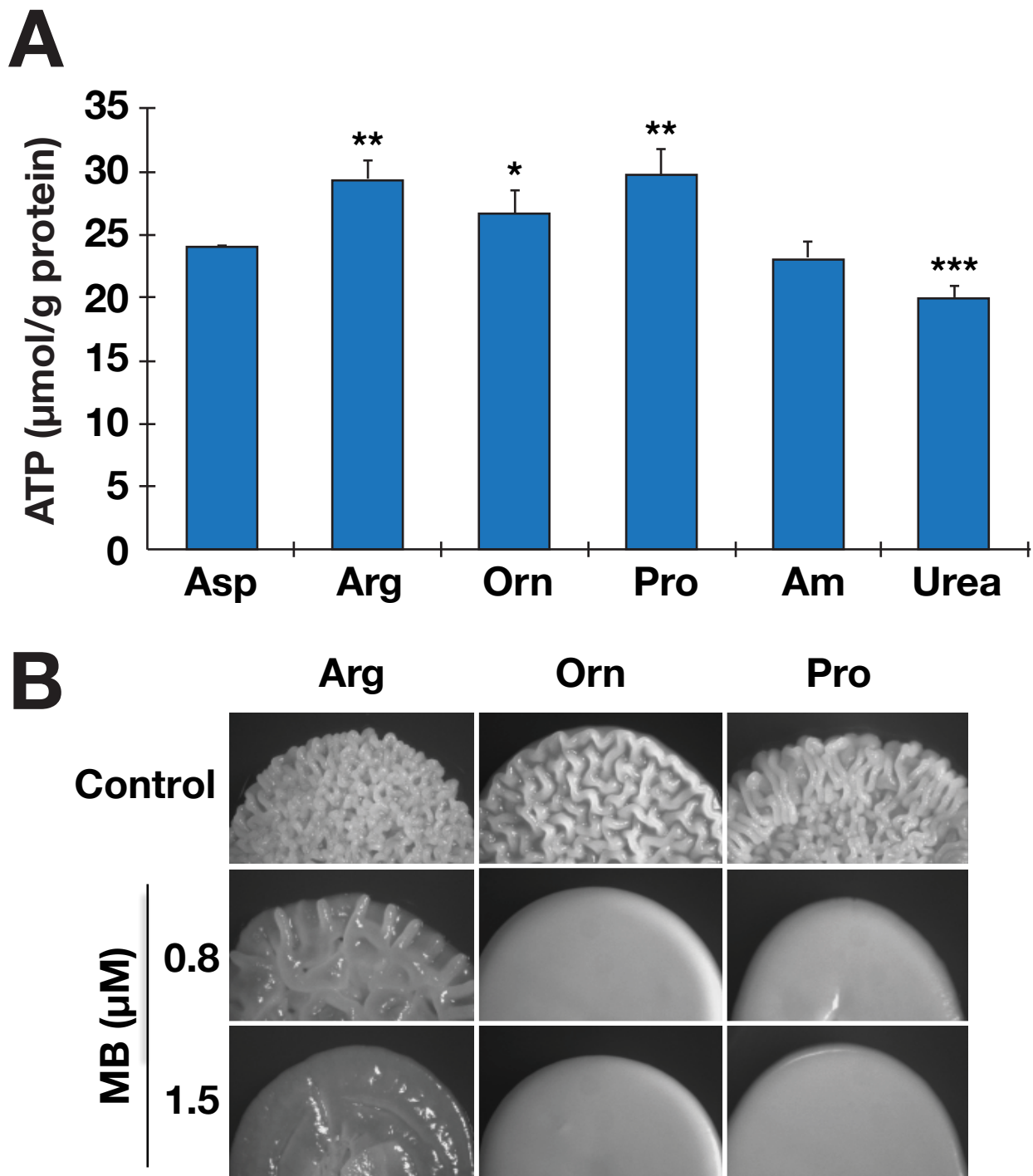


Fig. 3 Silao et al.

