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# Ras signalling in pathogenic yeasts

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**ABSTRACT The small GTPase Ras acts as a master regulator of growth, stress response and cell death in eukaryotic cells. The control of Ras activity is fundamental, as highlighted by the oncogenic properties of constitutive forms of Ras proteins. Ras also plays a crucial role in the pathogenicity of fungal pathogens where it has been found to regulate a number of adaptions required for virulence. The importance of Ras in fungal disease raises the possibility that it may provide a useful target for the development of new treatments at a time when resistance to available antifungals is increasing. New findings suggest that important regulatory sequences found within fungal Ras proteins that are not conserved may prove useful in the development of new antifungals. Here we review the roles of Ras protein function and signalling in the major human yeast pathogens *Candida albicans* and *Cryptococcus neoformans* and discuss the potential for targeting Ras as a novel approach to anti-fungal therapy.**

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***Abbreviations:***
*GEF – guanine nucleotide exchange, factor,*
*MAP – mitogen-activated protein,*
*MTL – mating type locus,*
*PKA – protein kinase A.*

## RAS PROTEINS – FORM AND FUNCTION

The Ras superfamily consists of small G-binding proteins that have been divided into five main groups on the basis of sequence and functional similarity: Ras, Rho, Arf/Sar, Rab and Ran [1]. As with all G proteins, Ras activation is dependent on the exchange of bound GDP for GTP and deactivation via hydrolysis of GTP to GDP. Cycling between active and non-active states is regulated by Guanine nucleotide exchange factors (GEF), dissociation inhibitor's (GDI) and GTPase Activating Proteins (GAP) accessory proteins [2]. The activation and deactivation cycle of Ras proteins couple a range of stimuli to effector proteins and as such these G-proteins serve as regulatory "switches" within a variety of cellular processes. The importance of Ras signalling is highlighted by the dramatic effects that can be observed upon inappropriate activation. For example mutations that lead to the constitutive activation of Ras signalling have been estimated to occur in ~50% of all tumours [3]. In addition, Ras proteins play important roles in the regulation of growth and adaption in fungal cells. In this review we will focus on Ras protein function in the major human yeast pathogens *Candida albicans* (*C. albicans*) and *Cryptococcus neoformans* (*C. neoformans*).

## RAS SIGNALLING IN THE FUNGAL PATHOGEN OF HUMANS *C. ALBICANS*

### *C. albicans* virulence and pathogenicity

*C. albicans* is a commensal organism that is commonly found on the mucosal surfaces of the oral cavity, gastrointestinal tract and genitourinary tract of healthy individuals [4, 5]. However *C. albicans* is also a well characterised opportunistic pathogen [4, 6] and a serious health-risk amongst immunocompromised individuals, such as those suffering from HIV infection, or persons living with indwelling medical devices such as catheters or voice prostheses [7, 8]. The infections caused by *C. albicans* range from superficial infection of mucosal and non-mucosal surfaces (candidiasis) to a full systemic infection (candidaemia) also affecting internal organs [4]. Superficial mucosal surface infections can be readily treated with a range of antifungals. For example, vulvovaginal candidiasis (VVC) is usually successfully treated with azole antifungals like fluconazole [9]. However, candidaemia is associated with a high mortality even when treated with a variety of classes of antifungal agents [10]. As with many *C. albicans* infections, weakened immune defences are a significant risk factor for developing candidaemia. In healthy individuals, neutrophils

provide suitable defence against *C. albicans*. As such, neutropenia, either as a result of particular blood cancers or treatment with immunosuppressants, significantly increases the risk of developing candidaemia. Furthermore, damage to the mucosa of the gastrointestinal tract, for example due to surgery, is also a risk factor as it enables the spread of *C. albicans* [11]. The symptoms of candidaemia range from fever and chills which do not abate following antibiotic treatment to severe sepsis or septic shock similar to that of bacterial septicaemia [9]. However, a lack of precise symptoms can lead to delayed diagnosis and required antifungal treatment leading to increased mortality [12]. It has been reported that even a delay of as little as 12-24h can double mortality rate [13]. Due to this, it has been suggested to prophylactically administer antifungals after any event which is likely to increase the risk of candidaemia, such as after abdominal surgery or bone marrow transplant [9]. Although the majority of cases of candidiasis and candidaemia are caused by *C. albicans*, there are other species within the *Candida* genus which are also pathogenic in humans. These include *Candida glabrata, Candida tropicalis, Candida dubliniensis and Candida parapsilosis*. *Candida* species have increasingly become associated with nosocomial infections [6]; in fact, *C. albicans* is recognised as the fourth most common cause of all hospital-acquired infections in the USA [4].

