

1 **Phosphate is the third nutrient monitored by TOR in *Candida albicans* and**
2 **provides a target for fungal-specific indirect TOR inhibition**

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13 Abstract

14
15 The TOR pathway regulates morphogenesis and responses to host cells in the fungal
16 pathogen *Candida albicans*. Eukaryotic TOR complex 1 (TORC1) induces growth and
17 proliferation in response to nitrogen and carbon source availability. Our unbiased
18 genetic approach seeking new components of TORC1 signaling in *C. albicans* revealed
19 that the phosphate transporter Pho84 is required for normal TORC1 activity. We found
20 that mutants in *PHO84* are hypersensitive to rapamycin and, in response to phosphate
21 feeding, generate less phosphorylated ribosomal protein S6 (P-S6) than wild type. The
22 small GTPase Gtr1, a component of the TORC1-activating EGO complex, links Pho84
23 to TORC1. Mutants in Gtr1, but not in another TORC1-activating GTPase, Rhb1, are
24 defective in the P-S6 response to phosphate. Overexpression of Gtr1 and of a
25 constitutively active Gtr1^{Q67L} mutant suppress TORC1-related defects. In *S. cerevisiae*
26 *pho84* mutants, constitutively active Gtr1 suppresses a TORC1 signaling defect but
27 does not rescue rapamycin hypersensitivity. Hence connections from phosphate
28 homeostasis to TORC1 may differ between *C. albicans* and *S. cerevisiae*. The converse
29 direction of signaling, from TORC1 to the phosphate homeostasis (PHO) regulon,
30 previously observed in *S. cerevisiae*, was genetically demonstrated in *C. albicans* using
31 conditional *TOR1* alleles. A small molecule inhibitor of Pho84, an FDA-approved drug,
32 inhibits TORC1 signaling and potentiates the activity of the antifungals amphotericin B
33 and micafungin. Anabolic TORC1-dependent processes require significant amounts of
34 phosphate. Our study demonstrates that phosphate availability is monitored and also
35 controlled by TORC1, and that TORC1 can be indirectly targeted by inhibiting Pho84.

36 37 Significance

38
39 The human fungal pathogen *Candida albicans* uses the TOR signaling pathway to
40 contend with varying host environments and thereby regulate cell growth. Seeking novel
41 components of the *C. albicans* TOR pathway we identified a cell-surface phosphate
42 importer, Pho84, and its molecular link to TOR complex 1 (TORC1). Since phosphorus
43 is a critical element for anabolic processes like DNA replication, ribosome biogenesis,
44 translation and membrane biosynthesis, TORC1 monitors its availability in regulating
45 these processes. By depleting the central kinase in the TORC1 pathway, we showed

46 that TORC1 signaling modulates regulation of phosphate acquisition. An FDA-approved
47 small-molecule inhibitor of Pho84 inhibits TORC1 signaling and potentiates the activity
48 of the gold-standard antifungal amphotericin B and the echinocandin micafungin.

49 50 **Introduction**

51
52 Organisms that fail to maximize growth in response to abundant nutrients can be
53 outcompeted by those that do. Conversely, organisms that fail to cease growth and
54 induce survival programs during stress and starvation lose viability. The Target of
55 Rapamycin (TOR) signaling pathway is conserved in eukaryotes and integrates multiple
56 channels of information regarding the cells' nutritional and physical environment, to
57 induce either growth and proliferation, or stress- and survival responses (1). In the
58 human fungal pathogen *Candida albicans*, TOR participates in regulating
59 morphogenesis (2-7) and responses to host cells (8). To control growth, *C. albicans*
60 TOR also integrates signals of carbon source availability from the protein kinase A
61 (PKA) pathway with nitrogen source status, its primary nutritional input (9). Similar TOR-
62 PKA intersections have been reported in the model yeast *Saccharomyces cerevisiae*
63 (10, 11).

64
65 In *S. cerevisiae*, TOR complex 1 (TORC1), which is susceptible to inhibition by
66 rapamycin, is activated by preferred nitrogen sources such as glutamine and leucine.
67 Leucine activates TORC1 through the Exit from G0 (EGO) complex by inducing GTP
68 loading of one of its subunits, the small GTPase Gtr1 (12). Leucine also promotes
69 TORC1 activity through leucine-tRNA synthase Cdc60, which physically interacts with
70 Gtr1 (13). Many transcriptional regulators responsive to *S. cerevisiae* and *C. albicans*
71 TORC1 pathways are conserved (4), but there are important differences between these
72 species as well.

73
74 A small Ras-like GTPase upstream of TORC1, Rheb in mammals (14) and Rhb1 in *S.*
75 *cerevisiae* (15), also responds to nutritional signals. Rheb is a central activator of
76 mammalian TORC1 (mTORC1) and is modulated by the TSC1/TSC2 complex (16, 17),
77 while in *S. cerevisiae*, Rhb1 seems to play a minor role in nutritional signaling. In *C.*
78 *albicans*, Rhb1 is required for normal tolerance to rapamycin and for phosphorylation of
79 ribosomal protein S6, a readout of TORC1 activation (9, 18). *C. albicans* Rhb1 co-

80 regulates expression of genes important in nitrogen source uptake (18) and in virulence
81 (19). Unlike *S. cerevisiae*, *C. albicans* also has a TSC2 homolog whose mutant
82 phenotypes are consistent with a conserved GTPase-activating activity of *C. albicans*
83 Tsc2 for Rhb1 (18).

