# Differential virulence of *Candida albicans* and *C. dubliniensis*: a role for Tor1 kinase?

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**Abstract**

*Candida albicans* and *Candida dubliniensis* are two very closely related species of pathogenic yeast. *C. albicans* is the most prevalent species in the human gastrointestinal tract and is responsible for far more opportunistic infections in comparison with *C. dubliniensis*. This disparity is likely to be due to the reduced ability of *C. dubliniensis* to undergo the yeast to hypha transition, a change in morphology that plays an important role in *C. albicans* virulence. We have recently shown that hypha formation by *C. dubliniensis* is specifically repressed by nutrients at alkaline pH. In this article, we present new data showing that this can be partly reversed by treatment with rapamycin, an inhibitor of the nutrient sensing kinase Tor1 (Target Of Rapamycin). We also provide a speculative model to describe why *C. albicans* filaments more efficiently in nutrient rich environments, citing recently described data on Mds3, a pH responsive regulator of Tor1 kinase activity.

Several yeast species of the genus *Candida* have evolved to colonise the human gastrointestinal tract. *C. albicans*, the most commonly recovered species, is highly adapted for growth on mucosal surfaces and even minor disturbances in the host's immunity can lead to superficial infection. Recent comparative genomic studies have identified a range of gene families in *C. albicans* with putative roles in adhesion and nutrient acquisition that may contribute to its greater success *in vivo* relative to other yeast species.[1] In addition to these factors, the ability of *C. albicans* to undergo a reversible switch from a budding yeast morphology to filamentous hyphal cells is widely regarded as an important contributory factor to its success as a commensal and opportunistic pathogen[2]. This morphological transition (termed morphogenesis) is crucial for virulence and hyphal forms have been shown to be highly adherent and express specific degradative enzymes and adhesins that may contribute to tissue invasion. Both yeast and hyphal cells can be recovered from mucosal surfaces, however, cells restricted to either phase of growth display reduced virulence, demonstrating the requirement for morphogenesis at different stages of infection.[3, 4] The only other member of the genus *Candida* that forms true hyphae (as opposed to pseudohyphae) is the closely related *C. dubliniensis*.[5] Despite the close relatedness of *C. albicans* and *C. dubliniensis*, epidemiological and virulence data show that there is a dramatic difference in the ability of these organisms to cause disease. Analysis of the indicence of *C. dubliniensis* in bloodstream infection shows that this organism is responsible for fewer than 2% of systemic *Candida* infections compared with over 60% by *C. albicans*.[6, 7] Data from animal models of infection confirm that *C. dubliniensis* isolates are less virulent than *C. albicans*. In the oral-intragastric murine infection model, *C. dubliniensis* colonised mice

62 less efficiently than *C. albicans* and were less able to establish systemic infection.[8] In
63 addition, mice infected systemically by tail vein inoculation with *C. dubliniensis* have
64 greater survival times relative to *C. albicans* infected mice.[8-10] Data from both models
65 indicate that *C. dubliniensis* forms fewer hyphae *in vivo*, which may partly account for its
66 reduced capacity to cause disease. Although genetically similar, comparative genomic
67 studies have shown that *C. dubliniensis* has undergone gene loss and extensive
68 pseudogenisation relative to *C. albicans*, indicative of reductive evolution, possibly as a
69 result of niche specialization by *C. dubliniensis.*[11] In addition, *C. albicans* has acquired
70 over 200 genes since its divergence from *C. dubliniensis*. These additional genes are
71 largely the result of gene duplication events. Several of the species-specific genes of *C.*
72 *albicans* are expressed exclusively in the hyphal form and have putative roles in virulence
73 such as *ALS3*, *HYR1*, *SAP5* and *SAP6*. *C. albicans* also has expansions in gene families of
74 unknown function such as the telomeric *TLO* genes and the *IFA* genes encoding a family
75 of leucine-rich repeat proteins.[11] The contribution of these genetic differences to
76 virulence and filamentation are currently under investigation in our laboratory.