**Morphogenesis, pathogenicity and Ras signalling in *C. albicans***

An important aspect of *C. albicans* biology, in terms of pathogenesis, is its ability to undergo morphogenesis from a yeast, to pseudohyphal or hyphal forms in response to environmental cues. The virulence of *C. albicans* is closely linked with the capacity to switch between these forms. Hyphal *C. albicans* cells are frequently located at sites of tissue invasion, moreover, cells which are unable to readily form hyphae exhibit reduced virulence [4]. However as strains that are incapable of growing in the yeast form also have less virulence it has been proposed that both the yeast and hyphal forms play important roles during infection [14, 15]. Ras signalling is crucial to the integration of environmental cues with morphogenesis and *C. albicans* possesses two Ras genes - *RAS1* and *RAS2*, which encode the Ras1 protein and a highly divergent Ras-like protein termed Ras2 [16]. The importance of Ras1 signalling to the virulence of *C. albicans* is demonstrated by the fact that mutants which lack Ras1 are defective in their ability to undergo hyphal transition and exhibit reduced virulence in mouse infection models [17]. Ras signalling is now known to mediate the induction of hyphal growth in response to a variety of environmental cues including growth at 37°C (via alleviation of Hsp90-mediated repression of the Ras1-cAMP-PKA pathway) [18, 19], exposure to high levels of $CO_2$ [20], N-acetylglucosamine [21] and serum exposure [22]. These environmental signals are transduced through the cyclic AMP-protein kinase A and a mitogen-activated protein (MAP) kinase pathways (Figure 1) [4]. These pathways culminate in the regulation of transcription factors which control the expression of hyphal-specific genes (HSGs) such as Als3 (adhesin) [23], Hwp1 (invasin) [24], Hyr1 (host immune response modulator) [25], and Hgc1 (hyphal-specific cyclin) [26].

Upon activation, Ras1 directly interacts with and activates Cyr1 (the *C. albicans* adenylate cyclase), causing an increase in the production of the second messenger cAMP [17]. cAMP causes the derepression of two isoforms of protein kinase A (PKA) by triggering the dissociation of the PKA regulatory subunit (Bcy1) from the catalytic subunits (Tpk1 or Tpk2). The activation of PKA stimulates several processes within the cell including the yeast-to-hyphae switch [27]. PKA is believed to phosphorylate the transcription factor Efg1 on threonine-206, thereby activating it and resulting in the expression of HSGs [28]. The Tpk1 and Tpk2 isoforms have some redundant functions in *C. albicans*, however, they also have specific roles in filamentation. For example, Tpk1 is necessary for the expression of genes encoding proteins involved in branched chain amino acid biosynthesis, and Tpk2 negatively regulates iron uptake genes and positively regulates those associated with trehalose degradation and water homeostasis [29].

A number of environmental signals promote the yeast-to-hyphae switch via the Ras1-Cyr1-PKA pathway. Some of these signals, including $CO_2$ interface directly with the Cyr1 adenylate cyclase to activate it [20]. $CO_2$ is able to do this because, unlike most signalling molecules, it is able to enter the cell by simple diffusion and is maintained in the cell as $HCO_3^-$ via conversion by a carbonic anhydrase encoded by *NCE103* [20]. It has recently been discovered that the expression of *NCE103* is controlled in response to $CO_2$ availability by the bZIP transcription factor Rca1; Rca1 is regulated in a $CO_2$-dependent manner by the Sch9 kinase via a cascade mediated by lipid/Pkh1/2 signalling [30]. A lysine residue at position 1373 is critical for $CO_2$ activation of Cyr1. This lysine residue is located in the C-terminal catalytic domain and makes up a receptor site which detects increased $HCO_3^-$ levels [31], leading to increased cAMP production and activation of PKA filamentation [20]. The response of *C. albicans* to $CO_2$ is of interest because within a mammalian host, the levels of $CO_2$ are approximately 150x that of normal air (~5% compared to 0.03%). It may be the case that high levels of $CO_2$, such as are found within the upper respiratory tract, may promote *C. albicans* colonisation. However, it is interesting to note that other pathogenic *Candida* species, including *C. dubliniensis*, *C. glabrata*, *C. paropsilosis* and *C. krusei*, do not undergo the yeast-to-hyphae transition in response to elevated $CO_2$ [20]. Although this does not rule out the fact that the adenylyl cyclase of the latter species is activated by carbon dioxide/bicarbonate; the physiological significance of $CO_2$ sensing with regards to *Candida* infection remains to be determined.

Muramyl dipeptide (MDP), the minimal biologically active subunit of bacterial peptidoglycan, also induces *C. albicans* filamentation by acting directly upon Cyr1 [32]. A further signal which can cause morphogenesis through direct interaction with Cyr1 are amino acids. Amino acids, when in the presence of glucose, activate Cyr1 via upstream signalling through the G-protein coupled receptor