84

85 Given the differences between the *S. cerevisiae* and *C. albicans* TORC1 pathways, we
86 employed a forward genetic approach to find new components of *C. albicans* TORC1
87 signaling. Using our mariner transposon mutant collection (20), we isolated a rapamycin
88 hypersensitive mutant in a *C. albicans* homolog of *PHO84*, the gene encoding the major
89 *S. cerevisiae* high-affinity phosphate transporter.

90

91 Having identified a connection between *C. albicans* Pho84 and the *C. albicans* TOR
92 pathway in a forward genetic screen, we characterized this link between phosphate
93 homeostasis and the cell's central growth control module. We found that we can
94 indirectly target *C. albicans* TORC1, using small-molecule Pho84 inhibitors one of which
95 is an FDA-approved antiviral drug, and that the antifungals amphotericin B and
96 micafungin are potentiated by Pho84 inhibitors.

97

98 **Results**

99

100 **A screen of haploinsufficient transposon mutants for altered rapamycin**
101 **susceptibility identified a *PHO84* ortholog.** We screened our heterozygous mutant
102 collection of mariner-transposon insertions marked with our dominant selectable marker
103 *NAT1* (20, 21) for altered rapamycin susceptibility. We isolated a transposon mutant
104 hypersensitive to rapamycin in which the transposon disrupts the promoter of orf19.655,
105 67 bp upstream of the predicted translational start site (Fig. S1A). This orf encodes a
106 protein with 66% amino acid identity to *S. cerevisiae* Pho84 and 55% amino acid
107 homology to the *Piriformospora indica* PiPT phosphate transporter whose crystal
108 structure was recently described (22) (Fig. S1B). According to Candida Genome
109 Database (CGD) nomenclature, we called this orf *C. albicans* *PHO84*, and used the
110 CGD sequence for further analysis (23). To confirm that rapamycin hypersensitivity of
111 the transposon mutant was linked to the disrupted *PHO84* locus, two independent
112 heterozygous deletion mutants and their homozygous null derivatives were constructed.

113 Mutant phenotypes in these two lineages were the same, and one was chosen for
114 further characterization (Fig. S1C). These mutants were also rapamycin hypersensitive
115 (Fig. 1A), confirming that *PHO84* is required for normal tolerance of rapamycin.

116
117 The cytoplasmic membrane protein Pho84 is the major high-affinity phosphate (Pi)
118 transporter in *S. cerevisiae* (24-26). *PHO84* expression is controlled by the PHO
119 regulon, a homeostatic system that maintains Pi availability for metabolism and growth
120 in fluctuating external Pi conditions (27). *C. albicans pho84* mutants, like those in the *S.*
121 *cerevisiae* homolog (28), failed to grow on medium without inorganic phosphate (Fig.
122 1B). Heterozygous and re-integrant cells, apparently haploinsufficient for rapamycin
123 tolerance (Fig. 1A), grew robustly on this medium (Fig. 1B), indicating that mechanisms
124 other than haploinsufficiency affect growth during Pi depletion, like the feedback loops
125 between expression of high- and low-affinity Pi transporters characterized in detail in the
126 *S. cerevisiae* PHO regulon (28). In liquid media with 1 mM Pi, growth of *pho84* cells was
127 close to wild type (Fig S1D). Expression of the *C. albicans PHO84* homolog restored
128 growth on medium lacking Pi to *S. cerevisiae pho84* mutants (Fig. S1E), indicating
129 functional orthology. Wild type cells secrete acid phosphatase in response to low
130 ambient Pi to mobilize covalently bound Pi from their environment, and this response
131 was used for decades in studies of the *S. cerevisiae* PHO regulon (29). *C. albicans*
132 *pho84*^{-/-} mutants, like those in *S. cerevisiae* (24), inappropriately de-repressed acid
133 phosphatase secretion in high ambient Pi (Fig. S1F), consistent with a conserved role of
134 *C. albicans* Pho84 in the PHO regulon.

135
136 ***C. albicans* Pho84 is required for the normal TORC1 response to Pi availability.**

137 We then examined the relationship between Pho84 and TORC1. To test whether
138 rapamycin hypersensitivity is due to decreased TORC1 kinase activity in the *pho84*^{-/-}
139 mutant, we monitored the phosphorylation state of ribosomal protein S6 (P-S6), which
140 we previously showed is controlled by TORC1 signaling (9). Null mutants in *PHO84* had
141 a weaker P-S6 signal than wild type during Pi refeeding at every Pi concentration of the
142 media, though they responded to increasing Pi concentrations with an increasing P-S6
143 signal (Fig. 1C). Pho84 therefore is required for normal anabolic TORC1 signaling, and
144 TORC1 activity responds to ambient Pi availability.

145

146 **Heterozygous and homozygous deletion mutants in *PHO84* are defective in**
147 **hyphal morphogenesis.** TORC1 regulates hyphal morphogenesis in *C. albicans* (2-7,
148 30), an important virulence determinant. Hyphal morphogenesis was defective in *pho84*
149 mutants on YPD agar medium containing 10% serum, Spider medium and RPMI 1640
150 (Figs. 1D and S2A), while equal growth of mutant and wild type was seen in these
151 media when the temperature signal for hyphal morphogenesis was absent (Fig. S2B).
152 While many signaling pathways converge on morphogenesis, these findings are
153 formally consistent with defective regulation by TORC1 (2).