77 The reduced capacity of *C. dubliniensis* to form hyphae *in vivo* has been
78 highlighted in several studies of virulence using murine models and these data have been
79 corroborated by *in vitro* studies of filamentation in alkaline liquid media (e.g. Lee's
80 medium, RPMI, serum supplemented broths) where *C. dubliniensis* generally forms far
81 fewer true hyphae than *C. albicans.*[8, 12, 13] Genomic comparisons have revealed that all of
82 the major transcriptional regulators and signalling pathways implicated in filamentation
83 at alkaline pH in *C. albicans* are highly conserved in *C. dubliniensis,* including the
84 cAMP-protein kinase A pathway and the pH responsive RIM101 pathway (Fig. 1). The

first studies to investigate these pathways for a molecular basis for differential morphogenesis focused on differences in the expression of Nrg1, a DNA binding protein that acts in a complex with the transcriptional repressor Tup1 to repress the expression of genes regulating morphogenesis (Fig 1). Morphogenesis in both species is associated with a reduction in *NRG1* transcription. *C. dubliniensis* forms abundant chlamydospores on several nutrient poor media such as Staib's medium, in contrast to *C. albicans* which grows solely as yeast cells.[14] Staib et al. showed that this enhanced chlamydospore formation by *C. dubliniensis* on Staib medium was due to species-specific downregulation of *NRG1* transcription.[14] Subsequent to this study, we hypothesised that differential *NRG1* transcription might also suppress hypha formation by *C. dublinienis*, as *NRG1* has been shown to repress *UME6*, a transcription factor required for hyphal extension (Fig 1).[15] However, although deletion of *NRG1* in *C. dubliniensis* could restore filament production under some conditons, the mutant grew mainly as pseudohyphae in liquid media and did not differ in virulence from the wild-type, suggesting that other Nrg1-independent factors are also involved in repressing hypha formation in *C. dubliniensis.*[15] More recently, O'Connor et al.[13] carried out a more thorough investigation of the environmental cues involved in differential filament production in *C. albicans* and *C. dubliniensis*. This study showed that efficient filamentation could be induced in *C. dubliniensis* in both wild-type and *nrg1*Δ cells by incubation in liquid media that were nutrient poor and alkaline (pH 7.0-7.5). For example, whereas *C. albicans* formed abundant hyphae in standard yeast extract peptone dextrose (YPD) broth supplemented with 10% bovine serum (YPDS), *C. dubliniensis* formed only pseudohyphae. However, in water supplemented with 10% bovine serum (WS), *C. dubliniensis* produced true

hyphae at levels equivalent to *C. albicans* in YPDS. The addition of 2% glucose to WS medium had little effect on filamentation in *C. dubliniensis*, whereas the addition of peptone up to 2% greatly inhibited filamentation (Fig 1). A combination of glucose and peptone also had a greater effect than peptone alone. These data suggested that nutrient starvation, in particular amino acid limitation, may be required for filamentation in *C. dubliniensis.*[13] Several mechanisms of peptide and amino acid sensing have been described in *C. albicans*. In liquid media, the presence of amino acids such as proline (10 mM) have been shown to stimulate hypha formation via the plasma membrane SPS amino acid sensing complex. In contrast, amino acid starvation on nutrient poor solid media such as synthetic low ammonium dextrose (SLAD) medium can also stimulate hypha formation in *C. albicans*. Filamentation under nitrogen starvation conditions requires the activity of two permeases, the amino acid permease *GAP1*[16] and the ammonium permease *MEP2*[17], which may act as sensors of nitrogen in the environment. Furthermore, in response to amino acid starvation, *C. albicans* activates the general amino acid control (GCN) response, which is activated by the transcription factor Gcn4.[18] In *C. albicans*, Gcn4 has also been shown to have an additional role in stimulating filamentation via interaction with the cAMP-PKA pathway, leading to activation of the transcription factor Efg1 (Fig 1). These nitrogen starvation responses are critical for filamentation of *C. albicans* on nitrogen poor solid media such as SLAD as the addition ammonium or amino acids acts to repress filamentation. However, the addition of serum to nitrogen-rich media can reverse this inhibition, indicating that the alkaline pH response can override nutrient repression. In contrast, in *C. dubliniensis* the addition of serum to amino acid rich medium such as YPD is not sufficient to stimulate true hypha

formation[13], indicating that perhaps the alkaline pH response alone cannot induce filamentation in nutrient rich environments. This phenotype is unexpected as transcript profiling of *C. dubliniensis* grown in alkaline medium (e.g. Lee's medium pH 7.5) shows that this organism posesses a robust alkaline pH response with all of the hallmarks of an active RIM101-mediated transcriptional response.[13] However, incubation of *C. dubliniensis* in WS, a nutrient-poor alkaline medium that promoted filamentation, activated a transcriptional response highly similar to that described during hypha formation in *C. albicans*, including expression of genes encoding septins, GTPases, DNA replication factors and cell surface proteins. Critically, nutrient depletion in *C. dubliniensis* resulted in significantly increased transcription of *UME6*[13], a transcription factor required for hyphal elongation in *C. albicans* that acts by inducing *HGC1*, encoding a component of a regulatory kinase complex required for filament extension (Fig 1).[19, 20] Addition of peptone to nutrient poor WS medium could sequentially decrease filament formation in *C. dubliniensis* and this was associated with a sequential decrease in *UME6* expression (Fig 1). Conversely, forced *UME6* expression in *C. dubliniensis,* either from a doxycycline-inducible promoter or by preculturing in peptone-free media could enhance filament formation in nutrient rich environments and increase subsequent adhesion and invasion of epithelial cells.[13] These data suggest a clear role for nutrients, specifically complex mixtures of amino acids, in supressing filamentation at alkaline pH in *C. dubliniensis*.