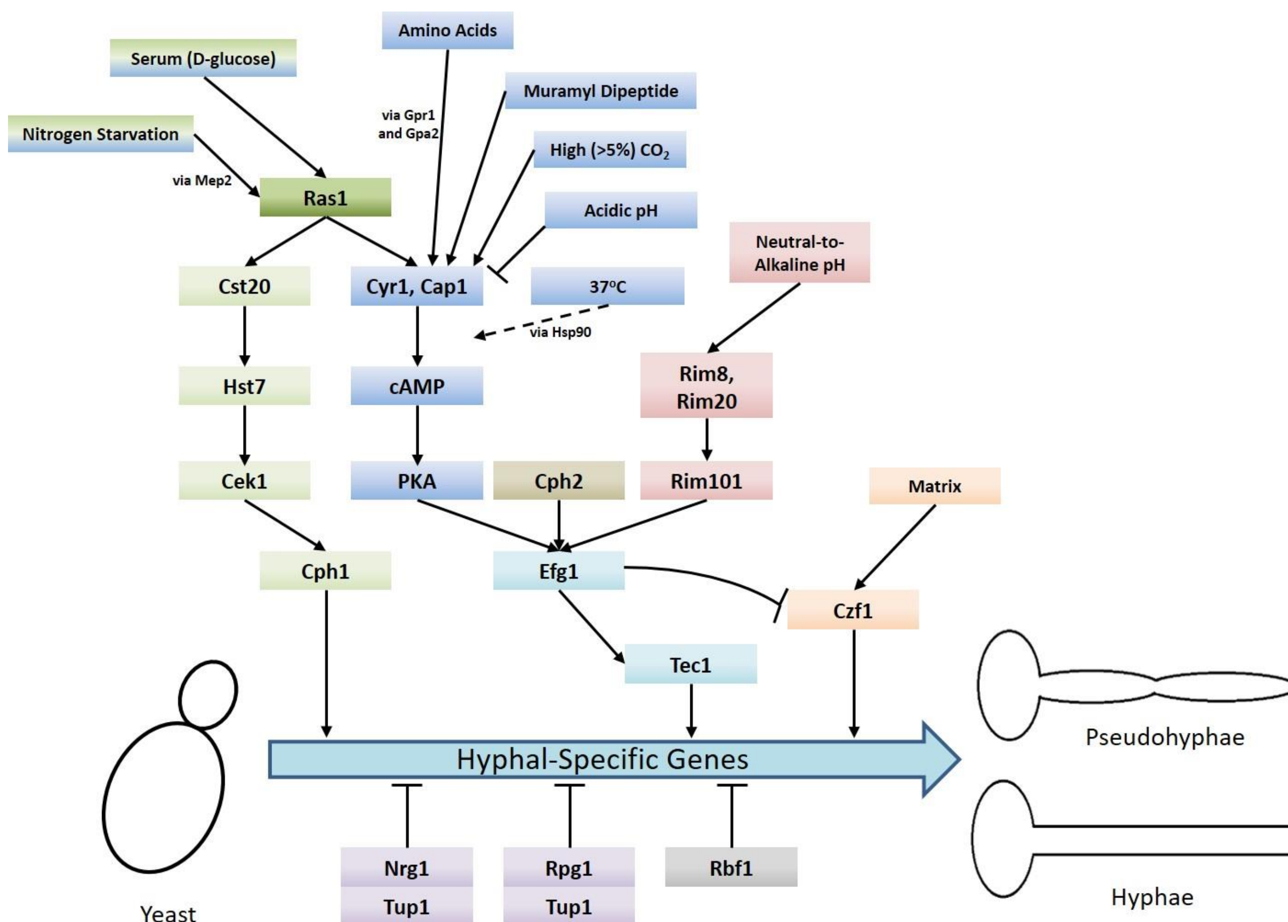


**FIGURE 1: Summary of the signalling pathways and stimuli which regulate the yeast-to-hyphae morphogenic switch in *Candida albicans*.** Several different pathways are responsible for inducing the yeast-to-hyphae switch: MAP kinase pathway (green), Cyr1-PKA pathway (blue), pH response pathway (red), and matrix response pathway (orange). There are also two pathways which negatively regulate the switch: Tup1-Nrg1-Rpg1 pathway (purple) and Rbf1 pathway (grey). The signalling pathways that engaged in response to different conditions to regulate the yeast-to-hyphae morphogenic switch in *C. albicans* are also indicated.

Gpr1 and its Gα protein Gpa2 [33]. Upon its activation by Gpr1, Gpa2 is believed to bind to a Gα domain on the Cyr1 adenylate cyclase thereby activating it [33]. This binding of Gpa2 to a fungal adenylate cyclase Gα domain has been demonstrated in fission yeast [34] but it is yet to be proved experimentally in *C. albicans*.

In contrast, acidic pH causes a reduction in signalling through the Ras1-Cyr1-PKA pathway via a Ras1-independent downregulation of Cyr1 activity [35]. *C. albicans* cells grown at pH 4 in hyphae-inducing conditions do not form hyphae, instead remaining as yeast or pseudohyphal cells and this is not reliant upon Ras1. It has also been observed that low extracellular pH results in fast and sustained decreases in intracellular pH which potentially contributes to reduced cAMP signalling through the reduction of intracellular bicarbonate levels [35].

The yeast-to-hyphae switch in response to exposure to serum relies on Ras1 signalling upstream of Cyr1 [22]. The component of serum principally responsible for the induction of hyphal growth is D-glucose which is able to activate both the Ras1-Cyr1-PKA pathway and the MAP-kinase pathway [22, 36]. The precise mechanism of Ras1 activation by D-glucose in *C. albicans* remains to be elucidated but in *Saccharomyces cerevisiae* (*S. cerevisiae*) it depends on both an intracellular phosphorylated form of D-glucose and a G-protein coupled receptor Gpr1 with its Gα protein Gpa2 [37]. Gpr1-type receptors have been characterised in *C. albicans* [33] and so a similar mechanism for Ras1 activation may exist in this pathogen. However, deletion of either CaGpr1 or CaGpa2 had no effect on D-glucose-mediated cAMP signalling, but deletion of CaCdc25 (the *C. albicans* Ras1 GEF) or CaRas1 eliminated this signalling [33]. These findings indicate Ras1 activation via Cdc25 is the primary mechanism by which D-glucose induces morphogenesis. The response of *C. albicans* to D-glucose is of physiological relevance because links between candida infection and hyperglycaemia [38] as well as insulin-dependent diabetes mellitus [39] have been reported. Moreover, *C. albicans* cells have increased resistance to oxidative and cationic stresses upon exposure to levels of glucose that may be found in the bloodstream [40].