154
155 **Pi content of cells lacking Pho84 is diminished.** We asked if *C. albicans* TORC1
156 activity may be downregulated in response to decreased intracellular Pi in *pho84*
157 mutants, analogously to the response of *S. cerevisiae* TORC1 to decreased intracellular
158 amino acids. Using *pho85* mutants as controls known to hyperaccumulate intracellular
159 Pi (31), we found that intracellular Pi concentrations were lower in *pho84*^{-/-} null than in
160 wild type cells in low and high Pi-containing media, though the difference was
161 substantially less than in the homologous *S. cerevisiae* mutant-wild type pair (Fig. 1E).
162 Diminished intracellular Pi concentrations of *C. albicans pho84*^{-/-} cells may be
163 responsible for, or contribute to the decreased TORC1 activation state, possibly in
164 addition to lack of a putative TORC1-activating function performed specifically by Pho84.

165
166 **Gtr1 links Pho84 to TORC1 in *C. albicans*.** Seeking a molecular link between Pho84
167 and TORC1 activity, we considered the possibility that Gtr1 may connect Pho84 to
168 TORC1. *GTR1* was first described for its functional and physical proximity to *S.*
169 *cerevisiae PHO84* (32, 33), and its product later was characterized as a component of
170 the TORC1-activating EGO complex (12, 13, 34, 35). We found that the P-S6 response
171 of *gtr1*^{-/-} cells to phosphate refeeding was blunted (Fig. 2A). To determine whether this
172 is an unspecific effect of decreased upstream TORC1 signaling, mutants in another
173 small TORC1-activating GTPase, *RHB1*, were tested. The *rhb1* mutants responded to
174 Pi refeeding like the wild type (Fig. 2B), suggesting that a Pi signal to TORC1 is
175 transmitted specifically through Gtr1.

176
177 If Gtr1 acts downstream of Pho84 in activating TORC1, its overexpression may
178 suppress *pho84*^{-/-} phenotypes. *GTR1* was overexpressed from the *ACT1* promoter in

179 wild type and *pho84*^{-/-} *C. albicans* cells. Compared with rapamycin hypersensitive
180 *pho84*^{-/-} cells transformed with the empty vector, the resulting *pho84*^{-/-} *pACT1-GTR1*
181 cells showed wild type tolerance to rapamycin, suggesting recovery of their TORC1
182 signaling activity (Fig. 2C, D). To investigate this possibility TORC1 activity was tested
183 directly by comparing the P-S6 signal of *pho84*^{-/-} cells transformed with the empty
184 vector with that of *pho84*^{-/-} *pACT1-GTR1* cells. Overexpression of *GTR1* recovered
185 Rps6 phosphorylation in *pho84*^{-/-} cells nearly to wild type levels (Fig. 2E). A *GTR1*
186 mutant encoding constitutively GTP-bound Gtr1^{Q67L}, homologous to *S. cerevisiae*
187 Gtr1^{Q65L} (12, 35, 36), was then constructed and overexpressed from the *ACT1* promoter.
188 This *GTR1*-GTP allele suppressed the TORC1 signaling defect of *pho84*^{-/-} cells
189 apparently equally to the overexpressed wild type *GTR1* (Fig. 2D, E). Overexpression of
190 *GTR1* or *GTR1*-GTP did not increase phosphorylation of S6 in wild type cells (Fig. 2E).
191 These findings are consistent with the model that in *C. albicans*, Gtr1 indirectly or
192 directly conveys a Pi signal to TORC1 and links Pho84 to TORC1 signaling.

193
194 We examined the relationship of Pho84 to TORC1 activity in the model yeast *S.*
195 *cerevisiae*. A *pho84* null mutant in the S288C genetic background (37) was
196 hypersensitive to rapamycin (Fig. S3A) at an intermediate ambient Pi concentration
197 (1mM). Of note the rapamycin phenotype was highly responsive to the Pi concentration
198 of pregrowth media. Rapamycin tolerance was not decreased by loss of *PHO84* in the
199 Σ 1278b background (not shown). Rapamycin hypersensitivity was not suppressed, but
200 the *S. cerevisiae* Sch9 phosphorylation state (36) (Fig. S3B) and P-S6 signal intensity,
201 which in *S. cerevisiae* also responds to TORC1 activation (Figs. 3 and S3C), were
202 recovered by constitutively active Gtr1 in *pho84* null cells. These findings suggest that
203 Pi homeostasis and TOR signaling are linked in *S. cerevisiae* as in *C. albicans*, though
204 specific molecular connections seem to have divergently evolved in these two fungi.

205
206 **TORC1 modulates the PHO regulon.** As TORC1 not only responds to nutrient
207 availability, but also directs nutrient uptake e.g. by regulating expression of amino acid
208 and ammonium transporters, we questioned whether it may play a similar role in
209 phosphate acquisition. Given known discrepancies between rapamycin exposure and
210 physiological TOR modulation (38-41), we examined this potential connection
211 genetically. Repressible *tetO* was used to control expression of *C. albicans TOR1* or of

212 a hypomorphic *TOR1*^{Δ₁₋₃₈₁} encoding a protein lacking the first 381 amino acids which
213 form protein-protein interaction HEAT repeat domains. The effect of *TOR1* depletion on
214 expression of *PHO84* was then examined.

