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# Ras-Mediated Signal Transduction and Virulence in Human Pathogenic Fungi

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## Abstract

Signal transduction pathways regulating growth and stress responses are areas of significant study in the effort to delineate pathogenic mechanisms of fungi. In-depth knowledge of signal transduction events deepens our understanding of how a fungal pathogen is able to sense changes in the environment and respond accordingly by modulation of gene expression and re-organization of cellular activities to optimize fitness. Members of the Ras protein family are important regulators of growth and differentiation in eukaryotic organisms, and have been the focus of numerous studies exploring fungal pathogenesis. Here, the current data regarding Ras signal transduction are reviewed for three major pathogenic fungi: *Cryptococcus neoformans*, *Candida albicans* and *Aspergillus fumigatus*. Particular emphasis is placed on Ras-protein interactions during control of morphogenesis, stress response and virulence.

**Keywords:** Ras; GTPase; Fungal morphogenesis; Fungal virulence

## Introduction

In recent years, a multitude of signal transduction pathways have been identified as regulators of morphogenesis in fungi. Due to their control over cell shape and response to environmental change, many of these pathways have also been associated with virulence attributes of the pathogenic fungi. Of the major signaling modules that fit this paradigm, Ras pathways have received considerable attention. The Ras super-family is composed of membrane-associated GTPase proteins serving as major signal transduction elements in eukaryotic cells [1]. Ras proteins are considered to be "molecular switches" existing in active and inactive states, bound to either Guanosine Triphosphate (GTP) or Guanosine Diphosphate (GDP), respectively. Ras is activated by interaction with Guanosine Nucleotide Exchange Factors (GEFs) and inactivated by interaction with GTPase Activator Proteins (GAPs). In mammalian cells, interaction of Ras proteins with specific GAPs, GEFs, and downstream effectors is, in part, controlled through sub-cellular localization and compartmentalization of Ras to distinct membrane signaling platforms [2]. Differential sub-cellular localization of mammalian Ras proteins is accomplished through post-translational farnesylation of the "CAAX" motif (C=cysteine, A=aliphatic amino acid, X=any amino acid) that serves as a recognition sequence for farnesyltransferase enzymes [3]. Once prenylated at the CAAX box cysteine residue, Ras is further processed by a Ras Converting Enzyme (RCE) on the endoplasmic reticulum. This maturation step catalyzes the proteolytic cleavage of the "–AAX" residues from the CAAX motif [4]. The carboxy terminus is then carboxymethylated by action of an isoprenylcysteine carboxymethyltransferase [5,6]. In mammalian cells, mature Ras proteins then follow one of two paths to the plasma membrane; 1) Ras homologs with a conserved cysteine residue in the hypervariable domain are palmitoylated by a palmitoyltransferase and trafficked through the secretory system [7-10], or 2) non-palmitoylated Ras homologs can traffic to the plasma membrane through a non-classical pathway and become associated with the plasma membrane via a poly-basic domain just upstream of the CAAX motif [11]. Ras palmitoylation is reversible in mammalian systems and plasma membrane localized Ras has been shown to cycle back to the endo-membrane system through the activity of a thioesterase located at the plasma membrane [12,13]. As subjects of both temporal and spatial control of activity, the Ras GTPases function as binary switches that, despite their apparent simplicity, control activity of multiple pathways within the mammalian cell.

Due to their central role in cancer biology, mammalian Ras proteins have been studied to a great extent and much is now known of the molecular mechanisms regulating Ras-mediated malignancy. The pivotal role of Ras in cancer formation and progression is, in large part, due to their multifunctional nature as regulators of cellular development and differentiation. The existence of functional Ras homologs in fungi was first realized in the model yeast organism, *Saccharomyces cerevisiae*, where two Ras proteins were identified: Ras1p and Ras2p. These two Ras homologs are thought to have evolved from ancient genome duplication [14]. As such, Ras1p and Ras2p are highly homologous at the amino acid level and appear to play overlapping roles in *S. cerevisiae* developmental processes. Ras2p is expressed at significantly higher levels than Ras1p, a theme present in other fungi containing two Ras homologs [15]. Ras2p was initially found to be necessary for growth on nonfermentable carbon sources, entry into the cell cycle during germination, and for completion of mitosis when deleted along with Ras1p [16,17]. Importantly, a conserved role for Ras in polarized growth processes among yeast organisms has also been observed. In response to either nitrogen starvation or excess glucose, diploid *S. cerevisiae* strains undergo a "filamentous growth" process whereby the yeast cells elongate and begin to bud in a unipolar fashion to form long strands [18,19]. *S. cerevisiae* haploid cells also convert to a polarized phenotype, called haploid invasive growth, after prolonged incubation on rich media [20]. These strands are not "true" hyphae, but they are considered a model for the sustained polarized

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growth seen in filamentous organisms. In *S. cerevisiae*, deletion of Ras leads to defects in the induction of filamentous growth under both conditions. Expression of a constitutively active form of Ras2p also causes cytoskeletal defects, evident by actin mislocalization, and induces the filamentous growth phenotype [21]. Like their human homologs, Ras2p localizes to the plasma membrane of *S. cerevisiae*. This specific localization is important for Ras signal transduction, as mislocalization of a constitutively activated Ras2p abrogates activated Ras2p phenotypes [22]. In the fission yeast, *Schizosaccharomyces pombe*, the sole Ras homolog, Ras1p, controls aspects of actin and microtubule assembly [23]. Therefore, Ras1p plays a major role in regulating cell morphology and size. *S. pombe ras1* null cells are sterile, display abnormal cell shape and lack the ability to undergo polarized growth [24].