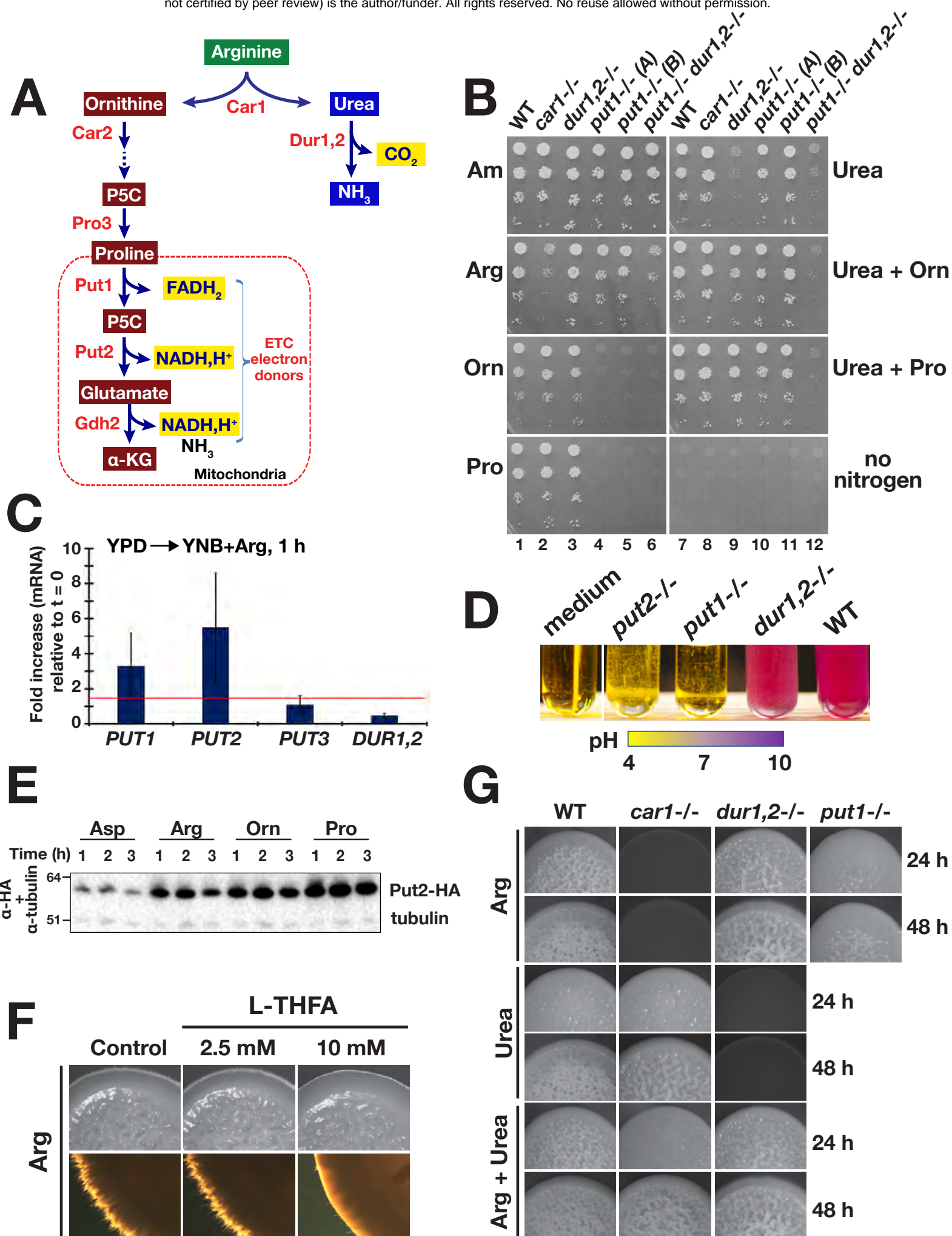


Fig. 4 Silao et al.

