

RESEARCH PAPER



Protein kinase A governs growth and virulence in *Candida tropicalis*

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ABSTRACT

Candida tropicalis is one of the most important human fungal pathogens causing superficial infections in locations such as the oral mucosa and genital tract, as well as systemic infections with high mortality. In its sister species *Candida albicans*, the cyclic AMP/protein kinase A (cAMP/PKA) pathway regulates fungal adhesion and dimorphism, both of which correlate closely with virulence. CaTpk1 and CaTpk2, the catalytic subunits of PKA, not only share redundant functions in hyphal growth, adhesion, and biofilm formation, but also have distinct roles in stress responses and pathogenesis, respectively. However, studies on PKA in the emerging fungal pathogen *C. tropicalis* are limited. Our results suggest that Tpk1 is involved in cell wall integrity and drug tolerance. The *tpk2/tpk2* mutants, which have no protein kinase A activity, have reduced hyphal growth and adhesion. In addition, the *tpk1/tpk1 tpk2/tpk2* double deletion mutant demonstrated delayed growth and impaired hyphal formation. In a murine model of systemic infection, both *TPK1* and *TPK2* were required for full virulence. We further found that *EFG1* and *HWP1* expression is regulated by PKA, while *BCR1*, *FLO8*, *GAL4*, and *RIM101* are upregulated in the *tpk1/tpk1 tpk2/tpk2* mutant. This study demonstrates that Tpk1 is involved in drug tolerance and cell wall integrity, while Tpk2 serves as a key regulator in dimorphism and adhesion. Both Tpk1 and Tpk2 are required for growth and full virulence in *C. tropicalis*.

ARTICLE HISTORY

Received 5 May 2017
Revised 29 November 2017
Accepted 1 December 2017

KEYWORDS

adhesion; *C. tropicalis*; hyphal growth; PKA; virulence

Introduction

Superficial and invasive fungal infections in humans are an emerging problem worldwide. Although the incidence of superficial infections is much higher than invasive infections, invasive infections are studied more frequently due to higher mortality rates [1]. Of the human pathogenic fungi, the genus *Candida* is the first or second most frequently isolated group from patients with invasive fungal infections [1]. *C. albicans* is the most prevalent causative agent of all forms of candidiasis, but more than 40% of *Candida* infections are caused by non-*albicans* *Candida* species (NACs) such as *C. tropicalis*, *C. glabrata*, and *C. parapsilosis* [2, 3]. Previous studies indicated that *C. tropicalis*, an evolutionary counterpart of *C. albicans*, is the most frequently isolated NAC from patients with candidiasis [2, 4]. Furthermore, the mortality rate of infections caused by *C. tropicalis* is higher than that of infections caused by other NACs or *C. albicans* in Brazil [5]. Recently, drug-tolerant or -resistant *C. tropicalis* isolates have been isolated from patients and environmental samples worldwide [6–11]. However, the molecular mechanisms of drug

tolerance and pathogenesis in *C. tropicalis* remain elusive.

Among the human fungal pathogens causing invasive infections, *Histoplasma capsulatum* as well as dimorphic fungi in general, undergo transitions between filamentous and yeast growth [12]. The morphological transition enables pathogens to adapt to the harsh environmental conditions within the host [12, 13]. In *C. albicans* and *C. tropicalis*, dimorphism is involved in the regulation of adhesion, biofilm formation, invasion, and virulence [12, 14–16]. Within the host, various environmental cues, such as nutrients (carbon, ammonium, or amino acids), N-acetylglucosamine (GlcNAc), pH, temperature, serum, and reactive oxygen species, trigger the morphological change between *C. albicans* yeast and hyphal forms via various signaling pathways, including the cyclic adenosine monophosphate/protein kinase A (cAMP/PKA), calcineurin, HOG, and MAPK pathways [12, 13].

The cAMP/PKA pathway receives much attention due to its crucial roles in morphological development and pathogenesis, not only in human fungal pathogens such as *C. albicans*, *Cryptococcus neoformans*, and *Aspergillus*

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fumigatus, but also in plant pathogenic fungi such as *Magnaporthe oryzae* and *Ustilago maydis* [17–21]. Upon receiving environmental cues, activated adenylyl cyclase converts ATP to cAMP. Being a secondary messenger, cAMP binds to the regulatory subunits of PKA and causes a conformational change that releases the PKA catalytic subunits. These then activate downstream transcription factors such as Efg1 and Flo8 in *C. albicans* and *Saccharomyces cerevisiae* via phosphorylation [18, 20, 22].

In the human fungal pathogen *C. albicans*, the cAMP/PKA pathway is activated by various environmental cues, such as serum and GlcNAc, and controls the morphological transition between the yeast and hyphal forms, which is important for pathogenesis [13, 15, 23]. In *C. albicans*, the cAMP/PKA pathway comprises adenylyl cyclase (CYR1/CDC35), two PKA catalytic subunits (TPK1 and TPK2), and a PKA regulatory subunit (BCY1) [18, 23]. Ras1 and Cyr1 have been shown to play important roles in sensing environmental cues, regulating cAMP levels, and governing the expression of Efg1, which is one of the major regulators of filamentation [24]. CaTpk1 and CaTpk2 have redundant roles in growth and dimorphic switching, but each has separate roles in filamentous growth in various filamentation-inducing conditions [25–29]. CaTpk1 contributes to filamentation on solid medium, while CaTpk2 is involved in hyphal growth in liquid medium. Interestingly, only CaTpk2 is required for virulence in murine models of systemic infection [29, 30]. In *C. albicans*, PKA activates downstream targets, for example Efg1, via phosphorylation, leading to a transition from yeast to pseudohyphal or hyphal growth, a key virulence factor [14]. Morphological switching also plays an important role in biofilm formation [30], which contributes to antifungal drug tolerance [31, 32]. As an evolutionary relative of *C. albicans*, *C. tropicalis* was reported to produce pseudohyphae in addition to true hyphae in culture media [2, 4, 16], suggesting that the cAMP/PKA pathway may also be involved in the morphological switching and pathogenesis of *C. tropicalis*. *C. tropicalis* PKA comprises two catalytic subunits (Tpk1 and Tpk2) and a regulatory subunit (Bcy1). Recently, Zhang *et al.* showed that loss of either TPK1 or TPK2 results in normal glucose-induced filamentation in *C. tropicalis*, indicating that the two catalytic isoforms have a redundant function in morphological transition [33]. However, the impact of PKA signaling pathway-regulated dimorphic switching on growth, drug tolerance, biofilm formation, and virulence remain unclear.

In the present study, we demonstrate that Tpk1 and Tpk2 not only have separate functions in hyphal growth, but also play distinct roles in stress tolerance. Tpk1 is required for cell wall integrity and drug tolerance, while

Tpk2 is involved in flocculation and biofilm formation. Furthermore, the *tpk1/tpk1 tpk2/tpk2* mutant exhibits severe defects in growth and morphological transition compared to the wild type and *tpk1/tpk1* or *tpk2/tpk2* mutants. Moreover, Efg1, Brg1, Hwp1, and Als3 might be involved in biofilm formation. Tpk1 and Tpk2 are redundant in pathogenesis, while the *tpk1/tpk1 tpk2/tpk2* mutant is avirulent in a murine model of systemic infection.

Results

Identification of Tpk1 and Tpk2 in *C. tropicalis*

To identify the genes encoding the PKA subunits in *C. tropicalis*, the amino acid sequences of *C. albicans* PKA catalytic Tpk1 and Tpk2 and *S. cerevisiae* Tpk1-3 were used for reciprocal BLAST searches. Each search resulted in the identification of the *C. tropicalis* TPK1 (CTRG_03315, 405 amino acids) and TPK2 (CTRG_01736, 425 amino acids) genes [34]. Sequence alignments showed that *C. tropicalis* Tpk1 shares 92% and 68% identity with the corresponding proteins in *C. albicans* and *S. cerevisiae*, respectively. Similarly, *C. tropicalis* Tpk2 has 89% and 78% identity over the full-length proteins with CaTpk2 and ScTpk2, respectively (Fig. 1A, above the diagonal line). Further analysis of the conserved PKA domain (accession: cd05580) revealed that the PKA domains of *C. tropicalis* Tpk1 and Tpk2 each share 99% homology with the corresponding proteins in *C. albicans* (Fig. 1A, below the diagonal line). A phylogenetic tree derived from the full-length proteins suggested that *Candida* Tpk2 proteins are more closely related to ScTpk2 than the Tpk1 proteins of *C. albicans* and *C. tropicalis*; however, the *Candida* PKA catalytic isoforms are more distantly related to ScTpk1 and ScTpk3 (Fig. 1B). These relationships suggest that the *C. tropicalis* catalytic isoforms (Tpk1 and Tpk2) might have similar functions in filamentation as the corresponding proteins in *C. albicans* and Tpk2 of *S. cerevisiae*.