Ras1 has also been shown to regulate hyphal transition in response to other environmental cues, such as nitrogen starvation [41], via MAP kinase signalling. As with the Cyr1/PKA pathway, the regulatory MAP kinase cascade is also activated by Ras1 [17] and consist of the kinases Cst20, Hst7 and Cek1 [42, 43, 44, 45, 46]. Ras1/MAPK signalling culminates in the phosphorylation and activation of the transcription factor Cph1, which in turn promotes the expression of HSGs (Figure 1) [33]. It has been shown that inactivation of the Ras1-Cyr1-PKA pathway inhibits filamentous growth in the majority of usual hyphae-inducing conditions, however, inactivation of the MAP-kinase pathway only prevents filamentous growth in only a specific subset of conditions [43].

The CaRas2 protein contains several variations in conserved motifs typically thought to be critical for Ras-related activities and is thus considered an unusual Ras protein. Sequence alignment using BLAST has shown that the *C. albicans* Ras2 protein only has 25-30% identity with all other fungal Ras proteins in the database except a Ras-like protein only found in *Candida dubliniensis* (80% identity) [47]. When *RAS2* is deleted in a *ras1*Δ/Δ background, intracellular cAMP levels are restored to approximately 30% of wild type levels (*ras1*Δ/Δ mutant has a 20x reduction in cAMP). Ras1 and Ras2 may therefore exhibit antagonistic roles in *C. albicans* [47]. This is intriguing since the deletion of *RAS2* in a *ras1*Δ/Δ background results in a significantly increased defect in hyphal morphogenesis. Nevertheless as *ras2Δ/Δ* mutants themselves exhibit normal hyphal development [47] the role of Ras2 in morphogenesis and pathogenesis in *C. albicans* has yet to be elucidated.

In addition to the signalling pathways which drive the yeast-to-hyphae switch, there are also negative regulators that are controlled by Ras signalling (Figure 1). Hyphal-specific genes are repressed by the global-repressor Tup1 [48] via the specific DNA-binding proteins Nrg1 [49][50] and Rfg1 [51]; the deletions of each of these three proteins results in *C. albicans* cells which are constitutively hyphal even under non-hyphal inducing conditions [48, 49, 50, 51]. Approximately half of the genes found to be upregulated during hyphal development in response to 37°C and serum are repressed by Tup1 and Nrg1 or Rfg1, suggesting that repression removal is a crucial step in the yeast-to-hyphae switch [52]. Consistently, it has been found that hyphal-inducing conditions such as serum exposure and growth at 37°C cause a reduction in the expression levels of *NRG1*, leading to the conclusion that one way in which repression of hyphal-specific genes is overcome during the yeast-to-hyphae switch is via down-regulation of the repressors [50]. Ras1-Cyr1-PKA pathway activation results in the prompt but short-term removal of Nrg1 from the promoters of hyphal-specific genes. The maintenance of this repression elimination, and hence hyphal development, is achieved through the subsequent recruitment of the Hda1 histone deacetylase which deacetylates a subunit of NuA4 histone acetyltransferase, causing it to also be removed from the promoter. This results in the coiling of the portion of the promoter containing the Nrg1 binding site, preventing the re-binding of Nrg1. It is important to note the removal of Nrg1 is an absolute prerequisite for the Hda1 recruitment [53].

**Ras signalling and white-opaque switching in *C. albicans***

*C. albicans* was traditionally considered to be asexual, only existing as an obligate diploid [54]. However, it has now been discovered that mating occurs between homozygous diploid mating type-like (*MTL*) **a** and α strains in this organism, producing an **a**/α tetraploid product [54, 55] which then undergoes 'concerted chromosome loss' to form diploid progeny [56]. *C. albicans* has also been reported to have a viable haploid state which can mate to restore the diploid form [57]. While this is similar to the mating program in *S. cerevisiae* (two haploid mating types; **a** and α which combine to generate an **a**/α diploid product) [58] it differs in several key respects.

Mating in *C. albicans* is reliant on a reversible phenotypic switch between two states termed 'white' and 'opaque'. Only the 'opaque' state is capable of mating efficiently; 'opaque' cells have been demonstrated to mate approximately $10^6$ fold more readily than 'white' cells [59]. 'White' cells are fairly round and form white, dome-shaped colonies on solid agar, they also express a specific set of genes. In contrast, 'opaque' cells tend to be larger and more oblong, forming darker colonies which grow flatter against solid agar. 'Opaque' cells also express a specific set of genes which differ from those expressed in 'white' cells [60].

This unusual mating program involving a reversible phenotypic switch hitherto seems to be unique to *C. albicans* as well as the very closely related fungal species *C. dubliniensis* [61]. It appears that 'white' cells are better suited for growth and survival within a mammalian host. Therefore, it is likely this unusual mating program has evolved to allow *C. albicans* to survive the variety of environments within a mammalian host while still being able to produce mating-competent cells [59].