215
216 When wild type cells were transferred from overnight cultures into fresh rich medium,
217 *PHO84* mRNA levels dropped, in accordance with the PHO regulon's response to
218 availability of fresh Pi sources. In cells depleted of either the wild type or the N-
219 terminally truncated *TOR1* allele, *PHO84* expression also decreased but to a
220 significantly lesser extent (Fig S4A). Full length *TOR1* permitted greater *PHO84*
221 expression than the *TOR1*^{Δ₁₋₃₈₁} allele, suggesting structural perturbation of TORC1 by
222 truncation of Tor1 affects its inhibitory as well as activating functions. Active TORC1,
223 signaling nutritional repletion, hence contributes input to the PHO regulon to
224 downregulate Pi starvation responses, while loss of TORC1 activity conveys a
225 starvation signal to dampen these responses (Fig. S4A). Similarly, overexpression of
226 Gtr1 and Gtr1-GTP blunted upregulation of secreted acid phosphatase in *pho84*^{-/-} cells
227 (Fig. S4B), supporting the model that in response to Pi TORC1 signaling downregulates
228 the PHO regulon to integrate its activity with availability of other nutrients.

229
230 **Small-molecule inhibitors of Pho84 repress TORC1 and potentiate antifungal**
231 **activity.**

232 *S. cerevisiae* Pho84 has been characterized as a Pi transceptor signaling to PKA,
233 through identification of point mutations and small molecules that preferentially perturb
234 transport, signaling or both (42, 43). Direct pharmacological inhibition of *C. albicans*
235 TORC1 with rapamycin incurs too high a cost on host immune function to be clinically
236 useful (44). We tested whether blocking Pho84 with its known small-molecule inhibitors
237 phosphonoformic acid (foscarnet, Fos) and phosphonoacetic acid (PAA) (42), which we
238 showed inhibit *C. albicans* growth in dependence on the presence of their target Pho84
239 (Fig. S5A, B), can indirectly inhibit *C. albicans* TORC1. Exposure of wild type cells to
240 the FDA-approved antiviral foscarnet inhibited Rps6 phosphorylation (P-S6) in a dose-
241 dependent manner (Fig. 4A), at Fos concentrations attained in human plasma during
242 antiviral therapy (45, 46). In heterozygous cells (*pho84*^{-/+}), whose haploinsufficiency
243 phenotypes likely reflect decreased copies of the drug target, Pho84, P-S6 was

244 hypersensitive to Fos (Fig. 4A). In cells lacking the target Pho84, exposure to Fos did
245 not further decrease the P-S6 signal (Fig. 4A). Pho84 inhibition with small molecules
246 also recapitulated the hyphal growth defect seen in cells genetically depleted of *PHO84*
247 (Figs. 1D, 4B and S5C).

248
249 Potentiating existing antifungals is a promising strategy (47-49). Fos at concentrations
250 reached in plasma during antiviral therapy (45, 46), and PAA, potentiate activity of the
251 antifungal amphotericin B, at concentrations of the latter far below those in serum or
252 tissue during standard dosing (50) (Fig. 4C, S5D). Activity of the antifungal micafungin,
253 belonging to the distinct drug class of echinocandins, was also potentiated (Fig. 4D).
254 Because Pho84 is not conserved in mammals, inhibition of Pho84 offers a novel
255 approach to fungal-specific TORC1 inhibition (Fig. 5) and to antifungal potentiation, as
256 shown in our proof-of-principle experiments with PAA and Fos.

257

258 **Discussion**

259

260 Eukaryotic cell mass consists to 0.5-1% of phosphorus (51). But phosphorus constitutes
261 only 0.07% of the earth's crust (51) and only ~15% of soil phosphorus is bioavailable in
262 a soluble form (52). Fungi, like plants and bacteria, have sophisticated regulatory
263 networks to manage Pi starvation. *S. cerevisiae* cells utilize the TORC1 and PKA
264 pathways to ensure orderly cessation of growth when nitrogen or carbon sources
265 become limiting (53, 54). *C. albicans* cells similarly use the TORC1 pathway for
266 calibrated responses to distinct nitrogen sources, with modulating input from PKA
267 according to carbon source type and concentration (9).

268

269 Our study shows that *C. albicans* TORC1 monitors not only nitrogen and carbon source-
270 but also Pi availability (Fig.1C; 2A,E). Whether Pho84 activates TORC1 indirectly by an
271 increased intracellular Pi content, directly in a transceptor role, or through a combination
272 of these inputs, remains an open question. Our results establish the small GTPase Gtr1,
273 first discovered in *S. cerevisiae* due to its mutant phenotypes' resemblance to those of
274 Pho84 (32), as an important element linking *C. albicans* Pho84 and TORC1 (Fig. 2).
275 How Gtr1, a subunit of the TORC1-associated vacuolar membrane-residing EGO
276 complex (55), receives information from Pho84 remains to be determined. Possible
277 models include an activating signal to the TORC1-activating SEACAT complex (55)

278 through a sensor of cytosolic Pi or vacuolar polyphosphate, or a physical interaction
279 between Pho84 and the EGO complex on endosomes while Pho84 is internalized
280 during ambient Pi abundance (56). We cannot exclude alternative explanations of our
281 findings, e.g. a TORC1 response only to intracellular Pi concentrations, and
282 upregulation of low-affinity Pi transporters during *GTR1* overexpression in *pho84*^{-/-} null
283 cells. If Gtr1 is activated by Pho84, this GTPase's role in the input to TORC1 regarding
284 the cell's Pi state appears to be specific (Fig. 2), as there was no perturbation of TORC1
285 activation during Pi refeeding in *rhb1*^{-/-} cells (Fig. 2B).