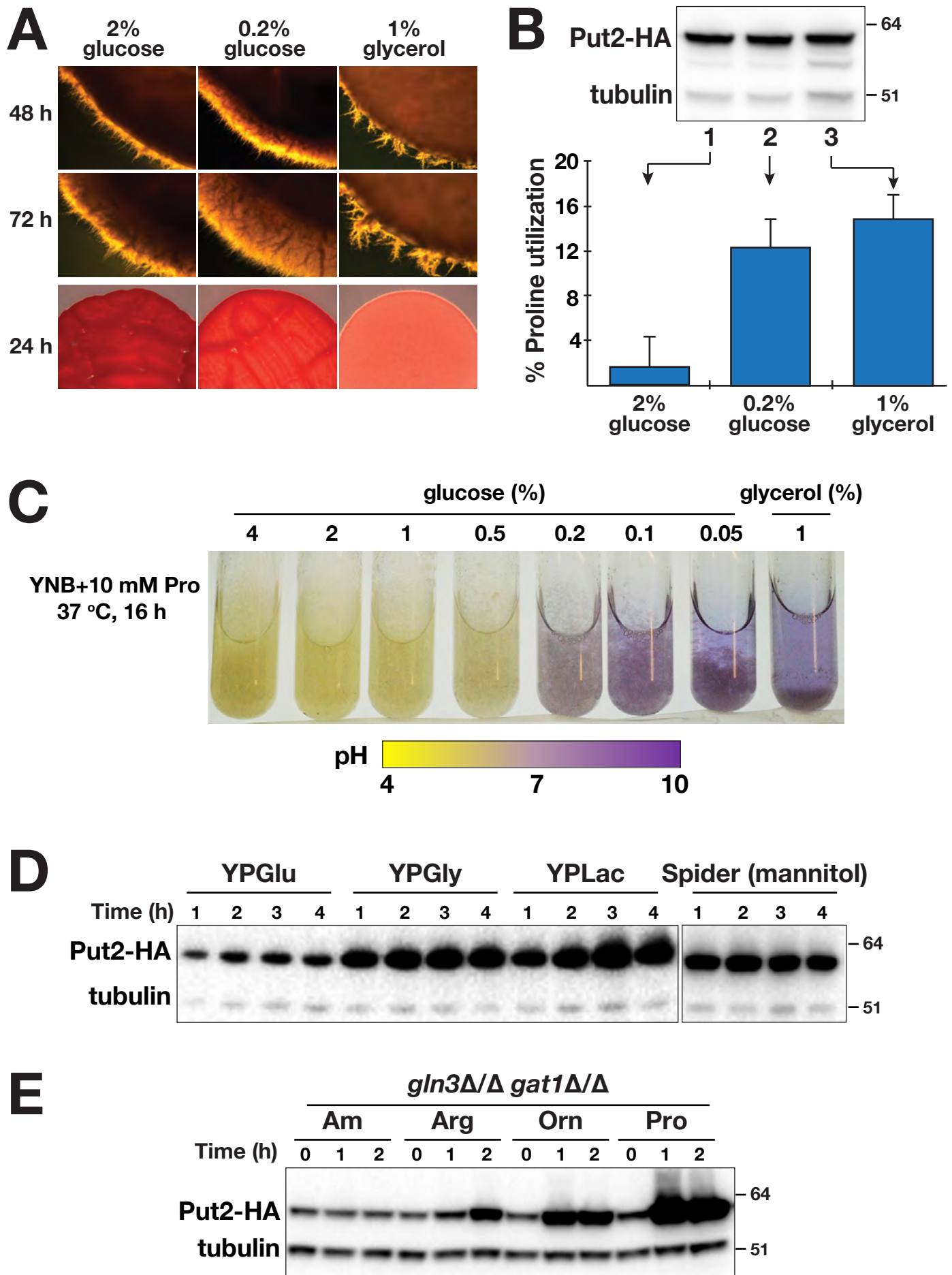


Fig. 5 Silao et al.