Tpk1 and Tpk2 have redundant functions in cell growth

The cAMP/PKA signaling pathway regulates a variety of cellular functions, and *C. albicans* PKA catalytic isoforms contribute to growth fitness [28]. To investigate whether *C. tropicalis* Tpk1 and Tpk2 are involved in growth fitness, we disrupted the TPK1 and TPK2 genes in *C. tropicalis* MYA3404 [34], resulting in two independent *tpk1/tpk1* (YC710 and YC757) and two independent *tpk2/tpk2* (YC681 and YC711) mutants. Both the *tpk1/tpk1* and *tpk2/tpk2* mutants grew as fast as wild type at 30°C

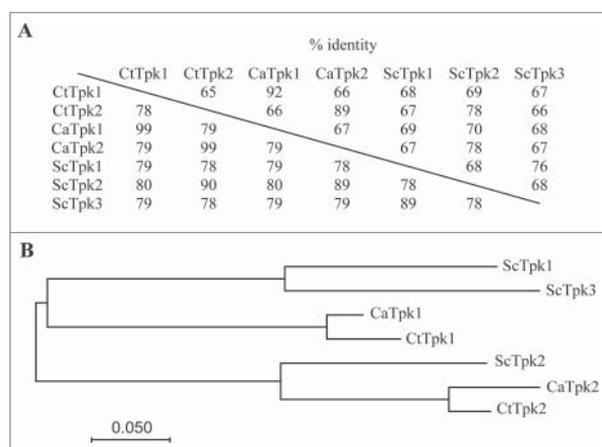


Figure 1. Identification of *C. tropicalis* PKA catalytic subunits (Tpk1 and Tpk2). (A) The amino acid identity of Tpk proteins from *C. albicans* (Ca), *C. tropicalis* (Ct), and *S. cerevisiae* (Sc). Numbers above the diagonal line indicate sequence identity between the full-length proteins, while numbers below represent the identity of PKA domain (accession:cd05580). (B) Phylogenetic tree of the PKA catalytic subunits of *C. albicans*, *C. tropicalis*, and *S. cerevisiae*. The amino acid identity and the multiple alignment were analyzed and constructed using the Clustal Omega program (<http://www.ebi.ac.uk/Tools/msa/clustalo/>). The phylogenetic tree was constructed in MEGA7 [68] software using Maximum Likelihood method based on the JTT matrix-based model [69]. The black bar indicates an evolutionary distance of 0.05 substitutions per site. Proteins and GenBank accession numbers: CaTpk1, XP_723574.1; CaTpk2, XP_714866.2; CtTpk1, XP_002549018.1; CtTpk2, XP_002547429.1; ScTpk1, NP_012371.2; ScTpk2, NP_015121.1; ScTpk3, NP_012755.1.

in liquid YPD medium (Fig. 2A). Furthermore, these mutants showed similar growth that was indistinguishable from wild type on solid YPD medium at various temperatures (Fig. 2B). These data suggest that neither Tpk1 nor Tpk2 alone are required for growth fitness.

Previous studies indicated that the PKA catalytic subunits are essential in yeast growth, as loss of all catalytic isoforms in *S. cerevisiae* or *C. albicans* result in loss of viability [28, 35–37]. However, PKA catalytic subunit-null mutants in *C. neoformans* and *A. fumigatus* are viable [38, 39]. To determine whether disruption of both *TPK1* and *TPK2* results in a growth defect or lethality in *C. tropicalis*, we deleted the *TPK2* gene in the *tpk1/tpk1* mutant (YC914) background (Table 1). In contrast to previous attempts by other laboratories [33], we obtained the *tpk1/tpk1 tpk2/tpk2* double deletion mutant (YEN1) and found that this mutant exhibits severely impaired growth compared to the wild type or *tpk1/tpk1* or *tpk2/tpk2* mutants at 30°C (Fig. 2A). In addition, the loss of both PKA catalytic isoforms abolished fungal growth at 25°C and 42°C and drastically reduced growth at 30°C and 37°C (Fig. 2B). The growth

defect of the *tpk1/tpk1 tpk2/tpk2* mutant was partially rescued by reintroducing either the *TPK1* or the *TPK2* gene (Figure S1). These results suggest that Tpk1 and Tpk2 have redundant function in fungal growth and temperature sensing.

In *C. albicans*, Tpk2 contributes more to PKA activity than the Tpk1 isoform [27, 40]. To investigate whether differential PKA activity is conserved in *C. tropicalis*, we extracted total protein from the wild type and mutants to determine PKA activity. PKA activity of the *tpk1/tpk1* mutants was slightly reduced compared to the wild type (Figure S2), and levels of PKA activity of *tpk2/tpk2* and *tpk1/tpk1 tpk2/tpk2* mutants were strongly reduced, indicating that Tpk2 contributes more to PKA activity than Tpk1 in *C. tropicalis* (Fig. 2C).

Tpk1 plays a key role in stress responses

To determine the roles of the *C. tropicalis* PKA subunits in the stress response, we first screened the *tpk1/tpk1* and *tpk2/tpk2* mutants on solid media containing various stressors. Loss of the *TPK1* gene resulted in impaired growth when exposed to antifungal drugs (micafungin, anidulafungin, or posaconazole) or cell wall-perturbing agents (sodium dodecyl sulfate [SDS] or dithiothreitol [DTT]) (Fig. 3). The *TPK1* complemented strain showed tolerance to SDS and antifungal drugs (Figure S3). In contrast, the *tpk2/tpk2* mutants showed similar growth to the wild type under various stresses except when exposed to 0.85 M MgCl₂, and growth of the *tpk1/tpk1 tpk2/tpk2* mutant was completely abolished (Fig. 3). Our results suggest that Tpk1 plays a major role in response to stresses in *C. tropicalis*, while Tpk2 might be involved in magnesium homeostasis.

PKA catalytic subunits play different roles in morphogenesis and adhesion

Tpk2 is involved in the regulation of cell-cell and cell-surface adhesion in *S. cerevisiae* [22] and loss of the *TPK2* gene in *C. albicans* results in defects in hyphal growth, adherence, and biofilm formation [27]. To determine the roles of *C. tropicalis* Tpk1 and Tpk2 in morphogenesis and adhesion, we performed a set of hyphal growth and adhesive assays. Hyphal growth was reduced in *tpk2/tpk2* mutants, but not *tpk1/tpk1* mutants, when grown in synthetic complete (SC) liquid medium with or without 10 mM GlcNAc or 10 mM 3AT (Fig. 4A). Interestingly, cell clumps and reduced hyphal growth were observed in the *tpk1/tpk1 tpk2/tpk2* mutant (Fig. 4A). On nitrogen source starvation (SLAD) solid medium, loss of *TPK2* resulted in delayed hyphal growth compared with the wild type and *tpk1/tpk1* mutants (Fig. 4B, upper panel). In

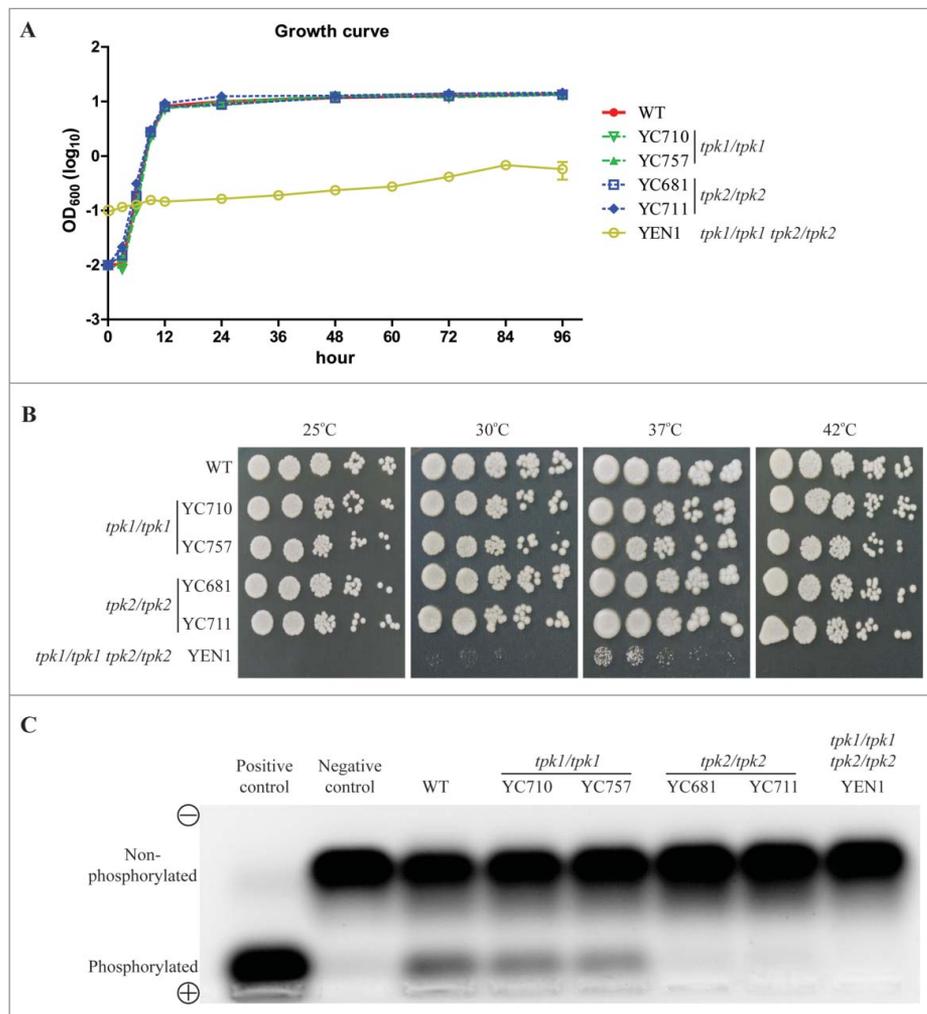


Figure 2. Protein kinase A is required for growth. (A) Growth curves of *C. tropicalis* wild type and mutants. Cells were grown overnight in YPD at 30°C, washed twice with dH₂O, diluted to 0.01 OD₆₀₀, except for the *tpk1/tpk1 tpk2/tpk2* mutant (YEN1), which was diluted to 0.1 OD₆₀₀ with fresh YPD medium, and incubated at 30°C and 200 rpm for four days. The OD₆₀₀ of strains was measured via microplate spectrophotometer at the indicated time. The experiments were performed in triplicate, and the values represent the mean ± the standard error of the mean. (B) Spot assay of the indicated strains at different temperatures. Cells were grown overnight in YPD at 30°C (except the *tpk1/tpk1 tpk2/tpk2* mutant, which was grown for two days), washed twice with dH₂O, and diluted to 0.2 OD₆₀₀ as the starting concentration. Cells were then five-fold serially diluted, spotted onto YPD medium, and incubated at 25°C, 30°C, 37°C, or 42°C for 48 h and photographed. (C) Protein kinase A activity of the indicated strains. Cells were grown overnight in YPD at 30°C (except the *tpk1/tpk1 tpk2/tpk2* mutant, which was grown for two days) and washed twice with dH₂O. Crude protein extracts of the samples were isolated. PKA activity assays were carried out using the PepTag assay for non-radioactive detection of cAMP-dependent protein kinase kit.

contrast, the *tpk2/tpk2* mutants showed hyper-filamentation on the carbon source starvation medium (Spider) compared with the wild type and *tpk1/tpk1* mutants (Fig. 4B, lower panel). As in the liquid medium, the *tpk1/tpk1 tpk2/tpk2* mutant exhibited no filamentation on either solid SLAD or Spider media (Fig. 4B).