286
287 In *S. cerevisiae*, Pho84 signals to PKA as a transceptor (42, 57). A signal from Pho84 to
288 TORC1 has not yet been described in *S. cerevisiae*. We found that in the genetic
289 background S288C loss of Pho84 leads to rapamycin hypersensitivity and TORC1
290 inactivation as it does in *C. albicans* (Fig. S3). Sch9 phosphorylation, like that of Rps6,
291 is known to correspond with the TORC1 activation state (36, 58). Overexpression of
292 constitutively active Gtr1 restores TORC1 activity in *S. cerevisiae*, as assayed by Sch9
293 and Rps6 phosphorylation (Fig. 3, S3B). But in contrast to *C. albicans*, constitutively
294 active Gtr1 did not suppress rapamycin hypersensitivity of *S. cerevisiae pho84* null cells,
295 indicating differences in the connections of PHO and TORC1 signaling between these
296 fungi. In *S. cerevisiae*, control of entry into quiescence (G0) by the Rim15 kinase is co-
297 regulated by the PHO pathway cyclin/cyclin-dependent kinase module Pho80/Pho85, as
298 well as by TORC1(59), suggesting multiple levels of cross-talk between phosphate-
299 specific and global nutritional signaling pathways in that organism, which remain to be
300 explored in *C. albicans*.

301
302 In addition to the signal from Pho84 to TORC1, we observed a signal in the opposite
303 direction, from TORC1 to expression of *PHO84* and to the classic readout of the PHO
304 regulon, the acid phosphatase (27) (Fig. S4A), though clearly input from TORC1
305 contributes to only a fraction of the PHO regulon responses. TORC1 is well known to
306 regulate proteins required for acquisition of other nutrients like amino acids and
307 ammonium in *S. cerevisiae* (60, 61). TORC1 input to the PHO regulon may fine-tune the
308 investment of energy and nutrients on Pi acquisition to match the overall state of the cell.
309 In *S. cerevisiae*, transcriptional regulation favoring Pi acquisition is achieved through the
310 transcription factor Pho4 (31). A *C. albicans* homolog of Pho4, required for stress

311 resistance (62, 63) and commensalism in a murine model (63), was recently shown to
312 control *C. albicans* *PHO84* expression (62). How *C. albicans* Pho4 may be co-regulated
313 by TORC1 remains to be determined.

314
315 While TORC1 monitors nutrient availability in mammals as in fungi, its relationship with
316 the PHO regulon may have diverged in these phyla. In fungi, H⁺-Pi symport is the major
317 form of Pi import, since the steep Pi concentration gradient at the plasma membrane
318 imposes a high energetic demand on transport, met by the electrochemical proton
319 gradient generated by the P-type H⁺ ATPase (52). In animals, Na⁺-Pi pumps
320 predominate (64). Humans largely consume Pi together with amino acids in food
321 consisting of other eukaryotes and their products, so that Pi starvation tends to occur
322 during starvation for protein (65). In contrast, Pi and nitrogen sources must be acquired
323 independently by unicellular organisms, so the connection we discovered between the
324 fungal H⁺-Pi symporter and the TORC1 pathway appears physiologically plausible.

325
326 We found that TORC1 is indirectly repressed during Pho84 inhibition with small
327 molecules (Fig. 4, S5). Since TORC1 components are conserved between fungi and
328 humans while Pho84 has no human homologs, this result provides an option for indirect,
329 fungal-specific TORC1 inhibition not previously explored in the search for new
330 antifungals. While TORC1 is only partially inhibited in this manner, other fungal-specific
331 indirect TORC1 activators may provide synergistic targets. More immediately, Pho84
332 inhibitors potentiate the antifungal effect of micafungin and of amphotericin B (Fig.
333 4C,D), possibly permitting lower dosing of the latter, “gold-standard” broad-spectrum
334 agent to obviate its often treatment-limiting toxicities (66). We used 2 of multiple Pho84
335 inhibitors previously characterized (42) (Fig. 4 and S5D), and more specific, non-
336 competitive inhibitors with more favorable therapeutic indices than Fos may be found
337 through screening efforts. Since Pho84 homologs are highly conserved among fungi,
338 potentiating amphotericin B activity through their inhibition may prove a viable
339 therapeutic strategy for other fungal species less amenable to other antifungal agents.

340

341 **Materials and Methods**

342

343 **Strains and culture conditions.** The *C. albicans* and *S. cerevisiae* strains, plasmids
344 and primers used are described in SI Tables 1-3. *C. albicans* strains were generated in
345 the SN genetic background using *HIS1* and *ARG4* markers (67), as well as the *CaNAT1*
346 selectable marker, as described in (20, 21). Two independent heterozygotes were used
347 to derive homozygous null and reintegrant mutants of *PHO84*, as well as *tetO-TOR1*
348 mutants. Auxotrophies were complemented so that only prototrophic strains, or strains
349 with identical auxotrophies, were compared in an experiment. Introduced mutations
350 were confirmed by PCR spanning the upstream and downstream homologous
351 recombination junctions of transforming constructs, and sequencing. Experiments with
352 defined ambient Pi concentrations were performed in YNB 0 Pi (ForMedium Ltd, Norfolk,
353 UK) with added KH_2PO_4 to stated concentrations. Other media were used as in (20).