Only *C. albicans* cells which are homozygous at the *MTL* locus (**a**/**a** or α/α) are capable of reversibly switching between 'white' and 'opaque' states, and are thus capable of efficient mating [55, 62]. This is because two homeodomain proteins called Mtl**a**1 and Mtlα2, encoded by the *MTL**a*** and *MTLα* alleles respectively, work together to inhibit white-opaque switching [59]. These proteins are both present in *MTL* heterozygous cells (**a**/α) and thus white-opaque switching cannot occur, only one of these two proteins is present in *MTL* homozygous cells, meaning white-opaque switching is not suppressed and mating (between **a**/**a** cells and α/α cells) can take place.

The transcription factor Wor1 is the master regulator of the white-opaque switch and acts in an all-or-nothing manner; it is virtually undetectable in white cells but highly expressed in opaque cells (expression is approximately 47-fold higher in opaque cells) [63]. Wor1 has been shown to control the expression of its own gene *WOR1* in either a positive feedback or double-negative feedback loop and drives the *C. albicans* cell into the opaque state [64]. Due to *WOR1* being repressed by the Mtl**a**1 and Mtlα2 proteins [63], Wor1 is not present in *MTL* heterozygous cells (**a**/α)

cells and ectopic *WOR1* expression in these cells causes them to undergo the white-opaque switch [64].

The Ras1-Cyr1-PKA pathway is known to have a role in the white-opaque switch. High levels of $CO_2$ can induce this switch; in 20% $CO_2$ the switch has been reported to occur with up to 105x more frequency compared to normal air [65]. In a *ras1*Δ/Δ mutant and a *cdc35*Δ/Δ mutant (which lacks the $CO_2$-responsive Cyr1 adenylate cyclase) white-opaque switching is reduced in both normal air and 1% $CO_2$ compared to wild-type but normal in 20% $CO_2$ [65]. This suggests signalling through the Ras1-Cyr1-PKA pathway is important for the switch in normal air and moderate $CO_2$ but not in very high levels of $CO_2$.

High N-acetylglucosamine levels also induce the white-opaque switch, and this switch in response to N-acetylglucosamine is significantly diminished from 90.5±3.8% cells in the wild-type to 11.2±1.5% in a *ras1*Δ/Δ mutant [66]. Likewise, in a *cdc35*Δ/Δ mutant, 8.0±3.5% of cells undergo the white-opaque response in the presence of N-acetylglucosamine compared to 86.9±4.3% in the wild-type [66]. These results suggest the N-acetylglucosamine switch occurs via signalling through the Ras1-Cyr1-PKA pathway. Furthermore, when the master switch regulator Wor1 is overexpressed in a *ras1*Δ/Δ, *cdc35*Δ/Δ, *tpk1*Δ/Δ or *tpk2*Δ/Δ background, the cells are driven into the opaque state. Conversely, a *wor1*Δ/Δ mutant does not switch in the presence of N-acetylglucosamine [66]. Wor1 contains a consensus PKA phosphorylation motif with a phosphorylatable threonine at residue 67 [64], and it has been demonstrated that this threonine is absolutely required for white-opaque switching in response to N-acetylglucosamine [66]. These results imply that the transcription factor Wor1 functions downstream of the Ras1-Cyr1-PKA pathway to induce white-opaque switching in the presence of N-acetylglucosamine.

In addition to Wor1 there are also other transcription factors which act as switch regulators, specifically; Efg1 (itself Ras1-regulated), Czf1, Wor2 [67] and Wor3 [68]. Binding sites for Wor1 have been identified upstream of *EFG1, CZF1* and *WOR2*, indicating that these transcription factors function in a regulatory circuit composing positive-feedback loops with the Ras1-regulated Wor1 in a central position [67].

## RAS SIGNALLING AND *C. ALBICANS* BIOFILM FORMATION

Biofilms are structured communities of microorganisms which are attached to either a living or non-living surface. The cells are often encased within a matrix of self-made extracellular polymeric substance (EPS); this EPS is composed of DNA [69, 70], lipids [69], proteins [69, 71] and polysaccharides [69]. Medically, biofilms are of particular importance because it is thought that a significant percentage of human microbial infections include biofilm formation [72, 73, 74]. Moreover, cells which reside within biofilms have distinctive phenotypes compared to planktonic cells, for example, they exhibit increased resistance to antibiotic and antifungal drugs. The reasons for this increased resistance are complex but include the presence of an extracellular matrix reducing the ability of antimicrobial agents to reach the cells, metabolic differences (such as modulation of glycolysis, ergosterol biosynthesis and mitochondrial respiration) [75] inherent to biofilms and upregulation of efflux pumps [76]. *C. albicans* biofilms are usually composed of a mixture of morphological forms; typically yeast, pseudohyphal and true hyphal cells are all present within a mature biofilm [6, 77, 78]. The formation of a biofilm is the result of a very precise and complex series of events that are divided into distinct stages; attachment, initiation, maturation and dispersal. Biofilm formation is therefore complex and highly regulated with more than 1000 genes found to be upregulated during biofilm development [79].

Hyphal cells are important for the formation of *C.albicans* biofilms, one reason for this is that the expression of several cell surface adhesins, such as Hwp1 and Eap1, is increased during hyphal growth. These adhesins are required for the initial attachment phase of biofilm formation, and as a result it means Ras signalling is strongly linked to their development [80]. This is highlighted by the finding that the hyphal-defective mutant *efg1*Δ/Δ is unable to form biofilms [81]. Rather than the true basal layer which wild-type *C. albicans* cells form, *efg1*Δ/Δ mutants produce very few surface-attached cells. Despite this the surface-attached mutant cells do display resistance to both fluconazole and amphotericin B [81]. These are important observations that suggest surface-adhesion is sufficient to induce an antifungal resistance response in biofilms [78].