Since publication of these data, we hypothesised that a nutrient-responsive mechanism must repress expression of *UME6* in *C. dubliniensis* and that perhaps in *C. albicans,* this repression can be removed by growth at alkaline pH. Recently, Bastidas et

al. (2009) showed that the nutrient regulated protein kinase Tor1 plays a novel role in regulating the expression of several cell wall genes and the transcriptional repressor Nrg1.[21] Tor1 is a multifunctional kinase conserved throughout the eukaryotic kingdom that coordinates cell growth and morphogenesis in response to nutrient-derived signals.[22] In nutrient rich environments, Tor1 activates transcription of genes involved in ribosome biogenesis and glycolysis and represses starvation responses, such as NCR (nitrogen catabolite repressed) genes and GCN responses (Fig. 2).[22] Bastidas et al showed that rapamycin, a specific inhibitor of Tor1 could reduce *NRG1* expression in Spider medium and induce extensive aggregation of *C. albicans* in an adhesin-dependent fashion.[21] As Tor1 kinase is activated by nutrients, particularly amino acids, we hypothesised that nutrient activated Tor1 could be involved in repression of filamentation in *C. dubliniensis*. In order to investigate this, we determined whether inhibition of Tor1 by rapamycin could enhance filamentation in *C. dubliniensis*. Addition of 20 nM rapamycin to YPDS at 37˚C significantly enhanced filamentation in *C. dubliniensis* Wü284 at 3 to 5 hours post inoculation compared to solvent (DMSO) treated cultures (2-way ANOVA, $p < 0.001$; Fig. 3a). The effect of rapamycin was greatest at 3 h post inoculation, with the production of filaments by 40 % (± 6.7 standard deviation) of cells compared to 9.7 % (± 1.9) of those incubated with DMSO alone (Fig. 3a). Rapamycin had no significant effect on *C. albicans* isolates in YPDS, however these isolates produced abundant filaments in the absence of rapamycin (data not shown). In total, 12 *C. dubliniensis* isolates examined exhibited an increase in filamentation in response to rapamycin (data not shown). The addition of rapamycin (20 nM) also reversed the inhibitory effects of peptone and glucose on filamentation in *C. dubliniensis* incubated in 10% serum (data not shown). These data

177 indicate that Tor1 may play a role as a sensor of nutrient rich conditions that acts to
178 inhibit filamentation in *C. dubliniensis*. We investigated whether rapamycin could
179 mediate similar effects on *NRG1* or *UME6* transcription in *C. dubliniensis*, thereby
180 promoting filamentation in YPDS. Exposure of *C. dubliniensis* to rapamycin in YPDS
181 prevented induction of *NRG1* transcription normally observed in YPDS and in addition,
182 we observed a ~2-fold increase in *UME6* expression (Fig. 3D). The effect of reducing
183 *NRG1* transcription and boosting *UME6* expression may provide a mechanism whereby
184 some cells in the population can undergo the yeast to hypha switch.

185 These preliminary data suggest that Tor1 kinase is at least partly responsible for
186 nutrient repression of filamentation in *C. dubliniensis*. As the Tor1 kinase pathway is
187 highly conserved in fungi, it is difficult to speculate why Tor1 activation does not also
188 repress filamentation in *C. albicans*. It appears that *C. albicans* forms filaments in media
189 containing serum, irrespective of nutrient availability. The addition of serum to growth
190 medium renders the pH of that medium alkaline, suggesting that perhaps *C. albicans* can
191 repress Tor1 functions at alkaline pH. The mechanism for this could involve a recently
192 described pH response pathway in *C. albicans* that acts in parallel to the traditional
193 Rim101 pH response pathway pathway.[23] Davis *et al*. first identified *MDS3* in *C.*
194 *albicans* as a novel pH response regulator with roles in morphogenesis, biofilm formation
195 and chlamydospore formation.[24] The *S. cerevisiae* orthologue of *MDS3* has roles in
196 induction of sporulation at alkaline pH.[25] Subsequently, Zacchi et al showed that a *C.*
197 *albicans mds3*Δ mutant, like *C. dubliniensis*, displays greatly reduced filamentation in
198 alkaline liquid media (M199).[23] In addition, deletion of Mds3 results in a Tor
199 'hyperactive' transcriptional response, with decreased expression of starvation response