In *S. cerevisiae* and *C. albicans*, hyphal growth is correlated with cell-cell and cell-surface adherence [27, 41]. We showed that in *C. tropicalis*, Tpk1 and Tpk2 play specific roles in filamentation under different conditions (Fig. 4). Therefore, we hypothesized that in *C. tropicalis* these two proteins may play

distinct roles in flocculation, adherence, and biofilm formation, which all require cell-cell or cell-surface adhesion. The *tpk2/tpk2* mutants had flocculation defects compared to the wild type and *tpk1/tpk1* mutants (Fig. 5A). Interestingly, the *tpk1/tpk1 tpk2/tpk2* mutant also exhibited sedimentation at the bottom of the glass tube, indicating that the *tpk1/tpk1* mutation is epistatic to the *tpk2/tpk2* mutation regarding the flocculation phenotype (Fig. 5A). In contrast to cell-cell adherence, loss of either PKA subunit has no effect on yeast adherence to plastic surfaces (Figure S4). However, the *tpk2/tpk2* mutants

Table 1. *C. tropicalis* strains used in this study.

Strain	Genotype	Background	Reference
MYA3404	Prototrophic wild type	Clinical isolate	[34]
YC680	<i>tpk1</i> Δ::SAT1-FLP/TPK1	MYA3404	This study
YC683	<i>tpk1</i> Δ::FRT/TPK1	YC680	This study
YC710 ^a	<i>tpk1</i> Δ::FRT/ <i>tpk1</i> Δ::SAT1-FLP	YC683	This study
YC914	<i>tpk1</i> Δ::FRT/ <i>tpk1</i> Δ::FRT	YC710	This study
YC708	<i>tpk1</i> Δ::SAT1-FLP/TPK1	MYA3404	This study
YC718	<i>tpk1</i> Δ::FRT/TPK1	YC708	This study
YC757 ^a	<i>tpk1</i> Δ::FRT/ <i>tpk1</i> Δ::SAT1-FLP	YC718	This study
YEN10	<i>tpk1</i> Δ::FRT/ <i>tpk1</i> Δ::FRT	YC757	This study
YEN43	<i>tpk1</i> Δ::FRT/ <i>tpk1</i> Δ::TPK1-SAT1-FLP	YEN10	This study
YC650	<i>tpk2</i> Δ::SAT1-FLP/TPK2	MYA3404	This study
YC670	<i>tpk2</i> Δ::FRT/TPK2	YC650	This study
YC711 ^b	<i>tpk2</i> Δ::FRT/ <i>tpk2</i> Δ::SAT1-FLP	YC670	This study
YC653	<i>tpk2</i> Δ::SAT1-FLP/TPK2	MYA3404	This study
YC672	<i>tpk2</i> Δ::FRT/TPK2	YC653	This study
YC681 ^b	<i>tpk2</i> Δ::FRT/ <i>tpk2</i> Δ::SAT1-FLP	YC672	This study
YEN20	<i>tpk2</i> Δ::FRT/ <i>tpk2</i> Δ::FRT	YC681	This study
YEN26	<i>tpk2</i> Δ::FRT/ <i>tpk2</i> Δ::TPK2-SAT1-FLP	YEN20	This study
YC928	<i>tpk1</i> Δ::FRT/ <i>tpk1</i> Δ::FRT <i>tpk2</i> Δ::SAT1-FLP/TPK2	YC914	This study
YSJ13	<i>tpk1</i> Δ::FRT/ <i>tpk1</i> Δ::FRT <i>tpk2</i> Δ::FRT/TPK2	YC928	This study
YEN1	<i>tpk1</i> Δ::FRT/ <i>tpk1</i> Δ::FRT <i>tpk2</i> Δ::FRT/ <i>tpk2</i> Δ::SAT1-FLP	YSJ13	This study
YEN38	<i>tpk1</i> Δ::FRT/ <i>tpk1</i> Δ::FRT <i>tpk2</i> Δ::FRT/ <i>tpk2</i> Δ::FRT	YEN1	This study
YEN40	<i>tpk1</i> Δ::FRT/ <i>tpk1</i> Δ::TPK1-SAT1-FLP <i>tpk2</i> Δ::FRT/ <i>tpk2</i> Δ::FRT	YEN38	This study
YEN42	<i>tpk1</i> Δ::FRT/ <i>tpk1</i> Δ::FRT <i>tpk2</i> Δ::FRT/ <i>tpk2</i> Δ::TPK2-SAT1-FLP	YEN38	This study

^aTwo independent *tpk1*/*tpk1* mutants.

^bTwo independent *tpk2*/*tpk2* mutants.

showed reduced biofilm formation on a polystyrene 12-well plate compared to the wild type and *tpk1/tpk1* mutants, whereas no biofilm formation was observed for the *tpk1/tpk1 tpk2/tpk2* mutant (Fig. 5B). Furthermore, the hyphal growth and biofilm defects of the *tpk2/tpk2* mutant were rescued by reintroducing the *TPK2* gene (Figure S5), suggesting that Tpk2 is required for hyphal growth in liquid medium and biofilm formation.

Both Tpk1 and Tpk2 are required for full virulence

The adherence of fungal pathogens to host cells is a key step in the establishment of infection [42, 43]. In *Candida* species, adherence often leads to invasion and biofilm formation [15, 43], and the cAMP/PKA pathway regulates the expression of adherence-associated genes in yeast cells [12, 44]. Our data show that both Tpk1 and Tpk2 are required for hyphal growth, supported by our findings that loss of either *TPK1* or *TPK2* does not affect yeast adherence to plastic surfaces and that *tpk2/tpk2* mutants have a defect in biofilm formation (Fig. 5B and S4). Hence, we analyzed whether loss of hyphal growth or biofilm formation of the PKA null mutants is associated with attenuated virulence in *C. tropicalis* in a murine model of systemic infection. The *tpk1/tpk1* (YC710 and YC757) and *tpk2/tpk2* (YC681 and YC711) mutants exhibited similar virulence to wild type, while the *tpk1/tpk1 tpk2/tpk2* mutant was avirulent in this infection model ($P < 0.001$, log-rank test) (Fig. 6A).

To analyze the colonization ability of *C. tropicalis* PKA mutants, we performed fungal burden analyses in the murine kidneys and brains. The *tpk1/tpk1* or *tpk2/tpk2* mutants had fungal burdens similar to wild-type, whereas the *tpk1/tpk1 tpk2/tpk2* mutant exhibited a significantly decreased fungal burden in the kidneys ($P = 0.016$) and brains ($P < 0.001$) compared to the wild type (Fig. 6B).

In the histopathological examination, infection with wild type, *tpk1/tpk1* mutants, or *tpk2/tpk2* mutants resulted in hyphal growth in the periodic acid-Schiff (PAS)-stained kidney tissues (Fig. 7). However, fungal cells were barely observed in the PAS-stained kidney tissues from mice infected with the *tpk1/tpk1 tpk2/tpk2* mutant. Similarly, necrotic tissues observed with hematoxylin-eosin (H&E) staining were only observed in mice infected with the wild type, *tpk1/tpk1* mutant, or *tpk2/tpk2* mutant, but not in those infected with the *tpk1/tpk1 tpk2/tpk2* mutant (Fig. 7). Similarly, brain tissue necrosis was observed in mice infected with the wild type, *tpk1/tpk1* mutant, or *tpk2/tpk2* mutant, while few yeast cells and no obvious necrosis were observed in the brains of mice infected with the *tpk1/tpk1 tpk2/tpk2* mutant (Fig. 7).

PKA regulates the expression of virulence-related genes

In the present study, we showed that the *tpk2/tpk2* mutants exhibited increased filamentation on solid Spider media (Fig. 4B), which is a key feature for invasion

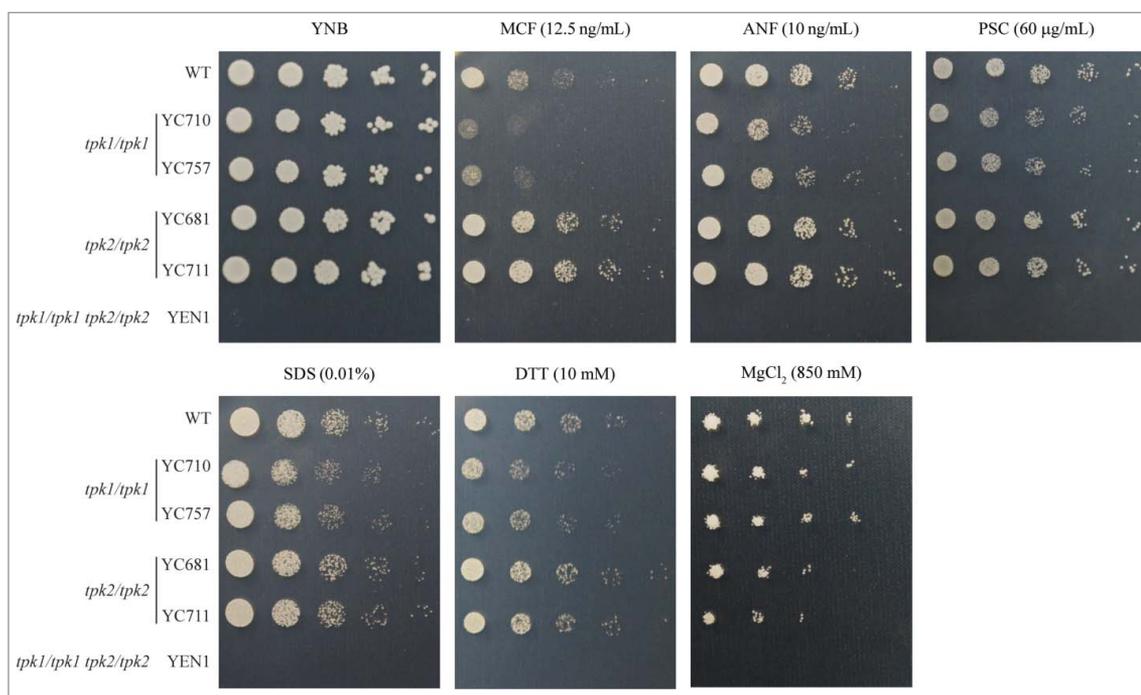


Figure 3. Tpk1 contributes to cell wall integrity and drug tolerance. Growth of the indicated strains exposed to cell wall-perturbing agents, antifungal drugs, and various stresses. Cells were grown overnight in YPD at 30°C (except *tpk1/tpk1 tpk2/tpk2* mutant, which was grown for two days), washed twice with dH₂O, and diluted to 0.2 OD₆₀₀ as the initial concentration. Samples were then five-fold serially diluted, spotted onto YNB medium containing the indicated chemicals, and incubated at 30°C for 48 h. MCF, micafungin; ANF, anidulafungin; PSC, posaconazole; SDS, sodium dodecyl sulfate; DTT, dithiothreitol.