Yeast Ras pathway components are primarily composed of Cdc42 and mitogen-activated protein kinase (MAPK) pathways in *S. cerevisiae* and *S. pombe*, as well as the cyclic AMP (cAMP) / Protein Kinase A (PKA) pathway in *S. cerevisiae*. In *S. cerevisiae*, Ras2p is activated by the GEF, Cdc25p, and is negatively regulated by the GAPs, Ira1p and Ira2p [25,26]. Active Ras2p proteins bind to and activate adenylate cyclase to produce cAMP, in turn activating the PKA pathway [27,28]. The *S. cerevisiae* PKA pathway has been implicated in numerous cellular activities and is overall known to be a negative regulator of the general stress response. Therefore, constitutive activation of Ras2p leads to the suppression of stress responses in *S. cerevisiae* causing increased heat shock and nitrogen starvation sensitivity [29]. However, the Ras2 null mutant displays a temperature sensitive actin polarization defect that cannot be suppressed by over-expression of PKA pathway components [29]. These findings suggest a PKA-independent, Ras-mediated actin control. To this end, Ras2p also regulates a MAPK pathway controlling the polarized morphogenesis observed in both diploid and haploid cells under stress. Here, Ras2p signals through Cdc42p to the Ste20 protein kinase [15,30]. Another mediator of Ras2p signal transduction is the GEF-like protein, Lte1p. The Lte1p protein is a regulator of mitotic exit, coupling mitosis to polarized morphogenesis in *S. cereivisae* [31]. Initially, the role of Lte1p was believed to be as a GEF for the mitotic exit network protein, Tem1p, but has since been reported to act as a direct inhibitor of Ras2p activity in areas of cell division [31]. These findings are very intriguing, as GEF proteins are normally considered to be positive regulators of Ras activity.

In contrast to *S. cerevisiae* Ras2p function, *S. pombe* Ras1p is not necessary for activation of the PKA pathway [24]. Instead, Ras1p regulates primarily a mating MAPK pathway including the kinases Byr2p, Byr1p, and Spk1p [32,33]. Ras1p also interacts with Scd1p, a putative GEF for Cdc42p [34]. Through Scd1p, Ras1p controls cytoskeletal change and cell shape likely by modulation of Cdc42p activity levels [23]. In *S. pombe*, Ras1p activity is positively regulated by two known GEFs, Ste6p and Efc25p, and as in *S. cerevisiae*, localization of the Ras1p is important for its regulation. For example, Ras1p that is restricted to endo-membranes interacts with Efc25p and signals through the Cdc42-mediated pathway to control cell morphology [35]. In contrast, Ras1p restricted to the plasma membrane is controlled by Ste6p and signals through the MAP kinase pathway to mediate the mating response [35]. Through identification of Ras-controlled molecular mechanisms, the yeast model systems laid the early groundwork required for investigation of Ras function in growth, response to stress and virulence of pathogenic fungi.

## Roles of Ras Proteins in Fungal Morphogenesis, Stress Response and Virulence

### *Cryptococcus neoformans*

*Cryptococcus neoformans* is a pleomorphic yeast and is a model organism for studying signal transduction in fungal growth and virulence. Infection with *C. neoformans* encapsulated yeasts primarily manifests in immunocompromised patients, although another species, *C. gattii*, is known to infect immunocompetent individuals and was recently associated with an outbreak in the pacific northwest [36]. In response to specific environmental signals, *C. neoformans* can undergo two forms of yeast-to-hyphal transition. For mating, strains of opposite mating types will form filamentous structures under nutrient deprivation [37]. In addition, strains of the MATα mating type can undergo a process termed "haploid fruiting", wherein filaments are formed during the production of basidiospores [38]. Signal transduction events regulating these processes have been the focus of intense study in fungal development [39].

The Ras1 gene of *C. neoformans* was originally identified by homology to known Ras proteins and was found to be a major regulator of high temperature growth, differentiation and virulence [40,41]. The Ras1 protein is a homolog of the proto-typical Ras proteins from mammalian cells, including H-ras, which has domains required for binding of GTP / GDP, association with GAP and GEF regulatory proteins, interaction with downstream effector proteins, and domains for post-translational modification with prenyl groups (CAAX box) and palmitoyl moieties (dual cysteine motif). Deletion of *C. neoformans Ras1* causes poor growth at 37°C and complete inhibition of growth at 39°C [40]. However, shorter periods of growth at high temperature showed that the *ras1* mutant cells are not inviable, but only growth arrested, after short incubations at high temperatures. Growth is recovered in the *ras1* mutant upon switching to incubation at 30°C [40]. Inhibition of growth at 37°C is associated with the inability of the *ras1* deletion mutant to properly polarize actin. The *ras1* mutant displays properly localized yet depolarized actin in large, unbudded cells at 37°C [42]. These findings suggested a role for *C. neoformans RAS1* in maintaining proper cell morphology under stress, likely through regulation of actin dynamics required for proper cellular morphology and bud formation. Since growth at high temperatures is required for *C. neoformans* virulence, the *ras1* mutant is also less virulent in a rabbit model of cryptococcosis [40]. However, *Ras1* deletion does not result in differences in capsule size or melanin production [40]. As these are major *C. neoformans* virulence factors, the lack of virulence in the *ras1* mutant is attributed mainly to decreased growth at 37°C. *Ras1* was later shown to be important for serum-induced capsule growth in vitro however, as many factors affect capsule induction in *C. neoformans*, this is likely not the major mechanism through which Ras1 signaling impacts virulence [43,44]. Although support of cellular growth under heat stress is considered the major role of *Ras1* during infection in mammalian models, the *ras1* mutant also displays decreased virulence at low temperature in the non-mammalian models *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Galleria mellonela* [45-47]. These findings suggest that, at least for these non-mammalian models, *Ras1* may play roles in *C. neoformans* virulence that go beyond the control of growth under temperature stress.