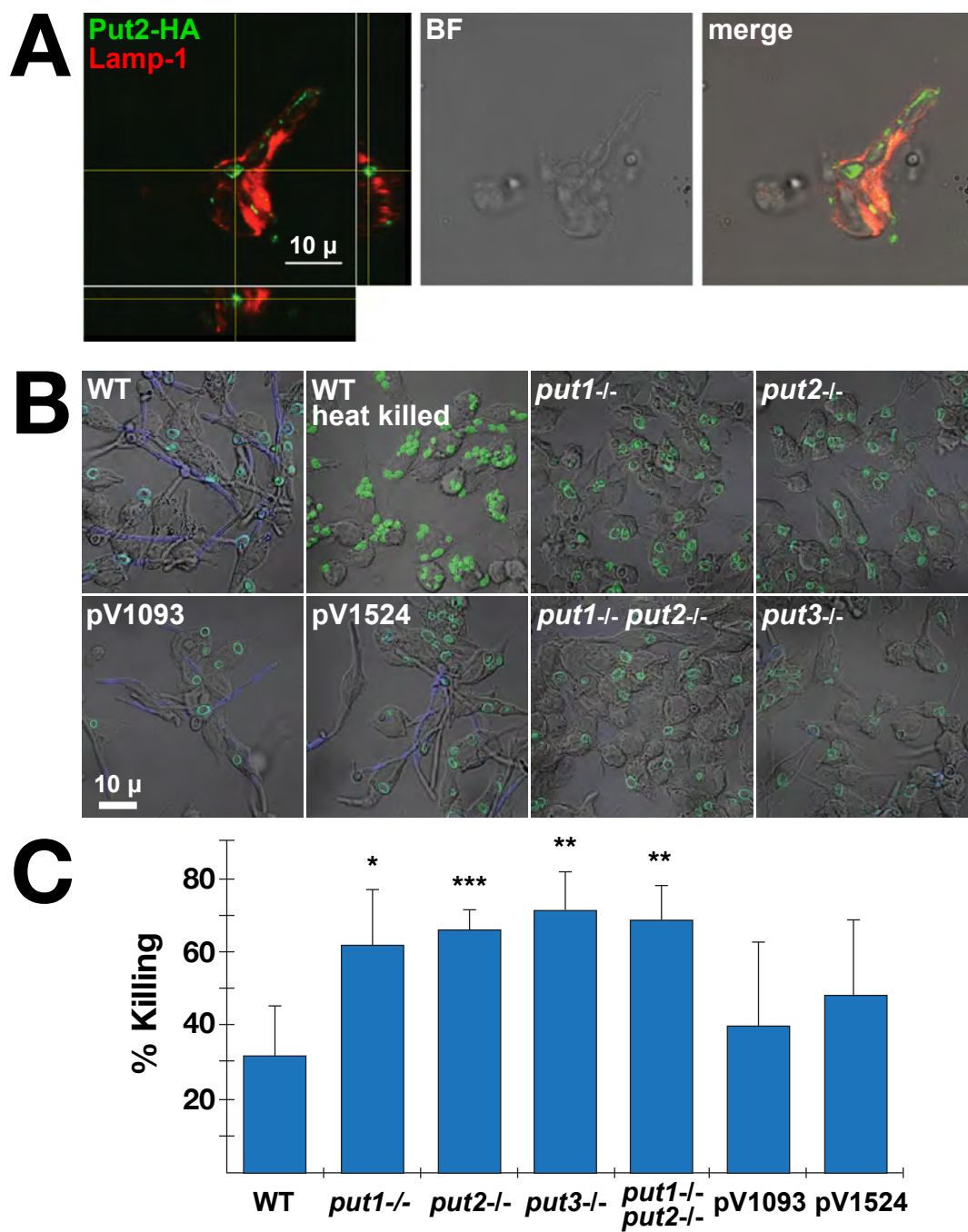


Fig. 6 Silao et al.