354 **Screening transposon mutants for altered rapamycin susceptibility.** Our
355 heterozygous mutant collection containing a mariner transposon marked with *CaNAT1*
356 (20) was used. Mutants were replicated to a 96-well plate containing 2xYPD with 8%
357 glucose and grown to saturation at room temperature, to minimize filamentous growth.
358 Cells were replicated to YPD agar medium with vehicle (90% ethanol) or 20 ng/ml
359 rapamycin. Clones showing less growth than the wild type (SC5314) were isolated as
360 rapamycin hypersensitive. The transposon insertion site was identified by vectorette
361 PCR as previously described (20).

362 **Growth Assays.** For cell dilutions spotted onto agar media as previously described (20),
363 saturated overnight cultures were diluted in 5-fold steps from an OD_{600} of 0.5. For
364 growth curves in liquid media, saturated overnight cultures in YPD were washed once in
365 0.9% NaCl and diluted to an OD_{600} of 0.15 in 150 μl medium in flat bottom 96-well
366 dishes. For growth assays including those during drug exposure, OD_{600} readings were
367 obtained every 15 min in a plate reader and standard deviations were calculated and
368 graphed in Graphpad Prism. Growth during drug exposure was assayed in SC medium.
369 Vehicle for Pho84 inhibitors PAA (Sigma, 284270) and Fos (Santa Cruz Biotechnology,
370 SC-253593A) was water and for amphotericin B (Sigma, A9528), DMSO.

371 **Western Blots.** Cell harvesting, lysis and Western blotting were performed as
372 described in (9). Antibodies are listed in SI Table 1. At least three biological replicates
373 were obtained. For densitometry, ImageJ (imagej.net/welcome) software (opensource)
374 was used to quantitate signals obtained on a KODAK Image Station 4000MM.

375 **Filamentous growth assay.** Cells were revived from frozen stocks on solid YPD

376 overnight, washed and resuspended in 0.9% NaCl to OD₆₀₀ 0.1. Variations between
377 single colonies and colony density effects were minimized by spotting 3 µl cell
378 suspension at 4 or 6 equidistant points, using a template, around the perimeter of an
379 agar medium plate, which then were incubated and imaged as in (20). For small-
380 molecule Pho84-inhibitor effects on filamentation, Spider and RPMI was used (TOKU-E,
381 Cat# R8999-04), the latter with 0.22mM KH₂PO₄ buffered to pH 7 with 50mM MOPS. At
382 least 3 biological replicates were obtained for each condition.

383 **Acid phosphatase assays.** As adapted from (25), overnight cultures in SC were
384 diluted to an OD₆₀₀ 0.05 into YNB medium buffered to pH4 with 50mM sodium citrate
385 containing 0 or 11mM KH₂PO₄ and grown overnight. They were washed thrice with
386 water, and p-Nitrophenyl Phosphate (Sigma, N4645) was added to a concentration of
387 5.62mg/ml. After 15 mins at room temperature the reaction was stopped with Na₂CO₃
388 (pH=11) to a concentration of 0.3g/ml, and OD₄₂₀ and OD₆₀₀ were measured. At least 3
389 biological replicates, with 3 technical replicates each, were obtained.

390 **Intracellular Pi assays.** Free and total Pi was measured by colorimetric molybdate
391 assay as described (68). Briefly, cultures were washed with distilled water twice,
392 resuspended in 500 µL 0.1% Triton X-100, and lysed by glass bead homogenization.
393 Lysate protein content was determined using a BioRad Protein Assay kit. Free Pi was
394 measured in unboiled lysate, then total phosphate was measured after boiling 3–30 µg
395 of whole cell lysate for 10 min in 1 N H₂SO₄. At least 3 biological replicates, with 3
396 technical replicates each, were obtained.

397 **RT-PCR expression analysis.** Cells were grown overnight in YPD medium with 5ng/ml
398 doxycycline, diluted into YPD with 30 µg/ml doxycycline, and harvested at time 0, 2 h
399 and 4 h. RNA was extracted with the Direct-Zol RNA miniprep kit (Zymo Research #
400 R2051). RT-PCR procedures were performed as indicated (69).