Much like *S. pombe Ras1*, the *C. neoformans Ras1* gene also plays important roles in mating and haploid fruiting, two processes requiring yeast-to-hyphal differentiation [24,40,42,48]. *C. neoformans* can be induced to mate when opposite mating type strains are cocultured

under nutrient starvation [49]. Under these conditions, yeast cells undergo a sustained polarized growth allowing opposite mating type filaments to fuse and form basidia. When the *ras1* mutant was cultured in mating conditions with a competent mating partner, no hyphal projections are observed and no recombinant basidiospores are formed [40]. These findings indicated that *Ras1* is essential for mating in *C. neoformans*. Subsequent mechanistic studies to address how *Ras1* impacts mating and hyphal growth showed that the *ras1* mutant is defective in both pheromone production and in response to a competent mating partner [40,50]. Further supporting a role in mating, Ras1 plays a critical function in transcriptional control of pheromone response genes, including: *MFα1*, encoding alpha mating pheromone; *CPRα1*, encoding pheromone receptor, a G-protein coupled receptor; and *GPB1*, encoding the Gβ protein subunit of the pheromone receptor [50,51-53]. Similar to the sexual differentiation that occurs in the presence of a mating partner, certain *C. neoformans* strains can also undergo asexual differentiation, called haploid fruiting, when cultured under nitrogen starvation conditions [38]. This differentiation again results in a yeast-to-hyphal transition, forming hyphae that are similar to, but distinct from, mating filaments. Deletion of *Ras1* causes the inability of *C. neoformans* to form hyphae on filament agar, whereas expression of a constitutively active *Ras1* (*Ras1*$^{Q67L}$) in the H99 genetic background leads to robust haploid fruiting [40,50]. In addition, hyphae formed by the *Ras1*$^{Q67L}$ mutant adhered to and invaded the agar to a greater extent than the wild type or *ras1* mutant strains [40]. Together, these data identified roles for *RAS1* in *C. neoformans* cellular morphology, specifically yeast cell shape and polarized morphogenesis during yeast-to-filament transitions. The *C. neoformans* genome also encodes a second Ras homolog, *Ras2*, which is expressed at very low levels compared to the *Ras1* gene [42]. Deletion of *RAS2* causes no notable defects, but deletion of both Ras genes causes defects more severe than deletion of either alone [42]. In addition, *Ras2* over-expression can partially suppress the *ras1* mutant growth and mating defects [42]. These findings suggested that, although Ras1 and Ras2 have both shared and a distinct role in growth, Ras1 is the predominant Ras protein controlling growth and virulence of *C. neoformans*.

Early attempts to identify Ras pathway signaling components revealed roles for cAMP and pheromone MAPK-pathway components in mating, but not haploid filamentation or growth at 37°C. Addition of exogenous cAMP or over-expression of *GPB1* or *MFα1* are each able to fully or partially suppress the *ras1* mating defect, whereas none of these conditions can rescue the *ras1* high temperature growth defect [40,50]. None of these conditions can suppress the *ras1* defect in haploid fruiting [50]. These data informed a model which places *C. neoformans* Ras1 upstream of the pheromone response pathway for control of mating, but did not identify Ras1 signaling components important for high temperature growth and, therefore, virulence. The initial clue towards uncovering Ras1 pathways regulating high temperature growth and morphology in a mating pathway-independent manner came from a multicopy suppressor screen that identified Rac1, a Rho-family protein, as a suppressor of the *ras1* high temperature growth defect [54]. Interestingly, Rac1 over-expression is also able to suppress the *ras1* mating defect [54]. Deletion of *RAC1* causes deficient filament production on filamentation agar and under mating conditions, and this hyphal deficiency is not associated with reduced pheromone production, ineffective cellular fusion, or basidium formation and sporulation [54]. Hyphae that are produced from *RAC1* mutant mating cell fusion display aberrant morphology without disrupted actin localization [54]. Together, these results suggested that Ras1 sends signals downstream to a second GTPase protein, Rac1, to control aspects of high temperature growth and polarized morphogenesis. Ras subfamily proteins have been shown to operate in GTPase signal transduction cascades to regulate gene expression and modulate cytoskeletal events for drastic morphogenetic changes in a variety of organisms, including fungi. These cascades commonly involve Rho-family proteins like Rac and Cdc42. In addition to Rac1, *C. neoformans* contains two Cdc42 paralogues; *CDC42* [previously *DCH2*] and *CDC420* [previously *CDC42*] [55,56]. Several lines of evidence have recently emerged demonstrating the importance for Ras-mediated Cdc42 function in high temperature growth and cellular morphogenesis of *C. neoformans*. First, Cdc24, a putative GEF for Cdc42, physically interacts with Ras1 in a yeast-two-hybrid assay in a GTP-dependent manner [56]. This finding indicates that the Ras1-Cdc24 interaction occurs preferentially when Ras is in an active state, suggesting a functional interaction. Deletion of Cdc24 causes decreased growth at 39°C, an actin polarization defect that mimics the *ras1* mutant at high temperatures, and loss of pathogenicity [56]. Ras1 has been shown to signal through Cdc24 as deletion of *CDC24* does not further exacerbate *ras1* phenotypes and over-expression of *Ras1* in the *cdc24* mutant does not abrogate the *cdc24* high temperature growth defect [56]. These epistasis experiments place Cdc24 downstream of Ras1 in a common signaling pathway. In addition, over-expression of either Cdc42 paralogue can at least partially suppress the *ras1* growth defect, while deletion of Cdc42, alone or in combination with Cdc420, causes phenotypes similar to the *ras1* mutant, including; decreased growth at 37°C [*cdc42Δ*, *cdc42Δ cdc420Δ*], increased sensitivity to latrunculin B [*cdc42Δ*, *cdc42Δ cdc420Δ*], defective actin polarization [*cdc42Δ cdc420Δ*], and loss of virulence [*cdc42Δ*] [55]. Taken together, these results support the hypothesis that the Rho-family proteins, Rac1, Cdc42, and Cdc420, function downstream of Ras1 to coordinate morphogenesis, high temperature growth and virulence in *C. neoformans*. What proteins function further downstream of these Ras1 effectors to mediate growth and virulence? Among as yet unidentified effectors, downstream signals are known to involve: 1) the protein-activated kinase homologs, Ste20 and Pak1 [54-56]; 2) septin proteins important for actin organization during cytokinesis [55,57]; and 3) Wsp1, a WASP protein important for the promotion of actin assembly [58,59].