and biofilm formation. To test whether hyperfilamentation of the *tpk2/tpk2* mutant is due to differential expression of key transcription factors, we performed qRT-PCR with the wild type and PKA null mutants with RNA isolated after incubation in Spider medium for 4 h. Expression of *EFG1* and *HWP1* was downregulated in *tpk1/tpk1*, *tpk2/tpk2*, and *tpk1/tpk1 tpk2/tpk2* mutants compared to the wild type (Fig. 8). In addition, loss of *TPK2* resulted in reduced expression of the major adhesin *ALS3* and transcription factor *BRG1* compared with the wild type and *tpk1/tpk1* mutants. Interestingly, the *tpk1/tpk1 tpk2/tpk2* mutant showed upregulation of four transcription factors (*BCR1*, *FLO8*, *GAL4*, and *RIM101*) in *C. tropicalis* (Fig. 8), all of which have demonstrated roles in adherence or biofilm formation in *C. albicans* [45–48].

Discussion

Roles of PKA in growth and stress responses of *C. tropicalis*

In the model organism *S. cerevisiae*, the cAMP/PKA signaling pathway is involved in the regulation of cell growth and cellular differentiation upon sensing nutrients and stressors from the environment [44, 49–51]. The

PKA catalytic subunits (Tpk1–3) of *S. cerevisiae* have overlapping functions in fungal growth, but a triple deletion mutant was not viable [35] with the exception of a temperature-sensitive *tpk2* mutant in the *tpk1 tpk3* mutant background [52]. In *C. albicans*, loss of either catalytic subunit (Tpk1 or Tpk2) resulted in growth similar to the wild type and, as in *S. cerevisiae*, the *tpk1/tpk1 tpk2/tpk2* mutant was not viable [28]. However, the conditional *tpk1/tpk1 tpk2/tpk2* mutant was viable but had severe growth defects [28]. Recently, Zhang *et al.* showed that *C. tropicalis* Tpk1 and Tpk2 had redundant functions in growth and, as previously reported, that the PKA catalytic null mutant could not grow. Here, our results showed that the *tpk1/tpk1 tpk2/tpk2* mutant was viable despite severe growth defects (Fig. 1), which is in contrast to the *C. albicans tpk1/tpk1 tpk2/tpk2* mutant, but similar to the corresponding *tpk1/PCK1p::TPK1 tpk2/tpk2* conditional mutant in *C. albicans* [28]. Our results are in line with previous studies in other fungi, such as *C. neoformans* or *A. fumigatus*, showing that PKA is not essential [38, 39]. Ding *et al.* identified a *C. albicans bcy1/bcy1* mutant, which had previously been attempted and unsuccessful [53], that is viable, and Bcy1 was found to play a role in filamentation [54]. Taken together, our findings suggest that PKA is not essential, at least in our tested conditions, in *C. tropicalis*.

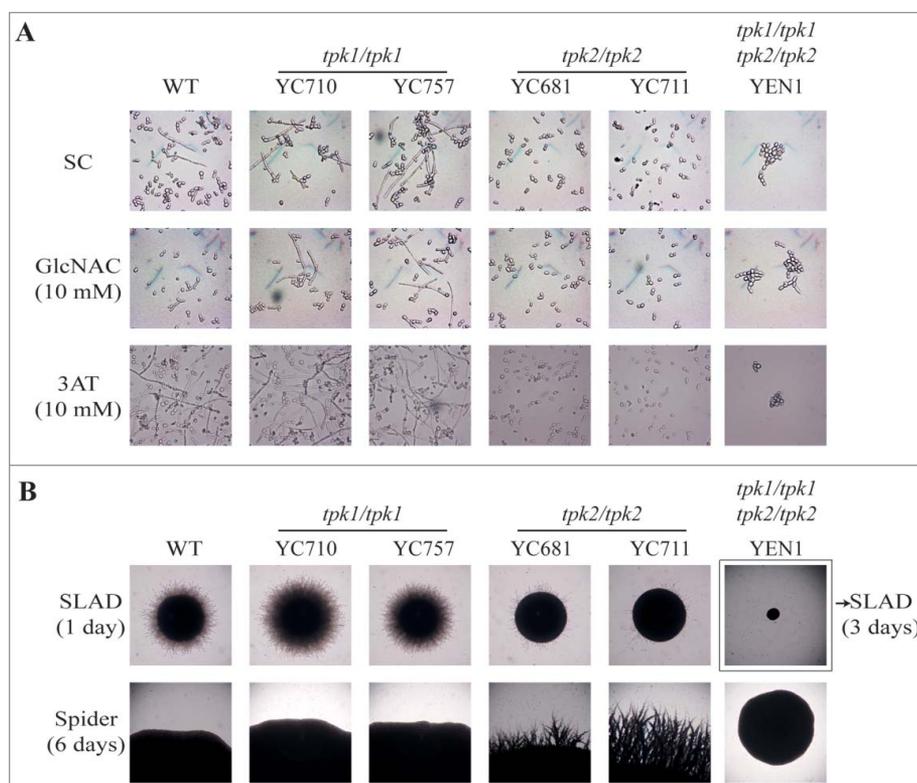


Figure 4. Tpk2, but not Tpk1, regulates hyphal growth. (A) Hyphal growth of the indicated strains in liquid media. Cells were grown overnight in YPD at 30°C (except *tpk1/tpk1 tpk2/tpk2* mutant, which was grown for two days), washed twice with dH₂O, diluted to 0.2 OD₆₀₀ with fresh SC medium or SC medium supplemented with 10 mM of GlcNAC or 3AT, and incubated at 37°C and 200 rpm. (B) Hyphal growth of the indicated strains on solid media. Cells were grown overnight in YPD at 30°C (except *tpk1/tpk1 tpk2/tpk2* mutant, which was grown for two days), washed twice with dH₂O, and diluted to 10³ cells/mL. Then, 50 μL containing ~50 cells were spread onto filament-inducing SLAD or Spider plates and incubated at 37°C for the indicated time.

The phylogenetic analysis of *S. cerevisiae*, *C. albicans*, and *C. tropicalis* PKA catalytic subunits further supports that CtTpk1 and CaTpk1 are evolutionary similar compared to ScTpk1 or ScTpk3. These data support findings from a previous study performed in *C. albicans* [28] and suggest that the cAMP/PKA pathway of *C. albicans* and *C. tropicalis* might have conserved functions in the regulation of morphogenesis and adhesion. In *C. tropicalis*, only the *tpk1/tpk1* mutant is sensitive to various stresses, such as cell wall-perturbing agents, amino acid starvation, and exposure to antifungal drugs. In contrast to *C. albicans* in which loss of *TPK1* results in increased susceptibility to osmotic, oxidative, and thermal stresses [26], *C. tropicalis tpk1/tpk1* and *tpk2/tpk2* mutants showed similar growth compared to wild type under osmotic, oxidative, and thermal stress (Fig. 2B and S6). Furthermore, *S. cerevisiae* PKA catalytic isoforms have overlapping functions in stress responses [52, 55]. CaTpk1 and CtTpk1 showed distinct phenotypes against various stresses, indicating that these two proteins might activate different downstream targets in order to survive under harsh conditions.

Roles of PKA in *C. tropicalis* hyphal growth and adhesion

The current understanding of the roles of the cAMP/PKA signaling pathway in filamentous growth and adhesion is based on studies of the *S. cerevisiae* and *C. albicans* dimorphic lifestyle [18, 22, 44]. In *C. albicans*, PKA activates downstream targets, for example Efg1, via phosphorylation, leading to a transition from yeast to pseudohyphal or hyphal growth, a key virulence factor [14]. Morphological switching also plays an important role in biofilm formation [30], which contributes to antifungal drug tolerance [31, 32]. A recent study in *C. tropicalis* suggested that the Efg1 transcription factor mediates hyphal growth as its counterpart does in *C. albicans* [56], indicating that *C. tropicalis* and *C. albicans* PKA, which is upstream of Efg1, may have conserved functions in hyphal growth and adhesion. Our results showed that *C. tropicalis* Tpk1 and Tpk2 have distinct effects on filamentation in liquid or on solid media (Fig. 4); however, the roles of *C. tropicalis* PKA in dimorphic switching are distinct from its relative *C. albicans*.