The transcription factor Bcr1, which is upregulated by Tec1 (Figure 1) is an important regulator of *C. albicans* biofilm formation. The *bcr1*Δ/Δ mutant is unable to form biofilms and also cannot switch to hyphal growth under certain conditions. However, when present within mixed biofilms formed using wild-type cells, *bcr1*Δ/Δ mutant cells can form hyphae [82]. Interestingly, *bcr1*Δ/Δ hyphal cells themselves are unable to adhere to surfaces and initiate biofilm formation. Bcr1 upregulates a number of genes which encode cell wall proteins, including the adhesins Als1, Als3, and Hwp1 [79]. It is likely therefore that hyphal associated cell wall composition is crucial for biofilm formation. Ras1-Cyr1-PKA signalling is important in this respect as it regulates the expression of adhesins, such as Als1, via its control of the activity of the key transcription factors Efg1, Tec1 and Bcr1 [79]. cAMP/PKA signalling is also likely to impact upon biofilm formation with respect to $CO_2$ levels. For example, a local accumulation of $CO_2$ within *C. albicans* colonies was sufficient to induce filamentous growth [31]. Although the precise roles have yet to be determined, it will be interesting to examine how $CO_2$ signalling contributes to biofilm establishment *in vivo*. The final stage of biofilm development is the dispersal stage in which a mature biofilm begins to 'throw' fragments off in order to establish additional biofilms elsewhere [78]. The transcription factor Nrg1, whose degradation is inhibited by the *C. albicans* quorum sensing molecule farnesol, has been shown to promote biofilm cell dispersion [83]. As Ras activation can influence Nrg1 levels, and as farnesol has

been shown to promote Ras1 degradation it will be of interest to investigate how Ras signalling influences the biofilm dispersion process.

## RAS SIGNALLING AND VIRULENCE IN *CRYPTOCOCCUS NEOFORMANS*

The prototypical species in the *Cryptococcus* genus is *C. neoformans* which is an encapsulated, pleomorphic yeast [84]. Similar to *C. albicans, C. neoformans* is an opportunistic human pathogen with infection primarily being associated with a compromised immune system; cryptococcal infections (cryptococcosis) are a particular a problem amongst HIV/AIDS patients [85]. The Centers for Disease Control and Prevention estimates the number of deaths attributed to cryptococcal meningitis in HIV/AIDS patients is as high as 181000 per year [86]. *C. neoformans* possesses two *RAS* genes, denoted *RAS1* and *RAS2*, which encode the Ras1 and Ras2 proteins respectively [87]. The Ras1 protein is highly conserved: it is a homolog of the traditional Ras proteins in mammalian cells such as *H-RAS* [84] and has been demonstrated to be essential for multiple processes in *C. neoformans* including growth at 37°C (Ras1 is not needed for growth at 30°C), mating, agar adherence and filamentation [88, 89]. Due to its crucial role in these processes, particularly thermotolerance, Ras1 is considered as an important virulence factor. Indeed, the Δ*ras*1 mutant strain is avirulent in a rabbit model of cryptococcal meningitis [88]. Moreover, introduction of a dominant active *RAS1* allele (*RAS1*$^{Q67L}$), which was constructed by introducing a point mutation in the active site of the GTPase domain of Ras1, resulted in significant increases in filamentation and agar invasion of haploid *C. neoformans* cells [88]. These are two properties which are very important for virulence, supporting the conclusion that Ras1 signalling is vital to the pathogenicity of *C. neoformans*. It is important to note that in other model systems, including *Caenorhabditis elegans* [90] and *Drosophila melanogaster* [91], the Δ*ras1* mutant strain exhibited decreased virulence at lower temperatures. This implies that, at least for non-mammalian model systems, Ras1 signalling may have significant functions in the pathogenicity of *C. neoformans* besides thermotolerance.

Ras signalling in *C. neoformans*, much like in mammalian cells, acts through and/or in concert with other Rho-type GTPases such as Cdc42 and Rac [89, 92, 93, 94, 95]. As is the case with *RAS*, *C. neoformans* possesses duplicate copies of *CDC*42 (*CDC*42 and *CDC*420) [92] and *RAC* (*RAC*1 and *RAC*2) [93]. The overexpression of any of these genes overcomes the thermotolerance deficiencies of the Δ*ras*1 mutant, supporting a model whereby these GTPases act downstream of Ras1 (Figure 2) [89, 95]. At 37°C, Δ*ras*1 *C. neoformans* mutants amass a number of defects in cell cycle progression, specifically concerning cell polarisation and cytokinesis. While neither is required for viability when the organism is not under stress, the two Cdc42 paralogues are essential for septin organisation and effective cytokinesis at 37°C [92]. Ras1 is also important in the activation of Cdc42 and Rac [94]. Indeed, Cdc24 which is the reported GEF for Cdc42, undergoes a GTP-dependent physical interaction with Ras1 [89, 96]. Once activated, Cdc42 organises the septin proteins Cdc3, Cdc10, Cdc11 and Cdc12, causing them to localise to the bud neck ready for cytokinesis [94]. In addition to septin protein organisation, Ras1 signalling through Cdc42 is also necessary for normal bud morphology [94]. Cdc42 appears to be the more important to *C. neoformans* virulence since it, and not Cdc420, is upregulated during temperature stress and is necessary for virulence in a mouse model of *Cryptococcus* infection [92]. As previously mentioned, Ras1 is not required for growth of *C. neoformans* at 30°C. The reason for this appears to be due to basal levels of Cdc42 activity even in the absence of Ras1 signalling which is sufficient to allow proliferation at this lower temperature [92, 94].