Fungal Ras proteins, like their human counterparts, contain a C-terminal CAAX motif for farnesyltransferase recognition and many also contain predicted palmitoylation motifs. For example, Ras proteins of *S. cerevisiae*, *S. pombe* and *C. albicans* contain a single cysteine, located just up-stream of the CAAX motif, together called "CCAAX", as a predicted palmitoylation site [60]. In contrast, *C. neoformans* and the mould organisms, like *A. fumigatus*, contain a dual-palmitoylation motif with tandem cysteines [60]. Among the human pathogenic fungi, studies with *C. neoformans* were the first to report the importance of properly localized Ras protein for activation of pathways required for growth and virulence. When intact, lipidation of the C-terminal domains aids the Ras1 protein in associating mainly with the plasma membrane [60]. Mutation of the prenylation residue, residing in the highly conserved CAAX-box, mislocalizes Ras1 to the cytosol where it is unable to complement the morphogenesis and mating defects associated with the *ras1* mutant [60]. In contrast, the dual palmitoylation residues present just upstream of the *C. neoformans Ras1* CAAX box are differentially important for Ras1 function in morphology, virulence, and mating. Whereas mutation of one palmitoylation residue has no observable effect on Ras function, mutation of both residues results in a Ras1 protein that is non-functional for growth at 39°C but is competent for development of mating filaments [60]. As expected

from its inability to suppress the *ras1* high temperature growth defect, the palmitoylation-deficient Ras1 protein is unable to support full virulence in a murine model of cryptococcosis [60]. Although the effect of mislocalization is profound, the exact mechanism behind the loss of Ras function remains unclear.

### *Candida albicans*

Ras subfamily proteins also play an essential role in morphogenesis and virulence in the yeast pathogen, *Candida albicans*. *C. albicans* is a human commensal yeast that can be associated with numerous forms of disease ranging from superficial to systemic infections, based largely on the immune status of the host. Unlike many yeasts, *C. albicans* has the unique ability to grow as budding cells, as strands of elongated yeast cells (pseudohyphae), and as "true" hyphae, containing parallel sidewalls and septa [61]. This variation in morphology is considered a virulence attribute, as yeast that cannot undergo the yeast-to-hyphae transition display reduced pathogenicity [62,63]. Multiple signal transduction pathways have been identified as regulators of the *C. albicans* yeast-to-hyphal transition and the Ras pathway is a major contributor.

The *C. albicans* Ras gene, *Ras1*, was cloned via its ability to suppress the viability defect of the *ras1/ras2 S. cerevisiae* mutant [64,65]. However, unlike *S. cerevisiae*, the *C. albicans* genome only contains one proto-typical Ras homolog [64,65]. The predicted *C. albicans* RAS1 gene product, Ras1p, contains conserved cysteine residues for prenylation and palmitoylation, making Ras1p more similar to *S. cerevisiae* Ras1p/Ras2p and *C. neoformans* Ras1, than to the *C. neoformans Ras2* gene product. Deletion of *Ras1* is not lethal in *C. albicans*, but the mutant exhibits slower growth and does not respond to serum by formation of hyphae or germ tubes [64,65]. In addition to loss of serum-induction, *Ras1* deletion causes the inability to form hyphae under multiple conditions that induce a yeast-to-hyphae transition in *C. albicans* [65]. In contrast, expression of an activated form of Ras1p encourages the morphological transition of *C. albicans* from yeast to hyphae. Early studies showed that a mutant expressing a dominant active *ras1G13V* mutation produces hyphal projections in a shorter time period than the wild type, when grown on solid agar under hyphal-inducing conditions [64]. Later studies revealed that constitutively active Ras1p induces constitutive hyphal growth, even under non-hyphal-inducing conditions [65]. Similar to *S. cerevisiae*, the activated Ras1p strain is also more sensitive to heat shock and displays decreased accumulation of glycogen [64]. In accordance with the *S. cerevisiae*, *S. pombe* and *C. neoformans* data, these early studies supported a role for the Ras pathway in morphological transitions to polarized growth. Since *Ras1* controls the dimorphic switch from yeast to hyphae, and the hyphal form of *C. albicans* is associated with pathogenicity, the *Ras1* null strain was further hypothesized to control virulence. This hypothesis was supported by studies that revealed increased survival of mice infected with the *ras1Δ* mutant in an intravenous mouse model of candidiasis [65]. *Ras1* null cells isolated from the kidneys displayed short deformed yeast while wild type and complemented strains were fully formed hyphae [65]. In addition, *in vitro* culture of the homozygous *ras1Δ* mutant with murine primary macrophages revealed the inability of *Ras1* null cells to form hyphae and destroy macrophages after ingestion [65]. These data indicate that, like *C. neoformans* Ras1, Ras1p regulates hyphal development and virulence in *C. albicans*. Mating in *C. albicans* requires a morphological switch from small, white yeast cells to a larger, more elongated opaque phenotype [66,67]. Although the opaque phenotype is unstable at 37°C *in vitro*, physiological levels of $CO_2$ in human tissue are able to stabilize the opaque phenotype at body temperature, arguing that mating may occur in the human host [68]. Recent studies have identified N-acetylglucosamine (GlcNAc), a carbohydrate produced by human gastrointestinal tract bacteria, as an inducer of the white-to-opaque phenotype switch in *Candida species* [69,70]. However, white yeast cells bearing a *Ras1* homozygous null mutation display greatly decreased rates of switching to the opaque phenotype, indicating that Ras1p is a major regulator of this morphological change [69]. Interestingly, GlcNAc is also an activator of Ras1p-dependent hyphal growth in *C. albicans* [71]. These reports highlight the versatility of the Ras1p pathway during response to environmental signals inducing either sexual differentiation or polarity.