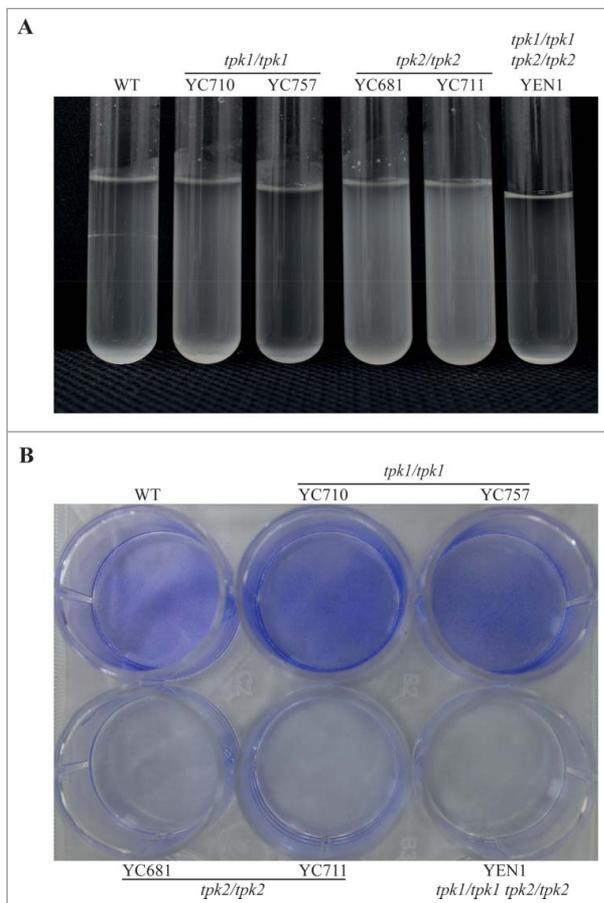


Figure 5. *Tpk2* is involved in flocculation and biofilm formation. (A) *Tpk2* is required for flocculation. Cells were grown overnight in SC medium containing 10 mM GlcNAc at 30°C (except the *tpk1/tpk1 tpk2/tpk2* mutant, which was grown 2 days in YPD, washed twice with dH₂O, transferred to SC medium containing 10 mM GlcNAc and incubated at 30°C overnight). The cells were then vortexed, centrifuged at 500 rpm for 1 min, and photographed. (B) *Tpk2* is critical for biofilm formation. Cells were grown in YPD medium overnight at 30°C (except *tpk1/tpk1 tpk2/tpk2* mutant, which was grown for two days), washed twice with dH₂O, and diluted to 0.5 OD₆₀₀ in SC medium. Then, 2 mL of each sample were inoculated into a 12-well plate for 90 min at 37°C and 200 rpm. The wells were then washed twice with PBS buffer, inoculated with 2 mL of fresh SC medium, and incubated for 24 h at 37°C and 200 rpm. The wells were then washed twice with PBS, stained with 0.05% crystal violet, and photographed.

Sonneborn *et al.* found that hyphal growth of a *tpk2/tpk2* mutant in *C. albicans* was compromised at 30°C but not at 37°C, suggesting that dimorphic switching on solid medium via *Tpk2* is temperature-dependent [29]. The same group further showed that loss of *TPK1* resulted in abolishment of filamentation at 37°C and that *TPK2* is required for hyphal growth in liquid medium [28]. In *C. tropicalis*, we showed that the *tpk2/tpk2* mutant had a filamentation defect both in liquid and on solid media at 37°C, while the loss

of *TPK1* did not affect hyphal growth at 37°C compared with the wild type (Fig. 4). These results suggest that *Tpk1* plays a divergent role in the yeast-hyphal transition of *C. tropicalis* and *C. albicans* upon sensing various environmental cues.

Dimorphism, biofilm formation, and adhesion are associated with pathogenicity in *Candida* species [15]. *Candida* biofilms consist of yeast cells attached to surfaces, hyphae or pseudohyphae and an extracellular matrix secreted by the cells [57]. Hence, both adherence and dimorphism are key determinants of biofilm formation in *C. albicans* [57]. Our data showed that the *C. tropicalis tpk2/tpk2* mutant had reduced ability to undergo dimorphic switching in specific conditions and subsequently exhibited defects in flocculation and biofilm formation, although the *tpk2/tpk2* mutant showed similar adherence to plastic surfaces as the wild type and *tpk1/tpk1* mutants. To date, the understanding of how PKA regulates biofilm formation in *C. tropicalis* is limited. *C. albicans* biofilm formation has been well characterized, and several transcription factors, including *Efg1*, *Flo8*, *Tec1*, *Ndt80*, *Rob1*, *Brg1*, and *Bcr1*, are known to be involved in this process [48]. Previous studies showed that *Efg1* is a direct target of the PKA signaling pathway in *C. albicans* and that it governs biofilm formation and adherence via upregulation *ALS3* [58, 59]. In addition, loss of *EFG1* results in abolishment of biofilm formation on plastic surfaces in *C. tropicalis* [56]. Our data showed that *tpk2/tpk2* mutants were unable to form a biofilm, indicating possible reduced expression of *EFG1* and *ALS3* as well as other adhesion-related genes. Although the two independent *tpk2/tpk2* mutants showed slightly different expressions of *EFG1* and *HWPI1*, it is clear that loss of *TPK2* resulted in low-level expression of *EFG1*, *BRG1*, *ALS3*, and *HWPI1* in comparison to the wild type, indicating that *C. tropicalis Tpk2* may regulate biofilm formation and adhesion via the transcription factors *Efg1* or *Brg1* and adhesins *Als3* or *Hwp1*. The different expression levels of *EFG1* and *HWPI1* might be due to experimental issues, such as a fitness change during the transformation procedure in one of the independent *tpk2/tpk2* mutants, as the transcription levels of other genes were similar between the two independent mutants. Interestingly, the *tpk1/tpk1 tpk2/tpk2* mutant exhibited cell aggregation but neither hyphal growth nor biofilm formation in the inducing conditions (Fig. 4A and 5B). We found high-level expression of adherence-related transcription factors such as *Bcr1*, *Flo8*, *Gal4*, and *Rim101* (Fig. 8), indicating that other signaling pathways, such as MAPK or RIM101, may be activated to

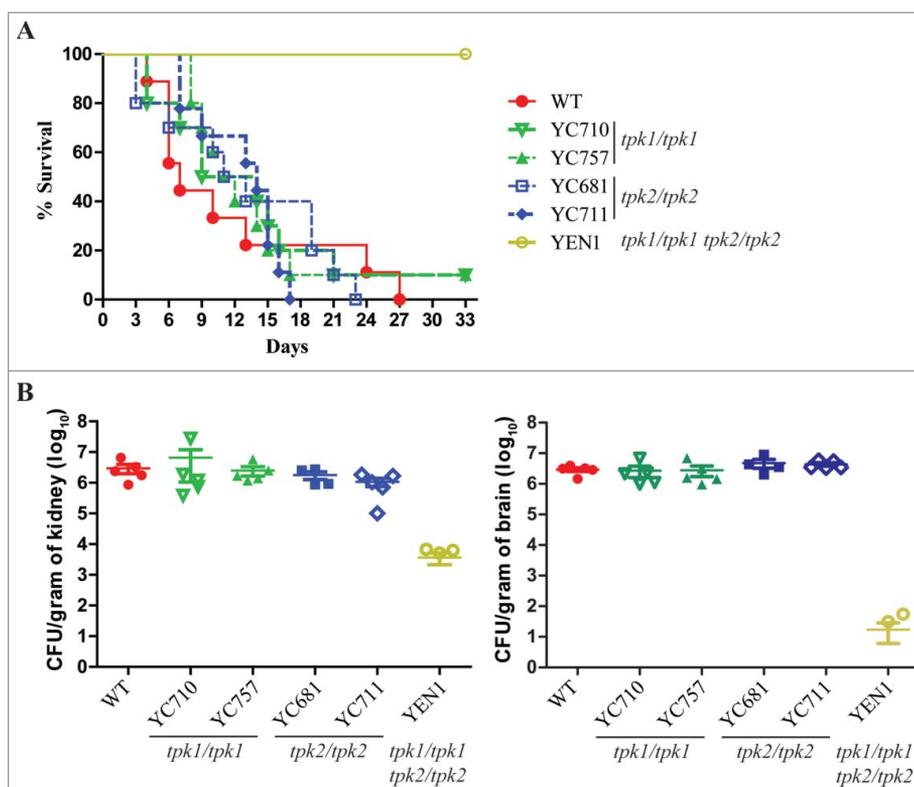


Figure 6. Both Tpk1 and Tpk2 are both required for full virulence. (A) Survival curves of mice infected with the indicated *C. tropicalis* strains. Cells were grown overnight in YPD medium (except *tpk1/tpk1 tpk2/tpk2* mutant, which was grown for two days), washed three times with PBS buffer (pH 7.4), resuspended in PBS buffer, and diluted to a concentration equal to 2.5×10^7 CFU/mL. Mice were inoculated with 5×10^6 *C. tropicalis* cells in 200 μ L and were monitored for 33 days. Ten mice per strain were used, except for the wild-type strain for which nine were used because one mouse died during the infection process. (B) The fungal burden in the kidneys and brains was measured on day 3 post-*C. tropicalis* infection in five mice per strain.

compensate for the loss of the cAMP/PKA pathway in the *tpk1/tpk1 tpk2/tpk2* mutant. The upregulation of these transcription factors might serve as a partial explanation for the aggregation observed in the *tpk1/tpk1 tpk2/tpk2* mutant.

Roles of PKA in *C. tropicalis* virulence

Previous studies of the PKA signaling pathway and virulence focused on *C. albicans*, *A. fumigatus*, and *C. neoformans* [17]. Though *C. albicans* Tpk1 and Tpk2 are involved in hyphal growth, only Tpk2 is required for virulence in a murine model of systemic infection [29, 60]. Our study indicated that in *C. tropicalis*, the PKA catalytic subunits play redundant roles in virulence. Loss of either Tpk1 or Tpk2 resulted in virulence similar to the wild type, while the *tpk1/tpk1 tpk2/tpk2* mutant was avirulent. Previous studies in *C. albicans* indicated that mutants with filamentation defects were avirulent [61] and found that a *tpk2/tpk2* mutant exhibited reduced hyphal growth and attenuated virulence [29]. A possible explanation for the full

virulence of the *C. tropicalis tpk2/tpk2* mutants is that these mutants have normal expression of hyphal-related genes such as *BCR1*, *FLO8*, and *RIM101* compared with the wild type and *tpk1/tpk1* mutants. In addition, *tpk2/tpk2* mutants exhibited similar tolerance as the wild type to various stresses such as cell wall perturbation and exposure to antifungal drugs, indicating that these mutants might survive under relatively harsh conditions within the host. The *tpk1/tpk1* mutants exhibited virulence similar to that of wild-type and the *tpk2/tpk2* mutants, but had increased susceptibility to the stressors mentioned above. Dimorphic switching and biofilm formation, which are critical for pathogenesis, may contribute to the virulence of the *tpk1/tpk1* mutant. Interestingly, the *tpk1/tpk1 tpk2/tpk2* mutant is avirulent, which may be explained by its severe growth defects. Taken together, our data suggest that PKA isoforms have distinct roles in stress responses, hyphal growth, and biofilm formation, but Tpk1 and Tpk2 coordinately regulate growth and virulence of the human fungal pathogen *C. tropicalis*.