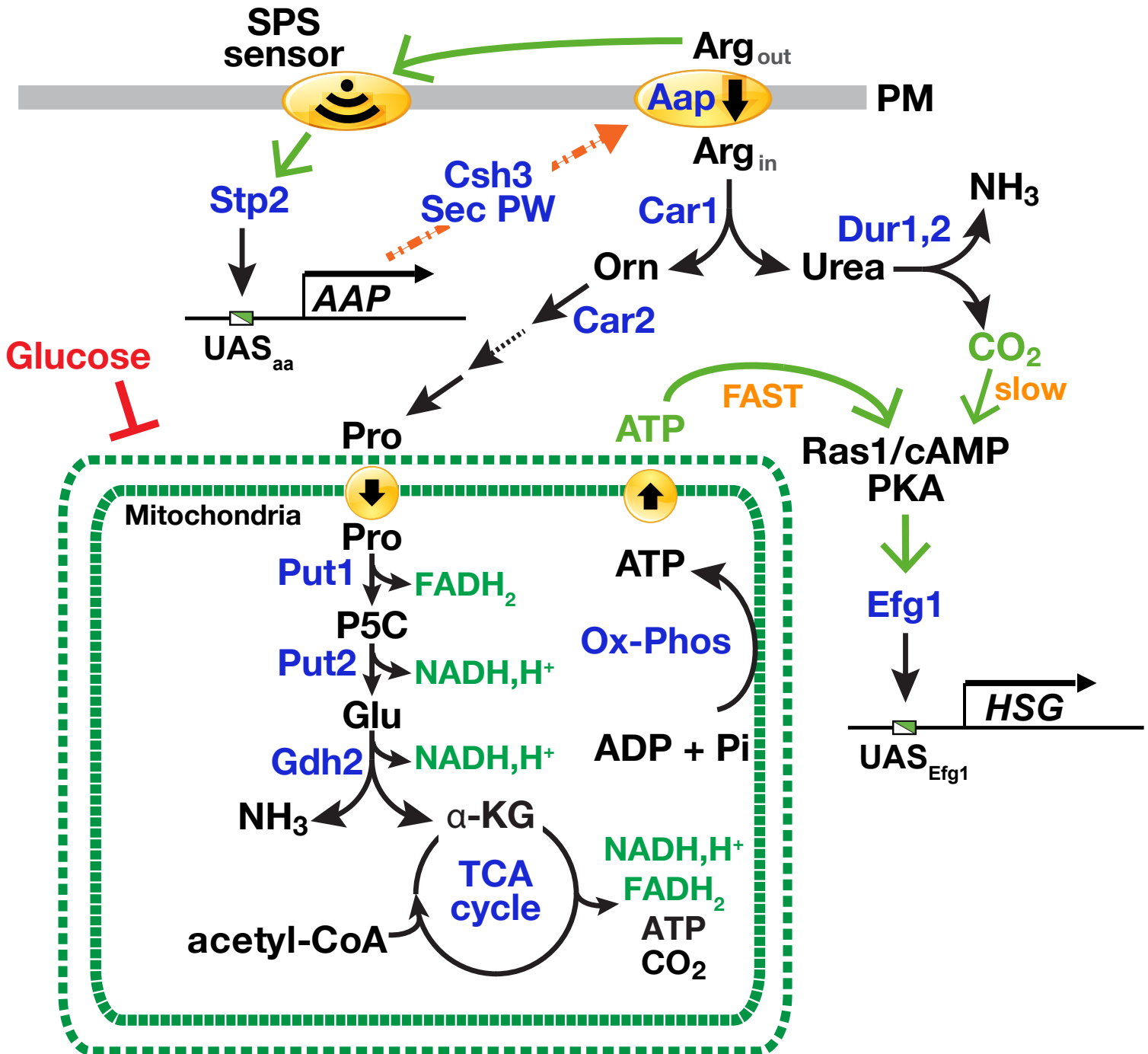


Fig. 7 Silao et al.