In contrast to *C. neoformans* Ras1 function, many of the phenotypic outcomes of Ras signaling in *C. albicans* are mediated by the cAMP-activated PKA pathway. This connection was originally made in *S. cerevisiae* and subsequently found to be conserved in *C. albicans*. Deletion of *CDC35*, the sole adenylate cyclase of *C. albicans*, leads to loss of detectable cAMP levels, slow growth and the inability to undergo the yeast-to-hyphae morphological transition [72]. In a strain lacking the both *CDC35* alleles, constitutive activation of Ras1p is unable to induce the yeast-to-hyphae transition, placing Ras1p upstream of Cdc35p and the PKA pathway [72]. In addition, deletion of *EFG1*, a major transcription factor downstream of the PKA pathway, blocks hyphal induction mediated by constitutively activated Ras1p [65]. The PKA protein complex, composed of two active subunit isoforms [Tpk1p and Tpk2p] and one regulatory subunit isoform [Bcy1p], is known to control response to stress and is a major regulator of *C. albicans* virulence [73]. Therefore, as a major regulator of cAMP production, Ras1p is a predominant mediator of morphogenesis and stress response in *C. albicans* via control of PKA activity. Interestingly, the Ras-cAMP-PKA pathway also controls the timing of Programmed Cell Death [PCD] in *C. albicans*. In response to environmental stressors, *C. albicans* cells can enter a pattern of PCD that bears some similarity to apoptotic patterns of mammalian cells [74]. Constitutive activation of Ras1p speeds up the transition to death, whereas a *Ras1Δ* strain is delayed in this transition [74]. Stimulatory or inhibitory mutations in adenylate cyclase and PKA have similar effects, suggesting that suppression of the general stress response pathway through over-activation of the Ras-PKA pathway accelerates PCD in *C. albicans* [74]. A connection between Ras signaling and PCD has not been reported in other human pathogenic fungi, though apoptotic-like PCD processes have been explored in *C. neoformans* and *A. fumigatus* [75-77].

*C. albicans* was recently reported to contain a second Ras homolog, *RAS2*. However, Ras2p is an atypical Ras homolog lacking the majority of the conserved G box elements found in proto-typical Ras proteins [78]. Despite the differences, Ras2p was found to be an active GTPase and may play an antagonistic role in cAMP production regulated by Ras1p [78]. No further studies have revealed potential interaction or crosstalk between Ras1p- and Ras2p-controlled pathways.

Similar to the other yeasts described above, Ras1p also signals through a Cdc42-dependent pathway to control morphogenetic changes in response to environmental signals. Cdc42p, and it's exchange factor, Cdc24p, are essential for vegetative growth in *C. albicans* due to a dual role in regulation of filamentation and mitosis [79,80]. However, using mutants previously described in *S. cerevisiae* that separate Cdc42's roles in these two processes, one study has generated filamentation-specific mutations that do not affect Cdc42p's role in completion of mitosis [80]. These mutant strains were found to have reduced expression of Efg1p transcription factor-dependent genes involved in the yeast-to-hyphae transition. Some of these

mutants also displayed reduced ability to damage endothelial cells, indicating the importance for Cdc42-mediated morphogenesis in *C. albicans* virulence [80]. Evidence of Ras-mediated control over Cdc42p signaling in *C. albicans* was shown when over-expression of *HST7*, a Cdc42-regulated MEK kinase, or *CPH1*, a Cdc42 pathway transcription factor, were able to partially suppress a *ras1Δ* filamentation defect [65]. In addition, deletion of either *HST7* or *CPH1* is able to block filamentation induced by expression of a constitutively active Ras1p [65]. These studies place Ras1p upstream of the Cdc42 pathway, similar to previous studies on Ras signaling in *S. cerevisiae*. One study has shown that Ras1p also directs actin patch and cable formation to regulate endocytosis and secretion during hyphal growth [81]. These processes are mediated through the formin, Bni1p, and the WASP homolog, Wal1p, in *C.albicans* likely through Cdc42p and have helped to inform a general model of how fungal organisms utilize Ras/Cdc42 GTPase signal transduction to support polarized morphogenesis [81]. Unlike *S. cerevisiae*, *C. albicans* contains a Rac homolog, Rac1p, and an accompanying GEF, Dck1p, that control filamentation in embedded agar [82,83]. Although a direct connection between Rac1p and Ras1p signaling has not been explored, embedded agar filamentation is a process that also requires Ras1p signaling. Rac1p and Dck1p, along with another protein, Lmo1p, have been shown to signal through both filamentation and cell wall integrity MAPK pathways to link these two important processes during growth [84]. However, Rac1 and Cdc42 appear to contribute different roles to morphogenesis in *C. albicans*, as *RAC1* over-expression cannot suppress *cdc42Δ* related defects [82]. An interesting question remains regarding the possibility that Ras1p may signal upstream of both Cdc42 and Rac1 pathways to coordinate proper morphogenesis in *C. albicans* during invasive infections.