Although functional redundancy may exist between Cdc42 and Rac, the two Rac paralogues in *C. neoformans* are predominantly involved in cell polarisation and polarised growth; particularly in hyphal development when mating and in the transport of vesicles during the yeast phase of growth [93, 95, 97]. The Δ*rac*1 mutant strain, while still able to grow at 37°C, displays a significant defect in haploid filamentation along with reduced mating [95]. Δ*rac*1 and Δ*rac*2 mutant strains have increased yeast cell size [93], in keeping with the phenotype exhibited by Δ*ras*1 cells when grown at 37°C [88] as well as that shown by polarity mutants of the prototypical budding yeast *S. cerevisiae* [98]. This highlights the important role Ras1 signalling through Rac1 and Rac2 plays in cell polarity and this precisely impacts hyphal development and yeast cell size.

As with *C. albicans*, the ability to sense $CO_2$ is of critical importance to *C. neoformans*. Capsule biosynthesis, which is a major virulence characteristic, is increased by high $CO_2$ levels and this is mediated by the *C. neoformans* adenylate cyclase Cac1 [20]. Importantly, it has been demonstrated that a fragment of CnCac1 can restore hyphal development in response to elevated $CO_2$ within a *cyr1*Δ/Δ *C. albicans* mutant. This implies the link between $CO_2$ sensing and cAMP could be a general feature of pathogenic yeasts since *C. neoformans* and *C. albicans* are evolutionarily distantly related but their adenylate cyclases are functionally highly conserved [20].

The *C. neoformans* Ras2 protein plays separable roles to those of Ras1. For instance, Δ*ras*2 cells do not exhibit reduced virulence in a mouse model of cryptococcosis [87]. Furthermore, Δ*ras*2 cells do not exhibit the same mating defects associated with the loss of *RAS1* [87]. *RAS*2 is also found to be expressed at low levels compared to *RAS*1 and Δ*ras*2 mutants do not display any differences in growth or differentiation when compared to wild-type [87]. However, it is worth noting that overexpression of *RAS*2 in a Δ*ras*1 mutant is able to rescue the mating defect and partially restores its growth at high temperature [87]. Moreover, the double Δ*ras*1Δ*ras*2 mutant has growth defects at all temperatures which are worse than with either single mutation alone [87]. These findings suggest that Ras1 and Ras2 protein may have some overlapping functions in growth, mating and virulence that have yet to be properly defined.