genes such as *GAP2* and the NCR response. Treatment of the *mds3*Δ mutant with the Tor1 inhibitor rapamycin could intiate starvation responses and restore filamentation in ~45% of the *mds3*Δ cells, similar to the level of restoration induced by rapamycin in *C. dubliniensis*. These data show that active Tor1 can inhibit filamentation and that Mds3 is required to reverse this inhibition at alkaline pH. Zacchi et al. have also shown that Mds3 modulates Tor1 activities via interaction with a phosphatase, Sit4, positioned downstream of Tor1 in the TOR pathway (Fig. 2).[23] In *C. albicans* Sit4 has been shown to be required for filament extension and stress responses in addition to starvation responses. Under nutrient rich conditions, Tor1 represses starvation responses by inactivating Sit4 (Fig. 2). Zaachi et al. have shown that *mds3*Δ and *sit4*Δ mutants are phenotypically similar and exhibit similar transcriptional changes indicative of Tor1 hyperactivity. In addition, Mds3 was shown to physically interact with Sit4, thereby providing the first evidence of a link between the pH response and Tor1 activity in *C. albicans.*[23] As described, deletion of *MDS3* prevented filamentation in alkaline M199 and prevented activation of Sit4 mediated starvation responses. Therefore, in alkaline hypha-inducing media, Mds3 may activate Sit4 to promote filamentation and this activation may occur irrespective of nutrient availability and Tor1 repression (Fig. 2). Zacchi et al. speculate that due to the presence of several Kelch repeats which are involved in protein-protein interactions, Mds3 may act as a scaffold to facilitate interactions between TOR pathway members.[23] It is not clear how Sit4 may activate filamentous growth, however it could involve interaction with the GCN response regulator Gcn2. In *S. cerevisiae*, amino acid starvation results in activation of Sit4 which activates the GCN regulator Gcn2, leading to enhanced translation of GCN4.[26] As described previously, *C. albicans GCN4* is also involved in

223 activating filamentous growth. In *C. albicans*, Gcn2 has also been shown to increase
224 *GCN4* transcript levels, providing a possible mechanism for Sit4 regulation of
225 filamentous growth (Fig 2).[27]

226 Due to the inability of *C. dubliniensis* to filament at alkaline pH in nutrient rich
227 conditions and the ability to rescue this phenotype with rapamycin (similar to *mds3*Δ) it is
228 our hypothesis that signaling of alkaline pH signals *via* the Mds3/Sit4 complex is
229 somehow defective. Alternatively, Tor1 may simply have greater kinase activity or
230 perhaps a lower activation threshold in *C. dubliniensis* relative to *C. albicans*, which acts
231 to repress downstream activities like filamentation. Interestingly, defective Sit4 in *C.*
232 *albicans* is also associated with increased sensitivity to NaCl and high temperatures, both
233 of which are characteristic traits of *C. dubliniensis*. Inspection of the orthologous *SIT4*
234 and *MDS3* genes in *C. dubliniensis* indicates high levels of homology in the encoded
235 proteins. Experiments are planned to determine whether *C. dubliniensis* Sit4 and Mds3
236 are functionally equivalent to their *C. albicans* orthologues. However, this phenotype
237 may be due to loss or or mutation of another protein in the Mds3-Sit4 complex. Several
238 genes encoding putative filamentous growth regulators (FGRs) are predicted to be
239 pseudogenes in *C. dubliniensis* and may account for this phenotypic disparity.[11]

240 It is difficult to speculate at this stage why these species have evolved such
241 different responses to nutrients and pH. Certainly, the ability of *C. albicans* to filament at
242 alkaline pH, irrespective of nutrients may allow this organism to colonise a wider range
243 of niches relative to *C. dubliniensis*. The reduced virulence of *C. dubliniensis* suggests
244 that nutrient concentrations in its *in vivo* niche may be sufficient to supress flamentous
245 growth in this organism, which may account for the attenuated virulence of *C.*

246 *dubliniensis* in the murine infection model. Although this morphogenetic defect may
247 result in a reduced capacity to cause disease, loss of filamentous growth under some
248 conditions could be part of a specialization process that may be advantageous in specific
249 niches. For example, filamentation and cellular invasion are likely to trigger
250 inflammatory responses and attract immune cells, and perhaps *C. dubliniensis* has
251 evolved to be predominantly non-filamentous *in vivo* to avoid triggering these responses.
252 Although this may have restricted the range of niches available to *C. dubliniensis*, it may
253 have allowed successful colonsation of new niche(s) that are under close surveillance by
254 the immune system.