Spatial regulation of Ras signaling has been recently studied in *C. albicans* as well. As expected, GFP-Ras1p localizes to the plasma membrane of both yeast and hyphal cells [85]. Similar to *S. cerevisiae*, *C. albicans* Ras1p contains a CAAX box for prenylation and only a single conserved cysteine (C287) for palmitoylation. Mutation of C287 decreases Ras1p abundance and causes mislocalization of GFP-Ras1p to endo-membrane structures, whereas mutation of the CAAX box cysteine (C288) causes cytosolic localization [85]. The Ras1p-C288S mutant is unable to support filamentous growth when cultured under hyphae inducing conditions, suggesting that prenylation of Ras1p is absolutely required for function in *C. albicans* [85]. Although the Ras1p-C287S mutant grows as wild type under most conditions, this mutant is also unable to form hyphae under conditions requiring activation of the Ras pathway [85]. However, constitutive activation of the Ras1p-C287S mutant suppresses this hyphal defect without affecting Ras1p localization or membrane dynamics [85]. Together, these data argue that, at least for *C. albicans*, Ras1p localization to the plasma membrane may be more important for activation than for interaction with specific effectors.

### *Aspergillus fumigatus*

Growth of *A. fumigatus*, like most filamentous fungi, involves progression through sequential steps of development. When cultured in the presence of a carbon and nitrogen source, growth begins with the process of conidial germination. This initial step is characterized by a period of isotropic swelling of conidia, initiation of mitosis and the establishment of polarized growth [86]. Continued growth requires the formation of interconnected hyphal networks through the maintenance of polarized extension and the formation of new growth axes. The *A. fumigatus* asexual developmental program concludes with the formation of conidiophores bearing conidial chains. In the immunocompromised host, *A. fumigatus* hyphae invade tissue and disseminate to distant sites via the bloodstream [87]. Therefore, the completion of proper morphogenetic processes is tightly linked to the ability to establish and maintain invasive disease.

In *A. fumigatus*, the major Ras homolog, RasA, plays critical roles in initiation of germination, polarized morphogenesis, cell wall integrity, and virulence. *A. fumigatus* mutants with decreased (dominant negative, DN*rasA*) or absent (Δ*rasA*) RasA activity display a lag in germination initiation, whereas mutants with constitutively activated RasA break dormancy in the absence of a carbon or nitrogen source [88-90]. This precocious germination caused by increased RasA activity is associated with an exaggerated isotropic growth phase and increased nuclear content [89,90]. Following germination, the Δ*rasA* mutant develops severe defects in hyphal morphogenesis characterized by slow growing hyphae incapable of maintaining polarity [88]. As proper polarized hyphae are a prerequisite for the establishment and maintenance of Invasive Aspergillosis (IA), the Δ*rasA* mutant is hypovirulent in a mouse model of IA [91]. Interestingly, increased RasA activity also produces decreased radial outgrowth [89,92]. However, unlike the Δ*rasA* mutant, hyphae produced under constitutive RasA activity develop hyper-vacuolated sub-apical compartments that spontaneously lyse [89]. These data support the importance of temporal regulation of RasA activity throughout polarized morphogenesis, as both increased and decreased activity levels are inhibitory for hyphal outgrowth [88,89]. This hypothesis is further supported by earlier studies with *Aspergillus nidulans* revealing that expression of dominant active or dominant negative *rasA* alleles can halt fungal growth during various stages of development [93,94]. Although *A. fumigatus* is the only mould for which a *rasA* deletion has been obtained, Ras-mediated growth and development in other human pathogenic moulds, *Penicillium marneffei* (a dimorphic pathogen) and *Mucor racemosus*, has been studied through dominant active and dominant negative mutational analysis [95,96]. In both of these fungi, proper regulation of RasA signaling is required for growth and morphogenesis. Similar to *C. neoformans*, the *A. fumigatus* genome contains a second Ras homolog, *rasB*, that does not contain the conserved palmitoylation motif seen in the proto-typical Ras homologs [90]. Cellular expression of *rasB* is lower than *rasA*, and deletion of *rasB* leads to decreased growth, increased hyphal branching and a moderate reduction in virulence [90,97,98]. Although differences in carboxy-terminal localization signals imply different cellular localizations of RasA and RasB, they may play overlapping roles in hyphal development and virulence [88]. Taken together, the current literature supports a conserved role for the RasA signaling pathway in polarized growth and morphogenesis of filamentous fungi. Since polarized morphogenesis is required for fulminant invasive disease, the RasA pathway is hypothesized to be an important virulence determinant for many filamentous pathogens.