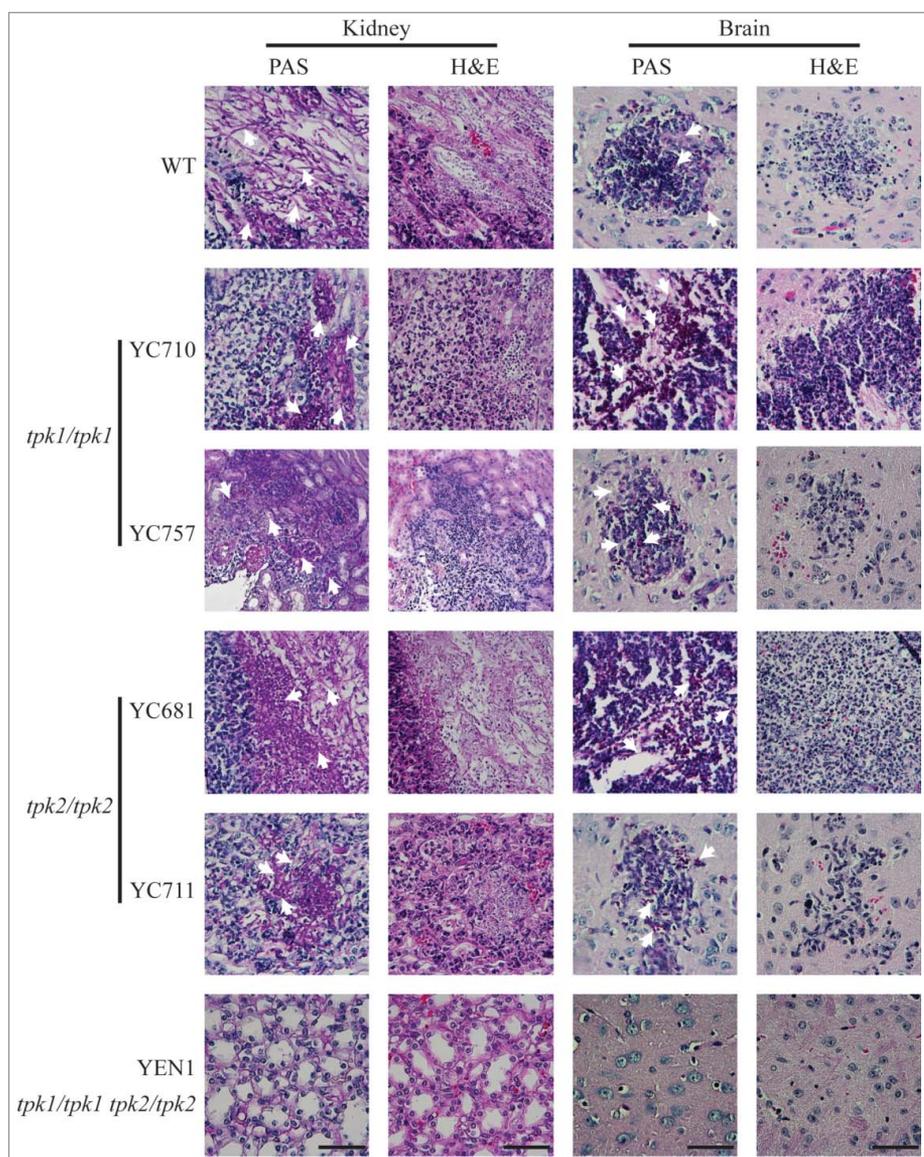


Figure 7. Both Tpk1 and Tpk2 are required for colonization and necrosis in kidneys and brains. Histopathological analyses of mouse kidney and brain organs obtained from *C. tropicalis*-infected mice at day 3. Organs were fixed in 10% formaldehyde solution, paraffin-embedded, and stained with PAS and H&E to observe *C. tropicalis* colonization and tissue necrosis, respectively. White arrowheads indicate fungal cells (hyphae or yeasts). Scale bar = 50 μ m.

In this study, we summarized and proposed a model to illustrate the distinct roles of Tpk1 and Tpk2 in different cellular process upon encountering various environmental conditions (Fig. 9). In contrast to CaTpk1, which regulates salt and oxidative stresses [26], CtTpk1 regulates cell wall integrity and drug tolerance. CtTpk2 has, at least in part, similar functions to CaTpk2 in the regulation of morphological switching under filamentation-inducing media in liquid or on solid media. CtTpk2 regulates flocculation and biofilm formation via expression of transcription factors and adhesins. However, unlike CaTpk2, CtTpk1 works together with CtTpk2 to reach full virulence in a murine model of systemic infection (Fig. 9).

Materials and methods

Yeast strains, media, and chemicals

C. tropicalis strains used in this study are listed in Table 1. The following media were used in this study: YPD (1% yeast extract, 2% peptone, 2% glucose); YPM (1% yeast extract, 2% peptone, 2% maltose); YNB (0.17% yeast nitrogen base without amino acids and $(\text{NH}_4)_2\text{SO}_4$, 0.5% $(\text{NH}_4)_2\text{SO}_4$, 2% glucose); SC (YNB with amino acids); SLAD (0.17% yeast nitrogen base without amino acids and $(\text{NH}_4)_2\text{SO}_4$, 50 μ M $(\text{NH}_4)_2\text{SO}_4$, 2% glucose); Spider medium (1% nutrient broth, 1% mannitol, 0.4% K_2HPO_4 , pH 7.2 with H_3PO_4). Agar (2%) was added to all solid media with the exception of

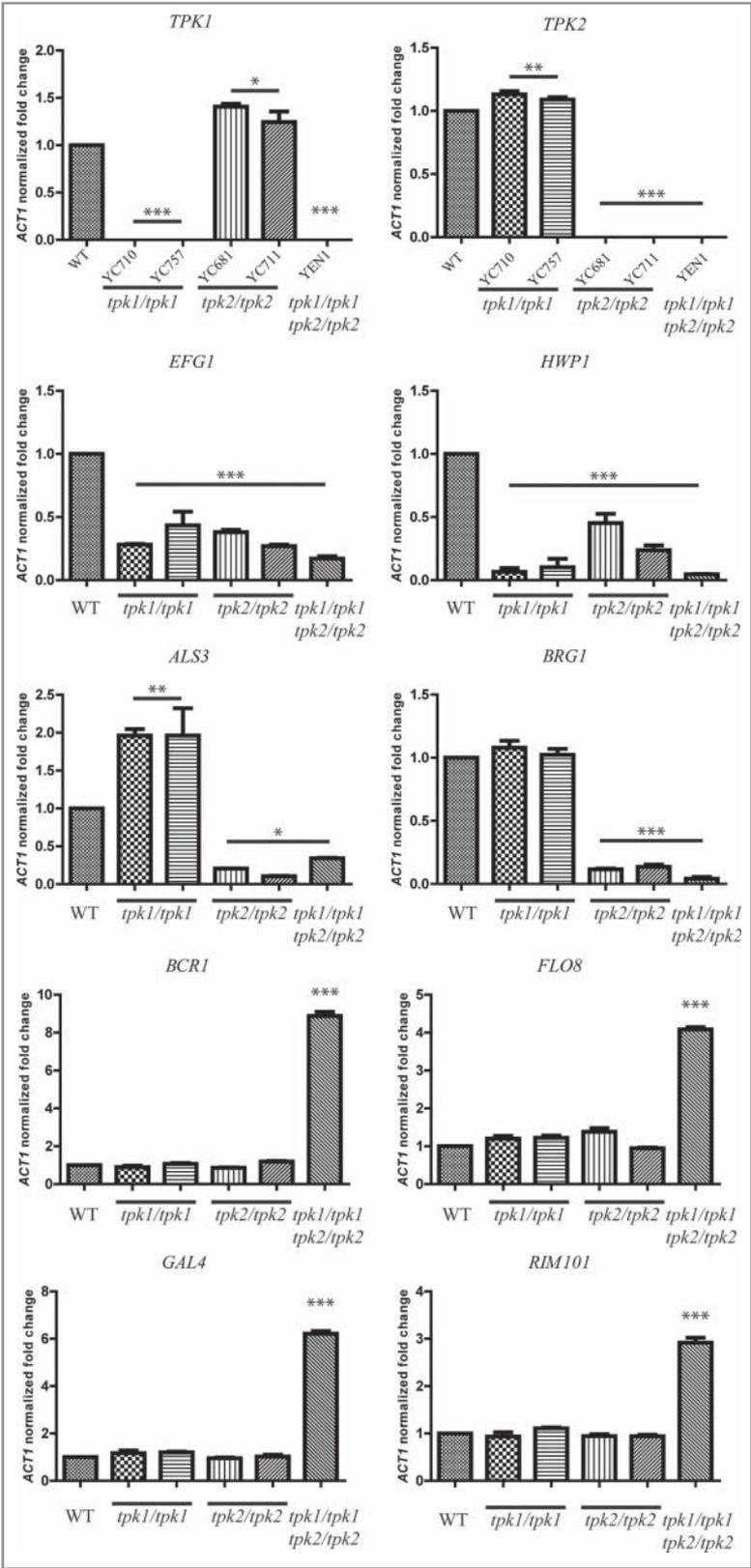


Figure 8. Tpk1 and Tpk2 are involved in the regulation of virulence-related gene expression. Cells were grown overnight in YPD at 30°C, washed twice with dH₂O, diluted to 0.1 OD₆₀₀ in 10 mL of Spider medium, and incubated for 4 h at 37°C. Total RNA was extracted using TRIzol and treated with the TURBO DNA-free™ Kit to remove genomic DNA. cDNA synthesis was carried out using 200 ng of RNA with the high-capacity cDNA reverse transcription kit. Transcript expression levels were analyzed by SYBR® Green PCR Master Mix and normalized to the *C. tropicalis* ACT1 gene using 2^{-ΔΔCt} method. Asterisks indicate statistically significant differences compared with the wild type using Dunnett’s multiple comparison test compared to wild type (*P < 0.05; **P < 0.01; ***P < 0.001).