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## Figure Legends

**Figure 1.** Flow diagram outlining the environmental signals and cognate transcription factors involved in induction of filamentous growth in *C. albicans* and *C. dubliniensis*. In *C. albicans*, growth in serum containing media induces a response initiated by the transcriptional regulators Efg1 and Rim101. Rim101 is activated by alkaline pH. Efg1 is the terminal transcription factor in a cAMP-Protein kinase A (cAMP-PKA) signalling pathway that is activated by growth at 37˚C, certain amino acids and $CO_2$. Production of long filaments requires the activation of Ume6. Transcription of *UME6* is probably activated by Efg1 (dashed line) and is negatively regulated by Nrg1. These elements are highly conserved in the *C. dubliniensis* genome. In *C. dubliniensis*, activation of Ume6 and/or Efg1 may be repressed by nutrient rich conditions, although the mechanism is unknown.

**Figure 2**. Speculative model outlining the role of the kinase Tor1 and its interactions with Mds3, based on our interpretation of the data of Zachi et al.[23] Tor1 is stimulated by nutrients, in particular amino acids and peptides. Rapamycin can inhibit nutrient stimulation of Tor1 via interaction with rapamycin binding protein Rbp1 (not shown). Down stream affects of nutrient activated Tor1 are the activation expression of genes involved in glycolysis and ribosome biogenesis via activation of the kinase Sch9 (not shown). In a paralell pathway, Tor1 inactivates the PP2A-like phosphatase Sit4, which has role in activating starvation responses and filamentation. Mds3, a protein required for filamentation at alkaline pH physically interacts with Sit4 and may promote Sit4 activity at alkaline pH, even when Tor1 is active. Our hypothesis at present is that this pH dependent Sit4 activation is *C. albicans*-specific.

**Figure 3.** (A) Effect of rapamycin on filamentation in *C. dubliniensis* in nutrient rich YPD supplemented with 10% bovine serum (YPDS). Cells of *C. dubliniensis* Wü284 were grown overnight in YPD broth at 30˚C and inoculated in YPDS at 37˚C (2 x $10^6$ cels/ml) in the presence of rapamycin in DMSO (20 nM) or DMSO alone. The percentage of cells forming unconstricted germ-tubes was assessed at the indicated time-points. Experiments were performed on four occassions. (B) and (C) Photomicrographs of *C. dubliniensis* Wü284 following 2 h incubation in YPDS plus (B) DMSO or (C) 20 nM rapamycin. (D) Expression of *UME6* and *NRG1* in *C. dubliniensis* YPDS cultures. Cells from overnight YPD cultures (30˚C) were inoculated in YPDS 37˚C with or without rapamycin (20 nM). RNA was extracted and QRT-PCR was carried out with Sybr Green (Applied Biosystems) as described by O'Connor et al. *UME6* and *NRG1* expression levels were normalised to *TEF1* to control for variations in template concentration. The graph shows the expression levels of normalised *UME6* and *NRG1* in rapamycin treated cultures relative to untreated cultures. The results are the average of three biological replicate experiments, with error bars showing standard deviations.