Roles for the Ras pathway in fungal cell wall formation have been reported for *C. albicans* where the *ras1Δ* mutant displays resistance to cell wall perturbation by calcofluor white and has increased Hog1p phosphorylation [98,99]. However, a surprising role for Ras pathway signaling in support of fungal cell wall integrity was first directly identified in *A. fumigatus* [88]. The fungal cell wall plays an essential role in protection against external stress and the proper morphogenesis of hyphae [100]. Because of this, inhibiting synthesis of cell wall components and disrupting the already assembled cell wall provide attractive antifungal strategies against *A. fumigatus* infections. When treated with the cell wall inhibitors Congo red or Nikkomycin Z, growth of the *A. fumigatus ΔrasA* mutant is severely inhibited, forming large swollen cells with no polarized growth axis [88,92]. Aberrant

phenotypes produced in the *ΔrasA* mutant by treatment with each stressor can be partially remediated by the addition of sorbitol to the growth medium, suggesting a cell wall defect [88]. The Δ*rasA* mutant also displays increased sensitivity to other cell wall stressors, including: caffeine, used to test cell wall integrity in yeast; SDS, a detergent that disrupts the cell membrane; and Fungin, a polyene that disrupts ion exchange through the cell membrane [88]. In addition to *rasA* deletion, constitutive activation of RasA leads to hypersensitivity to cell wall stress produced by caspofungin, a cell wall antifungal compound that inhibits B-glucan synthesis [92]. When considered with the morphogenetic analyses described previously, these data argue that temporal control of RasA activity is required for proper coordination of polarized growth and cell wall formation. Although *A. fumigatus* is the only mould organism for which Ras-mediated cell wall integrity has been identified, the *C. neoformans* Ras1 protein has recently been implicated in cell wall stress responses. Through a transcriptome analysis of Ras1-controlled genes, *C. neoformans* Ras1 was found to mediate expression of genes important for cell wall and membrane biogenesis [101]. Further analysis revealed a *Ras1* mutant hyperosmosensitivity that was even more pronounced than the *HOG1* deletion mutant [101]. The *C. neoformans* ras*1* null mutant was also found to be hypersensitive to fludioxonil and SDS, indicating the osmosensitivity is due to an underlying cell wall defect [101]. Taken together, these findings suggest that control of cell wall integrity and / or formation may be a common role for Ras pathways among fungi.

In contrast to the wealth of information generated for Ras signaling in *C. neoformans* and *C. albicans*, no studies have yet verified Ras pathway components in *A. fumigatus* and their potential roles in the phenotypes described above. However, it is reasonable to hypothesize that some combination of the highly conserved Cdc42, Rac, and PKA homologs of *A. fumigatus* are central to RasA signal transduction. Of these major downstream Ras effectors, the PKA holoenzyme and the single Rac homolog have been previously studied in *A. fumigatus*. The PKA enzyme complex consists of two catalytic subunits, encoded by one of two isoforms [*pkaC* or *pkaC1*], and two regulatory subunits, encoded by the single *pkaR* gene. PKA signaling in *A. fumigatus* regulates germination, metabolism, pigment production and virulence [102-106]. Although PKA is a major downstream effector of Ras signaling in yeast, exogenous cAMP cannot complement the Δ*rasA* defective growth phenotypes in *A. fumigatus* [88]. These results may indicate that, like *C. neoformans* Ras1, RasA signaling in *A. fumigatus* better fits a model where Ras is largely independent of the cAMP/PKA pathway. This model is also supported by work in *A. nidulans* showing that Ras and PKA signaling impact germination through distinct pathways [107]. Because RasA deletion causes aberrations of hyphal formation suggestive of cytoskeletal abnormalities, it is likely that the Rac and Cdc42 pathways are important downstream components. The single Rac homolog of *A. fumigatus*, RacA, has been shown to regulate hyphal morphology during vegetative growth [108]. Interestingly, the Δ*racA* mutant was also found to produce decreased levels of reactive oxygen species (ROS), a known regulator of apical dominance in *Aspergillus* hyphae [109], mimicking inhibitors of NADPH oxidase [108]. The contributions of the Cdc42 homolog, ModA, has not yet been addressed in *A. fumigatus* growth and virulence, but one study has explored the roles of both the Rac and Cdc42 homologs in the model fungus, *A. nidulans*. This study found that the *A. nidulans* ModA and RacA proteins share an essential role in growth, with ModA serving as the major GTPase controlling morphogenesis [110]. Although hyphal tip organization was unaffected in Δ*modA*, epistasis experiments suggest that ModA may promote the activation of the formin, SepA, to impact hyphal morphology [110]. A later study found that both ModA and RacA play roles in the localized production of ROS at the hyphal tips, providing another mechanism through which these GTPase cascades regulate hyphal morphogenesis [109]. Although not yet studied, it is interesting to speculate that RasA may be a shared upstream activator of ModA and RacA to control formin-mediated modulation of the actin cytoskeleton, as well as localized generation of ROS. Although much of the data from the study of individual signaling pathways within *A. fumigatus* and *A. nidulans* can be extrapolated to RasA function, more work needs to be accomplished to gain a clear understanding of the role RasA plays in activation of these putative effectors during *A. fumigatus* invasive growth.

As with the yeast pathogens discussed above, the spatial regulation of RasA is important for most RasA-pathway functions during *A. fumigatus* vegetative growth and pathogenesis. Expression of a GFP-RasA fusion in a Δ*rasA* mutant fully complements the growth deficiencies associated with loss of RasA [91]. The GFP-RasA protein localizes to the plasma membrane, but shows no enrichment at sites of polarity [91]. Similar to *C. neoformans*, the RasA carboxy-terminus contains a dual cysteine motif for palmitoylation-mediated association with the plasma membrane. Although mutation of either cysteine alone causes no observable defects in hyphal growth, ablation of both conserved cysteines leads to a largely non-functional RasA mutant. The palmitoylation-deficient RasA protien is mislocalized to internal membranes and the mutant strain exhibits polarized growth defects coupled with decreased cell wall integrity [91]. These findings are reminiscent of the Δ*rasA* mutant. In addition, virulence of the palmitoylation deficient mutant is reduced similar to the Δ*rasA* mutant [91]. However, the palmitoylation deficient mutant does produce a hyphal growth rate that is greater than the Δ*rasA* mutant, suggesting some level of functionality is retained by the mislocalized protein [91]. This partial functionality could be the result of inefficient activation of the palmitoylation-deficient RasA mutant due to inability to interact with the regulatory GAP and GEF proteins, or because of decreased interaction with specific downstream effectors that can only be engaged from the plasma membrane. The regulation of Ras localization and its role in activation of growth and virulence pathways is an area requiring further study in pathogenic fungi.