401

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403

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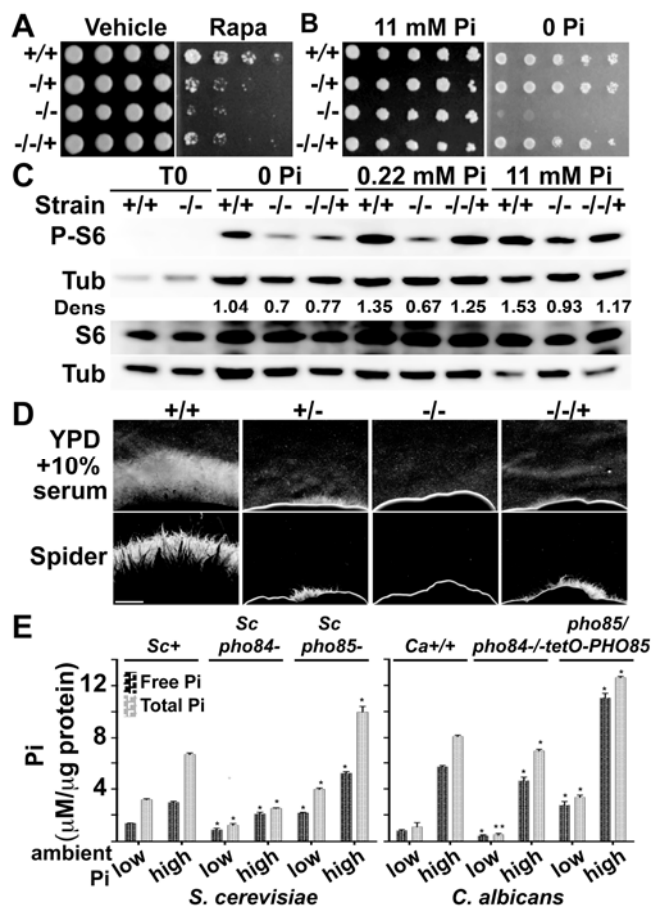
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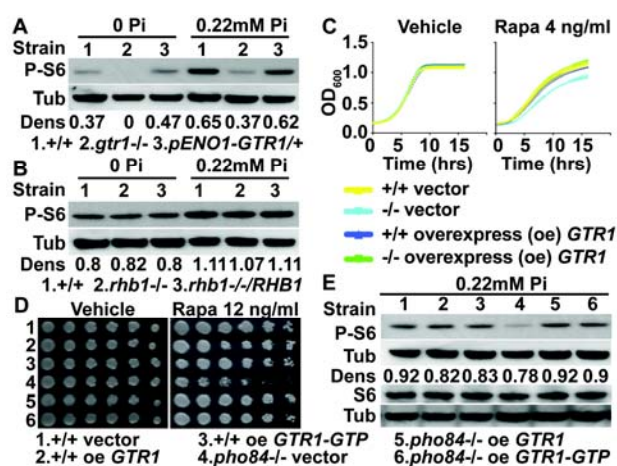
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613 **Figure Legends**

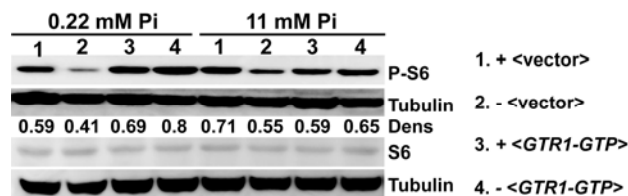


614
 615 **Fig. 1. *C. albicans* PHO84 is required for rapamycin tolerance, growth during Pi**
 616 **starvation, normal TORC1 activity and filamentation, and Pi homeostasis. A.** Cell
 617 dilutions of wild type (wt) and a mutant series in *PHO84* pinned onto YPD with vehicle
 618 or 12 ng/ml rapamycin. *PHO84*^{+/+}, JKC915. *pho84*^{+/+}, JKC1583. *pho84*^{-/-}, JKC1450.
 619 *pho84*^{-/-/+}, JKC1588. **B.** Cells as in A pinned onto YNB with 0 or 11 mM Pi. **C.** Separate
 620 Western blots of the same samples for P-S6, total Rps6 and tubulin of wt (+/+, JKC915),
 621 *pho84* null (-/-, JKC1450), and *PHO84* reintegrant (-/-/+, JKC1588) cells grown in YNB
 622 with 0, 0.22 mM or 11 mM KH₂PO₄ for 90 min. Dens: densitometry of P-S6 vs. tubulin

623 signal intensity. **D.** Strains as in (A) spotted at equidistant points around agar plates and
 624 spot edges imaged. Compare SI Fig. 2A. Bar 1mm. **E.** *S. cerevisiae* and *C. albicans*
 625 wild type and *pho84* null cells grown in SC medium with 0.22 mM (low Pi) or 11 mM Pi
 626 (high Pi) overnight, assayed for free and total Pi. *pho85* null cells as controls which
 627 hyperaccumulate Pi. *Sc+* (*S. cerevisiae* *PHO84 PHO85*), BY4741. *Scpho84-*, EY2960
 628 and *Scpho85-*, from (37). *Ca+/+* (*C. albicans* *PHO84/PHO84 PHO85/PHO85*), JKC915.
 629 *Capho84-/-*, JKC1450. *Capho85-/-*, CaLC1919, grown in 20 μ g/ml doxycycline overnight.
 630 * $p < 0.01$; ** $p < 0.05$. A-E represent at least 3 biological replicates; error bars SD of 3
 631 technical replicates.