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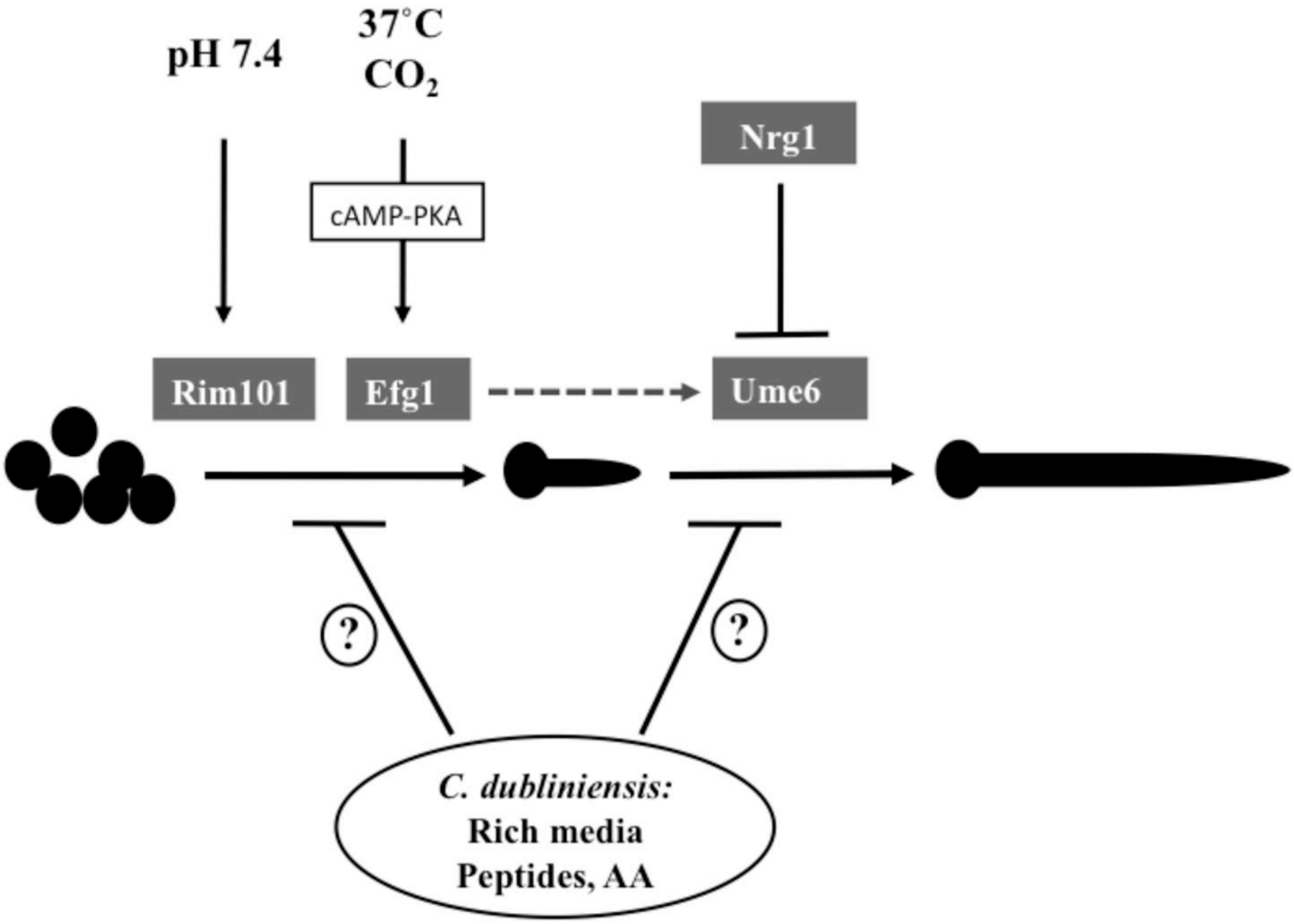

pH 7.4
37˚C
$CO_2$
Nrg1
cAMP-PKA
Rim101
Efg1
Ume6
?
?
*C. dubliniensis:*
Rich media
Peptides, AA