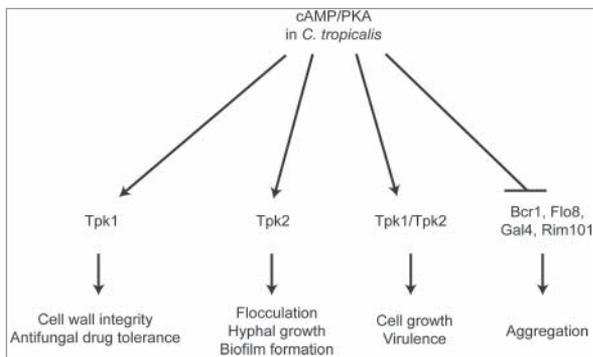


Figure 9. Proposed roles of PKA in *C. tropicalis*. The *C. tropicalis* PKA catalytic subunit Tpk1 controls cell wall integrity and drug tolerance, while Tpk2 is required for flocculation, hyphal growth, and biofilm formation. In addition, both proteins are not essential in *C. tropicalis* and have redundant roles in growth and virulence; loss of either gene results in growth and virulence similar to the wild type. In contrast, Tpk1 and Tpk2 negatively regulate expression of adherence-associated transcription factors (Bcr1, Flo8, Gal4, and Rim101), which were upregulated in the *tpk1/tpk2* mutant that showed cell aggregation.

Spider medium, which contained 1.4% agar. For selection assays, YPD medium was supplemented with 100 $\mu\text{g}/\text{mL}$ nourseothricin (Werner BioAgents, Jena, Germany). The following supplements were added to media at the indicated concentrations: 0.01% SDS (Biomax, New Taipei City, Taiwan), 60 $\mu\text{g}/\text{mL}$ posaconazole (Merck, Rahway, NJ, USA), 12.5 ng/mL micafungin (Astellas Pharma Inc., Deerfield, IL, USA), 10 ng/mL anidulafungin (Selleckchem, Houston, TX, USA), 10 mM DTT (Bioshop, Ontario, Canada), 10 mM 3AT (Sigma, St. Louis, MO, USA), 750 mM NaCl (Bioshop, Ontario, Canada), 750 mM KCl (Bioshop, Ontario, Canada), 500 mM CaCl_2 (MDBio, New Taipei City, Taiwan), 100 mM LiCl (MDBio, New Taipei City, Taiwan), 850 mM MgCl_2 (MDBio, New Taipei City, Taiwan), 0.005% H_2O_2 (Sigma, St. Louis, MO, USA), and 10 mM GlcNAc (Sigma, St. Louis, MO, USA).

Strain construction

Both alleles of the *C. tropicalis* *TPK1* and *TPK2* genes were disrupted with the *SAT1* flipper cassette [62] using the Frozen-EZ Yeast Transformation Kit (Zymo Research, Orange, CA, USA) or electroporation method [63]. To disrupt the *TPK1* gene, approximately 1-kb 5' (amplified with primers JC513/JC514, Table S1) and 3' (amplified with primers JC515/JC516) noncoding regions (NCRs) of *TPK1* were PCR-amplified from genomic DNA of the wild type strain MYA3404 [34]. The 4.2-

kb *SAT1* flipper sequence was amplified from plasmid pSFS2A [62] with primers JC17/JC18. These three PCR products were treated with ExoSAP-IT (USB corp., Ohio, USA) to remove additional primers and dNTPs, and then combined in a 1:3:1 molar ratio (5' *TPK1*^{NCR} : *SAT1* flipper:3' *TPK1*^{NCR}) to generate the disruption allele by overlap PCR using nesting primers JC517/JC518 (~100 bp closer to *TPK1* compared with JC513/JC516, respectively), resulting in an ~6-kb *TPK1* disruption fragment with 5' *TPK1*^{NCR}-*SAT1* flipper-3' *TPK1*^{NCR}.

The first allele of the *TPK1* gene was disrupted in the wild-type strain MYA3404 by transformation with 0.2 to 1 μg of gel-purified disruption DNA using the Frozen-EZ Yeast Transformation Kit. Two independent heterozygous nourseothricin-resistant mutants (YC680 and YC708, Table 1) were obtained from two separate transformations. Liquid YPM medium was used to activate the expression of the FLP recombinase under the control of the *C. albicans* *MAL2* promoter. The *SAT1* flipper was then excised, leaving a FLP recombination target (FRT) sequence. This resulted in nourseothricin-sensitive *TPK1/tpk1* mutant strains (YC683 and YC718). Despite multiple attempts, the second allele of the *TPK1* gene could not be disrupted with the same overlap-PCR disruption allele. Therefore, we amplified 5' *TPK1*^{NCR} with JC513/555 and 3' *TPK1*^{NCR} with JC556/516 from the second wild-type *TPK1* allele of 683 and YC718 and mixed these with the *SAT1* flipper to produce an overlap-PCR *TPK1* disruption allele specific for the second allele of the *TPK1* gene. Eventually, two independent nourseothricin-resistant homozygous *tpk1/tpk1* mutants (YC710 and YC757) derived from two separate transformations were obtained.

A similar approach was employed to disrupt the *TPK2* gene, with ~1-kb 5' and 3' NCRs for homologous recombination. To generate the ~6.0-kb *TPK2* disruption allele, the overlap PCR DNA products 5' *TPK2*^{NCR} (amplified with primers JC521/JC522), *SAT1* flipper (amplified with primers JC17/JC18), and 3' *TPK2*^{NCR} (amplified with primers JC523/JC524) were mixed in a 1:3:1 molar ratio and amplified with primers JC525/JC526 (~100 bp closer to the *TPK2* ORF compared with JC521/JC524, respectively). A similar approach was used to disrupt the second allele of the *TPK2* gene. We amplified the 5' *TPK2*^{NCR} with JC521/JC545 and 3' *TPK2* NCR with JC546/524 from the second *TPK2* allele of the heterozygous *TPK2/tpk2* mutant. We obtained two independent nourseothricin-resistant *tpk2/tpk2* mutants (YC711 and YC681, Table 1) from two separate transformations. Correct integration of the disruption cassettes was confirmed by PCR using primers for the ORF and the 5'/3' NCRs.

To obtain the *tpk1/tpk1 tpk2/tpk2* double deletion mutant, YC710 mutant (*tpk1/tpk1::SAT1-FLP* mutant) was cultured in YPM media to excise the *SAT1* flipper, leaving an FRT sequence and resulting in a nourseothricin-sensitive *tpk1/tpk1* mutant (YC914). In contrast to the fusion PCR method, the plasmid pYSJ10 was constructed to carry the *TPK2* disruption cassette. A ~1-kb 5' *TPK2*^{NCR} containing *KpnI* and *ApaI* enzyme cutting sites with primer JC758/759 and ~1 kb 3' *TPK2*^{NCR} containing *SacII* and *SacI* enzyme cutting sites with JC760/761 were amplified. Then, a *SacII/SacI*-digested 3' *TPK2*^{NCR} fragment was cloned into the *SacII*- and *SacI*-digested pSFS2A to generate pYSJ6. The 5' *TPK2*^{NCR} fragment was then subcloned into *KpnI*- and *ApaI*-digested pYSJ6 to generate pYSJ10 (Table S2).

The *TPK2* disruption cassette isolated from pYSJ10 by *SacI* and *KpnI* digestion was transformed into the *tpk1/tpk1* mutant (YC914) using electroporation to obtain the *tpk1/tpk1 tpk2::SAT1-FLP/TPK2* mutant (YC928). As described previously, the *SAT1* flipper of YC928 was excised to generate a nourseothricin-sensitive *tpk1/tpk1 tpk2/TPK2* mutant (YSJ13). Because the second *TPK2* allele could not be deleted using the same disruption fragment, we amplified ~700 bp of the 5' *TPK2*^{NCR} containing *KpnI* and *ApaI* enzyme cutting sites with JC782/JC783 and ~1 kb of the 3' *TPK2*^{NCR} containing *SacII* and *SacI* enzyme cutting sites with JC784/JC785 from the YSJ13 mutant. Afterwards, a *SacII/SacI*-digested 3' *TPK2*^{NCR} fragment was cloned into the *SacII*- and *SacI*-digested pSFS2A to generate pYSJ25 and the 5' *TPK2*^{NCR} fragment was then subcloned into *KpnI*- and *ApaI*-digested pYSJ25 to generate pYSJ29 (Table S2). The *tpk1/tpk1 tpk2/tpk2* double mutant (YEN1) was obtained by transforming the *TPK2* disruption cassette from pYSJ29 into the *tpk1/tpk1 tpk2/TPK2* mutant (YSJ13). All strains were confirmed by amplifying the ORF and 5' and 3' NCR integrations of the disruption cassettes, as well as by qRT-PCR to detect expression of *TPK1* or *TPK2* ORFs.