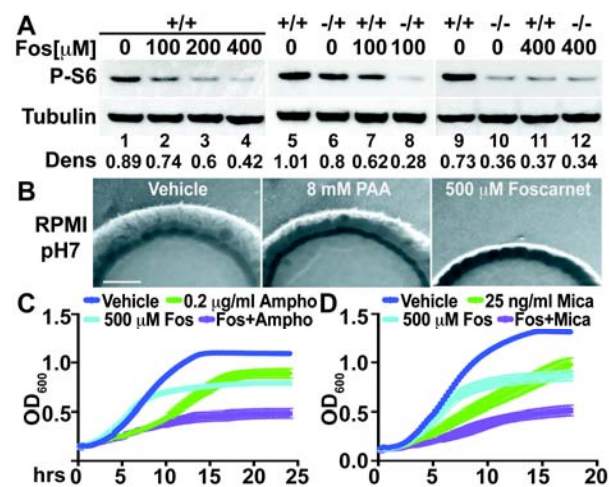
632 **Fig. 2. Gtr1 links Pho84 to TOR in *C. albicans*.** **A.** Western blot of wild type (wt)
 633 (SC5314), *gtr1*^{-/-} (YGM367) and *pENO1-GTR/GTR1* (YGM365) cells, grown in YNB
 634 with 0 and 0.22 mM KH₂PO₄ for 90 min. Dens: densitometric ratio of P-S6 vs. tubulin
 635 signal intensity. **B.** Western blot of wt (SC5314), *rhb1*^{-/-} (CCT-D1) and *rhb1*^{-/-}/*pADH1-*
 636 *RHB1* (CCT-OE1) cells, grown in YNB with 0 or 0.22 mM KH₂PO₄ for 90 min. **C.** Growth
 637 in YPD with 4 ng/ml rapamycin or vehicle. OD₆₀₀ monitored every 15 minutes. Yellow:
 638 wt with vector (JKC1594); blue: wt overexpressing *GTR1* (JKC1596); cyan: *pho84*^{-/-}
 639 with vector (JKC1598); green: *pho84*^{-/-} overexpressing *GTR1* (JKC1600). **D.** Cell
 640 dilutions pinned onto YPD with vehicle or 12 ng/ml rapamycin. Strains, (1) wt with vector
 641 (JKC1594), (2) wt overexpressing *GTR1* (JKC1596), (3) wt overexpressing *GTR1-GTP*
 642 (JKC1619), (4) *pho84*^{-/-} with vector (JKC1598), (5) *pho84*^{-/-} overexpressing *GTR1*
 643 (JKC1600) and (6) *pho84*^{-/-} overexpressing *GTR1-GTP* (JKC1616). **E.** Western blot of
 644 cells grown in YNB with 0.22 mM KH₂PO₄ for 90 min. Strains 1-6 as in (D). A-E
 645 represent at least 3 biological replicates.
 646



647

648 **Fig. 3. A connection between Pho84 and TORC1 is conserved in *S. cerevisiae*.**

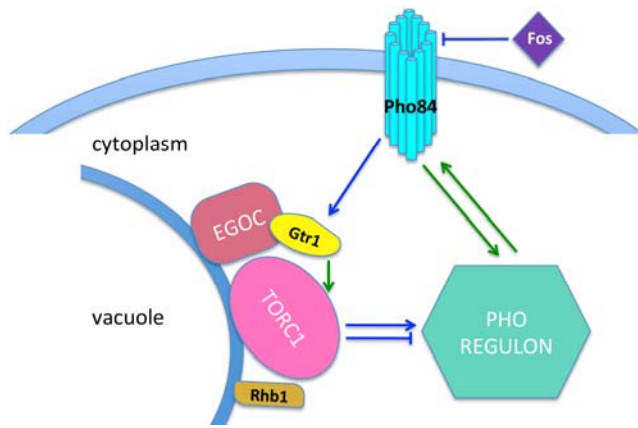
649 Western blot of (1) *PHO84* with vector (Y1597), (2) *pho84* with vector (Y1599), (3)
650 *PHO84* with *GTR1-GTP GTR2-GDP* (Y1596) and (4) *pho84* with *GTR1-GTP GTR2-*
651 *GDP* (Y1604) cells, grown in SC (-His-Ura) containing 0.22 mM and 11 mM KH_2PO_4 for
652 90 min, probed for P-S6, S6 and tubulin. Representative of 3 biological replicates.



653

654 **Fig. 4. Small-molecule inhibition of Pho84 represses TORC1 and filamentous**
655 **growth, and potentiates amphotericin B and micafungin. A.** Western blot of wild

656 type (wt) (SC5314) cells with (1) vehicle, (2) 100 μM foscarnet (Fos), (3) 200 μM Fos,
657 (4) 400 μM Fos, and strains (5, 7, 9, 11) *PHO84*^{+/+} (JKC915), (6, 8) *pho84*^{+/+}
658 (JKC1583) and (10, 12) *pho84*^{-/-} (JKC1450), grown in standard SC (7.3 mM Pi) for 60
659 min, probed for P-S6 and tubulin. **B.** Wt (SC5314) spotted at equidistant points around
660 RPMI agar (0.22 mM KH_2PO_4 , pH7) containing vehicle, 8mM PAA, or 500 μM Fos,
661 grown at 37°C, and spot edges imaged. Compare SI Fig. 2A. Bar 1mm. **C.** Wt (SC5314)
662 exposed to vehicle, 500 μM Fos, 0.2 μg/ml amphotericin B, and amphotericin B plus
663 Fos; OD₆₀₀ in SC with 0.5 mM KH_2PO_4 at 30°C monitored every 15 minutes. **D.** Wt
664 (SC5314) exposed to vehicle, 500 μM Fos, 25 ng/ml micafungin, and micafungin plus
665 Fos; OD₆₀₀ in SC with 0.5 mM KH_2PO_4 at 30°C monitored every 15 minutes.



666

667 **Fig. 5. Pho84 activates TORC1 via Gtr1, and TORC1 in turn modulates the PHO**
668 **regulon. Foscarnet inhibits Pho84 and thereby indirectly blocks TORC1 activity.**

669 Signaling events with known molecular mechanisms in *S. cerevisiae* are shown as
670 green lines. Blue lines represent predicted activities based on our findings.

671