Fig. 2

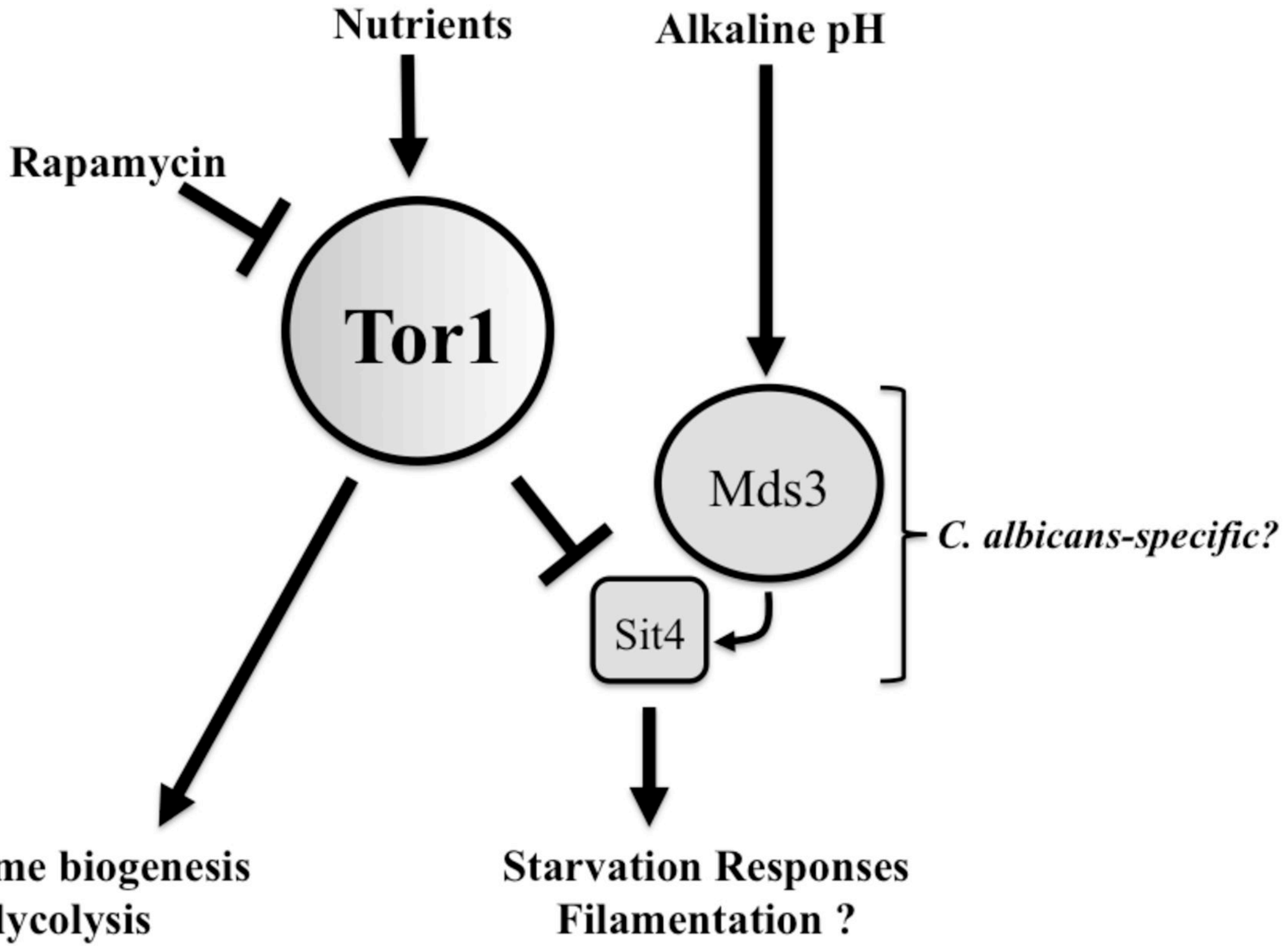
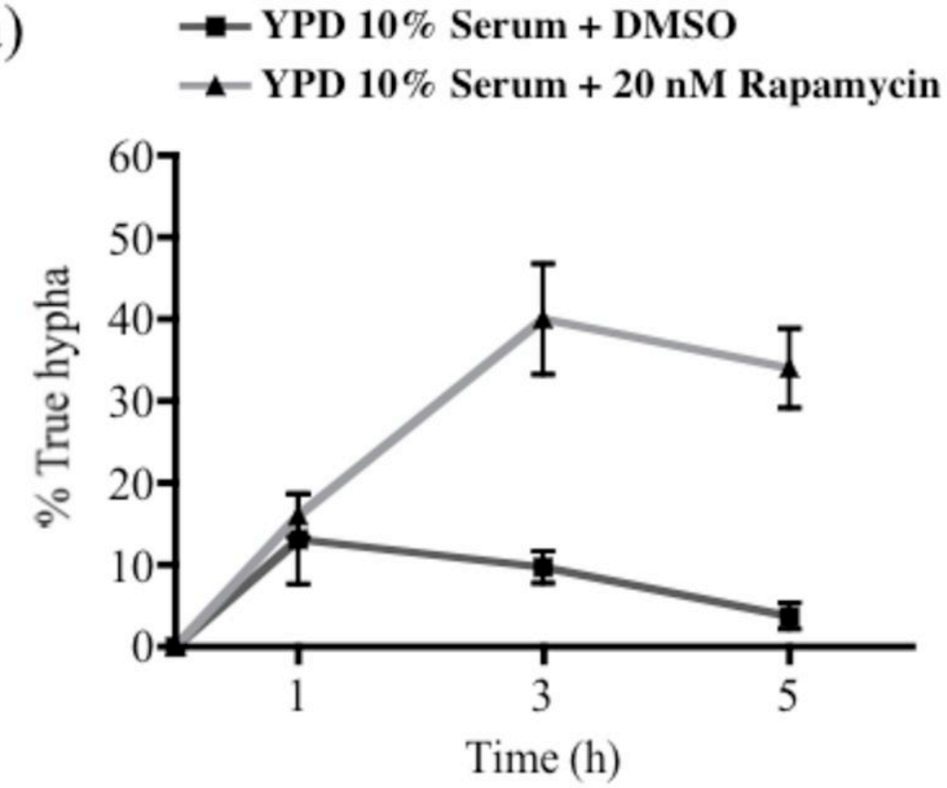
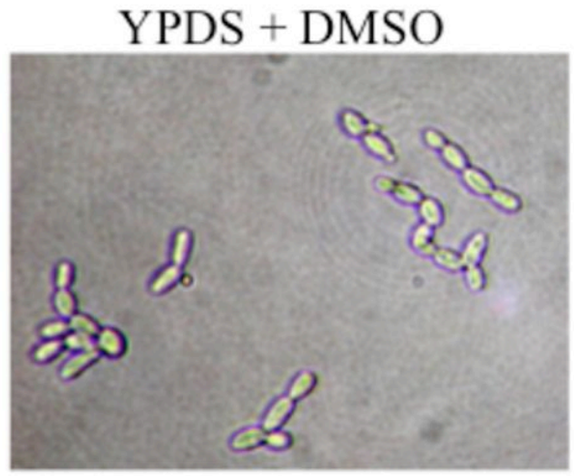