Similar to the deletion cassette, the *SAT1*-flipper marker was also used to obtain the revertant construct. First, a ~1.8-kb fragment carrying the 5' NCR and ORF of *TPK1* with *ApaI* and *XhoI* restriction sites and a ~1-kb fragment with the 3' NCR of *TPK1* containing *SacII* and *SacI* sites were amplified with primers JC1006/1007 and JC1004/1023, respectively. Then, a *SacII/SacI*-digested 3' *TPK1*^{NCR} fragment was cloned into pSFS2A and digested with the same enzymes to generate pYEN37 (Table S2). Similarly, the *TPK1* 5' NCR and ORF were cloned into *ApaI* and *XhoI*-digested pYEN37 to generate pYSJ122. The *TPK1* complementary cassette containing the ~7-kb 5'NCR-ORF-*SAT1-FLP*-3'NCR

digested with *ApaI* and *SacI* from pYSJ112 was transformed into the *tpk1/tpk1* pop-out mutant (YEN10) to generate the *tpk1/tpk1::TPK1-SAT1* complemented strain (YEN43) and into the *tpk1/tpk1 tpk2/tpk2* pop-out strain (YEN38) to obtain the *tpk1/tpk1::TPK1-SAT1 tpk2/tpk2* complemented strain (YEN40).

The same procedure was also used to construct the *TPK2* revertant construct. The *TPK2* complementary cassette was obtained by cloning a ~2.3 kb of the 5' NCR and ORF of *TPK2* amplified with primers JC998/999 into *KpnI*- and *ApaI*-digested pYSJ6 to generate pYEN2. The *TPK2* revertants *tpk2/tpk2::TPK2-SAT1* (YEN26) and *tpk1/tpk1 tpk2/tpk2::TPK2-SAT1* (YEN42) were obtained by transforming the *TPK2* complementary cassette from pYEN2 into the *tpk2/tpk2* pop-out mutant (YEN20) and *tpk1/tpk1 tpk2/tpk2* pop-out mutant (YEN38), respectively.

Cell adhesion assay

The cell adhesion assay was performed as previously described with modifications to mimic the adherence step of the biofilm assay [64]. Briefly, cells grown in YPD medium overnight were washed twice with PBS and then adjusted to a concentration of OD₆₀₀ = 0.5 with SC medium. Then, 200 μ L of cells were inoculated into a 96-well plate and incubated for 90 min (37°C and 200 rpm). Afterwards, the wells were washed twice with PBS to remove non-adherent cells. The adhered cells were vigorously resuspended in water, serially diluted (2,000-fold), plated on YPD medium, and incubated for two days at 30°C. The colony forming units (CFU) were then determined.

Serial dilution growth assays

Cells were grown in YPD broth at 30°C overnight (except *tpk1/tpk1 tpk2/tpk2* mutant, which was grown for two days), washed twice with dH₂O, and diluted with dH₂O to an OD₆₀₀ of 0.2 as the starting concentration. Then, 3 μ L of five-fold serial dilutions in dH₂O of the indicated strains were spotted onto solid agar plates with a multichannel pipette, incubated at 25°C, 30°C, 37°C, or 42°C for 48 h, and photographed.

Growth kinetics and protein kinase A activity assays

For growth kinetics assays, cells were grown in YPD broth at 30°C overnight (except the *tpk1/tpk1 tpk2/tpk2* mutant, which was grown for two days), washed twice with dH₂O, diluted to 0.01 OD₆₀₀ (0.1 OD₆₀₀ for the *tpk1/tpk1 tpk2/tpk2* mutant) in fresh YPD medium, and

incubated at 30°C with shaking at 200 rpm. The OD₆₀₀ of cultures was measured at 0, 3, 6, 9, 12, 24, 48, 72, and 96 h via microplate spectrophotometer (Spectra MAX 190, Molecular Devices, CA, USA). The experiments were performed in triplicate, and the data were plotted using Prism 5.03 (GraphPad Software, CA, USA).

The PKA activity assay was carried out using PepTag Assay for non-radioactive detection of cAMP-dependent protein kinase kit (Promega, Madison, WI, USA) according to the user manual. Briefly, strains were grown in 10 mL of YPD broth at 30°C overnight (except the *tpk1/tpk1 tpk2/tpk2* mutant, which was grown for two days) and washed twice with dH₂O. Crude protein extracts were purified using 500 μL of PKA extraction buffer (0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 25 mM Tris-HCl [pH 7.4], and 1 μg/mL leupeptin and aprotinin), and the total protein concentration was measured by Bradford assay [65]. Then, 4 μg of the samples were mixed with PKA reaction buffer (1 μM cAMP, 1 mM ATP, 10 mM MgCl₂, and 20 mM Tris-HCl [pH 7.4]) and incubated at room temperature for 30 min. The reaction was stopped by incubating the tube at 95°C for 10 min. The samples were loaded and separated on a 0.8% agarose gel in 50 mM Tris-HCl (pH 8.0) at 100 V for 25 min, and the gel was photographed.

Flocculation and biofilm formation assays

The *C. tropicalis* flocculation assay was performed as previously described [66] with modifications. The wild type and PKA mutants were inoculated in SC liquid medium containing 10 mM GlcNAc and incubated at 30°C overnight (except the *tpk1/tpk1 tpk2/tpk2* mutant, which was grown 2 days in YPD, washed twice with dH₂O, transferred to SC medium and incubated at 30°C overnight). The cultures were vortexed, centrifuged for 1 min at 500 rpm, and photographed.

The biofilm formation assay was performed as previously described [48] with modifications. *C. tropicalis* strains were grown in YPD at 30°C overnight (except the *tpk1/tpk1 tpk2/tpk2* mutant, which was grown for two days), washed twice with dH₂O, adjusted to 0.5 OD₆₀₀ in 2 mL of SC medium, and inoculated into the non-tissue culture treated 12-well plate (Corning, Durham, NC, USA). The inoculated plate was incubated for 90 min at 37°C and 200 rpm, washed with PBS buffer twice, and re-filled with fresh SC medium. The plate was incubated for 24 h at 37°C and 200 rpm. Then, the culture medium was removed and the wells were washed twice with PBS buffer. Biofilm formation was visualized by applying 2 mL 0.05% crystal violet solution for 5 min. Excess stain was removed and the plates were washed twice with 2 mL of dH₂O and photographed.

qRT-PCR

C. tropicalis strains were grown in YPD at 30°C overnight (except *tpk1/tpk1 tpk2/tpk2* mutant, which was grown for two days) and washed twice with dH₂O. Then, 0.2 OD₆₀₀ cells were transferred to 10 mL of fresh liquid Spider medium and incubated for 4 h at 37°C. Total RNA was then extracted using TRIzol (Ambion, Carlsbad, CA, USA) and treated with TURBO DNA-free™ Kit (Thermo Fisher Scientific, Vilnius, Lithuania) to remove genomic DNA. cDNA synthesis was carried out using 200 ng of total RNA with the high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Vilnius, Lithuania) in a PCR machine (Biometra, Jena, Germany) following the manufacturer's manual. Transcript expression levels were analyzed by SYBR® Green PCR Master Mix (Thermo Fisher Scientific, Vilnius, Lithuania) in a StepOnePlus machine (Applied Biosystems, Foster City, CA, USA) and normalized to the *ACT1* gene using the 2^{-ΔΔCt} method [67]. Accession numbers of the analyzed genes are *ACT1* (CTRG_03626); *ALS3* (CTRG_02293); *HWPI* (CTRG_00477); *EFG1* (KP314278); *BCR1* (CTRG_00608); *RIM101* (CTRG_03710); *BRG1* (CTRG_04523); *FLO8* (CTRG_02751); and *GAL4* (CTRG_01928). The primers for qRT-PCR are shown in Table S1.

Murine model of systemic infection

Four- to five-week-old outbred male ICR mice were purchased from the bioLASCO Taiwan Co. (Taipei, Taiwan) (n = 10 for each group, except n = 9 for the wild type). The *C. tropicalis* wild-type strain (MYA3404), *tpk1/tpk1* mutants (YC710 and YC757), and *tpk2/tpk2* mutants (YC711 and YC681), were grown overnight in YPD broth at 30°C (except *tpk1/tpk1 tpk2/tpk2* mutant, which was grown for two days), washed three times with PBS buffer (pH 7.4), resuspended in PBS buffer, and diluted to a concentration equal to 2.5 × 10⁷ CFU/mL using a hemocytometer (Paul Marienfeld GmbH&Co. KG, Lauda-Königshofen, Germany). Then, 200 μL (5 × 10⁶ cells) were used to infect mice by lateral tail vein injection. CFU and cell viability were verified by incubating cells on YPD agar plates 48 h at 30°C. Survival was monitored twice daily for 33 days and data were statistically analyzed by log-rank test using Prism 5.03.

To determine fungal burden, mice (n=5 for each group) were infected intravenously with 5 × 10⁶ *C. tropicalis* wild type or mutant cells (in 200 μL) and were sacrificed three days post-infection. Kidneys and brains from the mice were dissected into two parts and weighed. One part of each organ was soaked in 10 mL PBS buffer and homogenized for 2 min at 1,750 rpm with 2010 Geno/Grinder (SPEX SamplePrep, Metuchen, NJ, USA).

The tissue homogenates were serially diluted on YPD solid agar plates containing chloramphenicol (100 µg/mL) and incubated at 30°C for 24 to 48 h (96 h for the *tpk1tpk1 tpk2/tpk2* mutant) to determine the CFU per gram of kidney or brain. The identities of the colonies recovered from the organs were further confirmed by PCR. The significance of differences in fungal burden was determined using the unpaired *t*-test compared to wild type. For histopathological analysis, the other sections of the organs were fixed in 10% formaldehyde solution (Sigma, St Louis, MO, USA), paraffin-embedded, and staining with hematoxylin-eosin (H&E) or periodic acid-Schiff (PAS) was performed by the School of Veterinary Medicine, National Taiwan University. After slide preparation, *Candida* colonization (PAS) and tissue necrosis (H&E) were examined by microscope.

Ethical statements

All experimental procedures were carried out according to NIH guidelines and were approved by the Institutional Animal Care and Use Committee at National Taiwan University (approval number NTU104-EL-00009).

Acknowledgments

This work was financially supported by grants MOST 102-2320-B-002-041-MY2, 104-2320-B-002-063-MY3, and 106-2923-B-002-001-MY3 from the Ministry of Science & Technology (MOST), Taiwan. The authors thank Ms. Cecelia Wall for language editing.

Funding

Ministry of Science & Technology (MOST), Taiwan; MOST 102-2320-B-002-041-MY2, 104-2320-B-002-063-MY3 and 106-2923-B-002-001-MY3

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