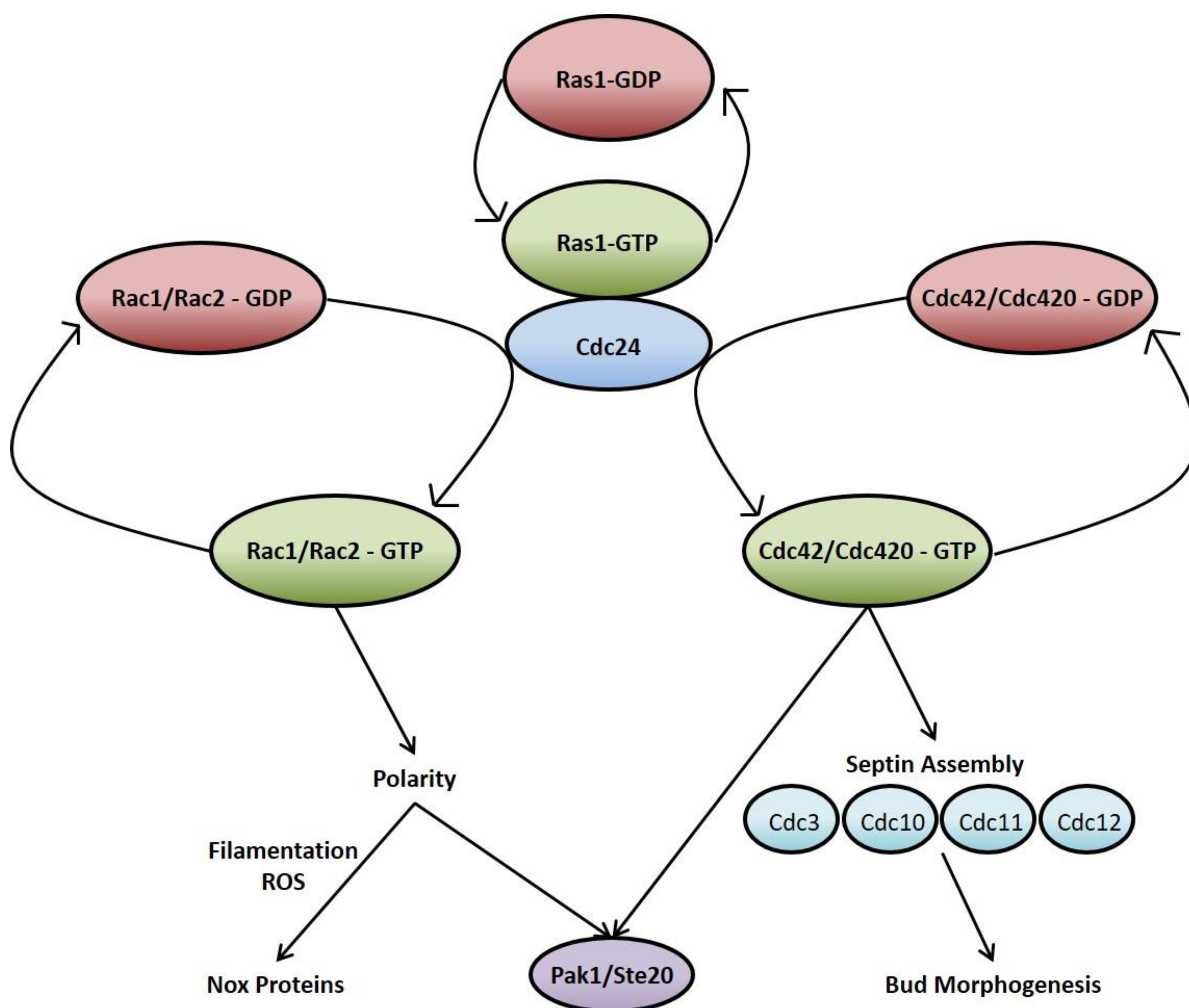


**FIGURE 2: Ras1 signals via Cdc42 and Rac proteins to regulate many processes within the *C. neoformans* cell.** Upon activation Ras1 associates with Cdc24 and subsequently activates the Rho-type GTPases Rac1/Rac2 and Cdc42/Cdc420. These proteins are involved in multiple cellular processes such as cell cycle progression, mating and morphogenesis.

## TARGETING RAS TO COMBAT YEAST PATHOGENESIS

Given its role in the virulence and pathogenic properties of multiple yeast (and other fungal) species, it would seem to have potential as anti-fungal drug target. Additionally, it has been shown that the manipulation of Ras signalling is an important control point in the activation of apoptosis in the budding yeast *S. cerevisiae* [99, 100, 101] and in *C. albicans* [102]. These findings present the possibility that the pharmacological manipulation of Ras signalling may be useful in the induction of yeast cell death. Some current antifungals do exert an effect on fungal Ras signalling but this is indirect. For example, amphotericin B inserts into ergosterol-containing fungal membranes to form aqueous pores [103] which cause a local thinning of the bilayer. This thinning of the bilayer has been proposed to force lipid-anchored Ras proteins into sterol-rich lipid rafts, promoting its interactions with downstream proteins and thus activating signalling [104]. Enhanced Ras signalling through pharmacological manipulation induces yeast apoptosis and oxidative damage via the cAMP-PKA pathway, indicated by the fact *S. cerevisiae* Δ*ras1,* Δ*ras2*, Δ*tpk1*, Δ*tpk2* and Δ*tpk3* mutants have reduced amphotericin B-induced reactive oxygen species production and hence are unaffected by the lethal effect of amphotericin B [105]. Other fungicides such as miconazole and ciclopirox have been reported to cause fungal cell death via a similar mechanism [105].

Ras proteins have proven difficult to directly target pharmacologically and because the GTPase domain is very highly conserved any attempt to target a fungal Ras may result in unfavourable side effects upon the host. Encouragingly a recent discovery does suggest that fungal Ras proteins may be targetable after all. A recent study demonstrated that the RasA protein of the human fungal pathogen *Aspergillus fumigatus* possesses a short N-terminal tail domain which is missing from Ras homologs in higher eukaryotes [106]. This domain takes the form of a

short length of amino acid residues which terminates in an arginine and has been dubbed the invariant arginine domain (IRD). Through sequence alignment, it has been reported that the IRD is present in many different fungal pathogens including *C. albicans* and *C. neoformans*, as well as non-pathogenic fungi such as *S. cerevisiae* and *S. pombe* [106]. Mutation of the *A. fumigatus* RasA IRD decreased the activation of PKA as well as reducing the interaction of RasA with Cdc42 [106] which controls polarity and thus is essential for normal growth and cell division [107]. In line with this, mutational analyses confirmed that the IRD is necessary for polarised morphogenesis, a characteristic strongly linked with pathogenesis in *A. fumigatus*, and asexual development [106]. These findings point towards the possibility of designing drugs to specifically target the IRD as a new Ras-based pan-antifungal therapy. As an extension of this it may also be possible to target regions within the C-terminal variable domain of fungal Ras proteins to modulate its activity.

## CONCLUSIONS

Ras signalling has proven to be an important component of the growth and adaptability of fungal cells, as is indeed the case in higher eukaryotes. It is also crucial to the virulence and pathogenic properties of fungal species. Ras signalling therefore represents an interesting therapeutic target if fungal specific targets can be found. This may be particularly effective when used in combination with other antifungal agents. A full understanding of the Ras signalling network and its effectors will be required to achieve this aim. As an example, it may be possible to identify a small molecule that can activate fungal Ras in *C. albicans*. This may, at first glance, seem an unlikely intervention given the role of Ras signalling in promoting growth. However, as we now also know that the activation of Ras sensitizes cells to cell death, such an approach may prove particularly effective when used in combination with existing antifungals. Given the rise of antifungal resistance and our limited number of existing targets such "knowledge based" approaches will doubtless prove crucial in future therapy development.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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