Nutrients
Alkaline pH
Rapamycin
Tor1
Mds3
C. albicans-specific?
Sit4
Ribosome biogenesis
Glycolysis
Starvation Responses
Filamentation ?

Fig. 3

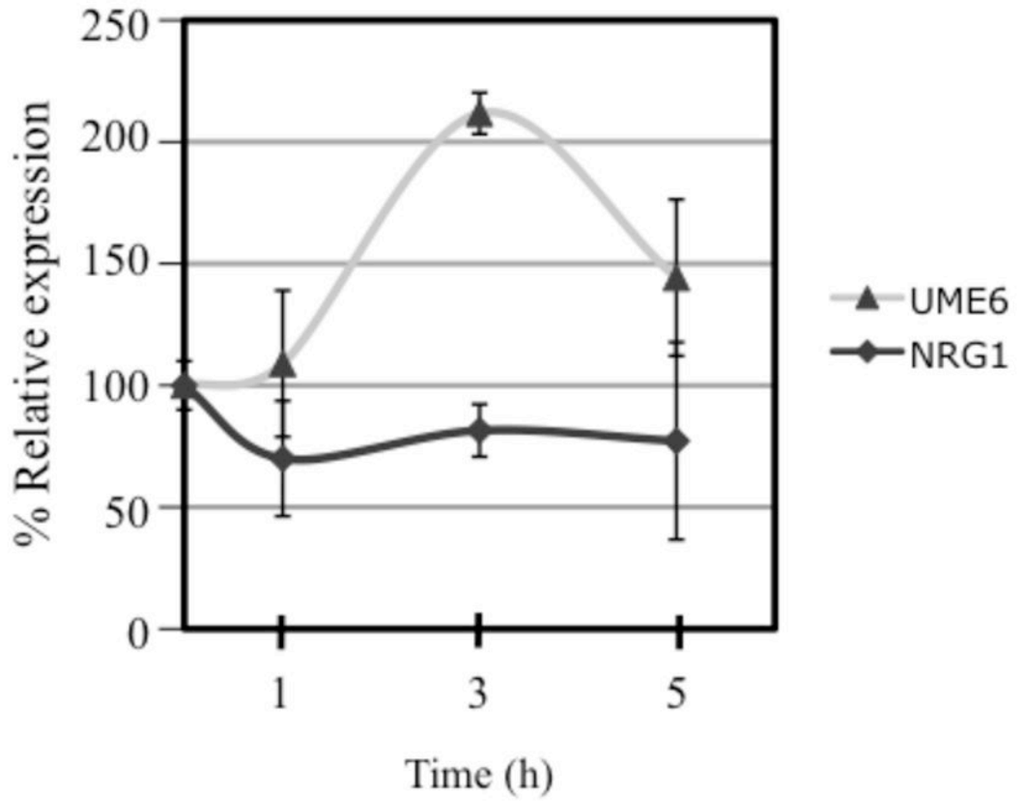
(a)




(b)




(d) Expression in Rapamycin treated vs untreated




(c) YPDS + 20 nM Rapamycin