## A Future for Ras in Pathogenic Fungi: Ras Pathways as Targets for Antifungal Therapy

In summary, the current data support a model where fungal Ras proteins play conserved roles in polarized growth and virulence, at least in part, through modulation of the GTPase signaling partners Cd42 and Rac, leading to gene expression, localized ROS production, and modulation of the actin cytoskeleton. In addition, Ras-mediated growth and virulence through PKA stress signaling appears to play a larger role in *C. albicans* than in *C. neoformans* or *A. fumigatus*. The Ras pathway data collected thus far have aided in the development of greatly improved models for fungal growth, differentiation and virulence of pathogenic fungi. It is clear that, although the precise mechanisms are not fully understood, spatiotemporal control of Ras pathway activation is key to achieving the appropriate signals for growth and, therefore, virulence. Can we take advantage of this information for therapeutic benefit? Are there aspects of Ras pathways that may serve as useful antifungal targets? The fact that inhibition or ablation of Ras activity decreases virulence in the pathogenic fungi discussed above argues that this signaling pathway may represent a rich landscape of potential targets. Although any novel Ras pathway inhibitor may make an excellent stand-alone antifungal therapy, an obvious area of

exploration would be combination therapy of Ras inhibition coupled with antifungal agents targeting the cell wall and / or membrane or with agents targeting parallel signaling pathways controlling morphogenesis. Such combinations have the potential to cause complete inhibition of fungal growth or even cell death. For example, additive effects are seen when loss of Ras activity is coupled with cell wall inhibition in *A. fumigatus* and *C. neoformans* [88,91,92,101], or with Amphotericin B treatment in *C. neoformans* [101], or with calcineurin inhibition in *A. fumigatus* and *C. neoformans* [92,111].

To approach the inhibition of Ras protein signaling, the most readily apparent targets lie in the enzymes governing Ras protein maturation and localization. Inhibition of Ras localization has been an area of major interest for anti-cancer therapies for some time. Individual targets include the inhibition of Ras protein farnesyltransferases, palmitoyltransferases, CAAX proteases and isoprenylcysteine carboxymethyltransferases [112-115]. Although many of the farnesyltransferase inhibitors developed as cancer therapies have failed clinical trials for multiple reasons [112], the post-translational prenylation of Ras is still one area of interest for novel antifungal therapies. For example, early Ras pathway studies in *C. neoformans* revealed an inhibitory effect of farnesyltransferase inhibitors [FTI's] on fungal growth [111]. In addition, a recent in depth study of farnesyltransferase protein structure in *C. neoformans* has provided valuable insight into the potency of FTI's against this fungus and highlighted possibilities for rational design of new FTI's to inhibit fungal Ras localization [116]. Although a mechanistic understanding of palmitoylation has only recently been described and novel inhibitors of palmitoylation are in their infancy, the mutational analyses performed thus far argue that inhibition of Ras palmitoylation may be an interesting avenue for future antifungal therapies. Recent studies in *A. fumigatus* have shown that the palmitoylation inhibitor, 2-bromopalmitate, causes mislocalization of RasA from the plasma membrane and inhibits hyphal growth in a dose-dependent manner [91]. In addition, although the exact mechanism is not understood, exogenous conjugated linoleic acid appears to inhibit the yeast-to-hyphae transition through mislocalization of the Ras1p protein in *C. albicans* [117]. Whatever form it may take, inhibition of Ras protein maturation is an area deserving future attention in the pathogenic fungi.

Ras-effector interactions are also areas requiring further exploration. Both upstream activators and downstream effectors of Ras signal transduction could be areas of potential antifungal targets. Through the studies described above, we have collected a large amount of information on the downstream effectors employed by Ras to generate proper fungal morphology for growth and virulence. Although there are no reports of the direct inhibition of Ras-effector interactions in pathogenic fungi, a collection of studies in *C. albicans* has shown that molecules linked to carrier peptides can be targeted to CRIB-domains of Cdc42 effector proteins, inhibiting their interaction *in vitro* [118]. In addition, the inhibition of Cdc42-effector interactions via the CRIB-domain could be specifically targeted to fungal proteins with minimal effects on their human counterparts [118]. These studies make a strong case that the inhibition of signal transduction may be a very promising future for antifungal therapies. A similar methodology could be employed against Ras pathway interactions if further study can highlight fungal-specific aspects of Ras signal transduction. Compared to what we now know of Ras signal transduction to downstream effectors in pathogenic fungi, we know very little about how Ras activity is regulated by GAPs and GEFs, and how exactly this regulation takes place in time and space within the cell. In mammalian cells, Ras activity is activated by GEF proteins that are recruited to the membrane via an activated receptor tyrosine kinase [119]. Fungal genomes do not encode identifiable receptor tyrosine kinase homologs, therefore, regulation of the GEFs controlling Ras activation is less clear. Studies in model fungi have revealed that the *S. pombe* Ras homolog, Ras1p, is differentially regulated by two GEFs; one that controls morphogenesis and one that is transcriptionally up-regulated in response to pheromone, driving Ras pathway signaling towards mating control [120]. Early studies in yeast suggested that Ras GAPs may be involved in the response to intracellular acidification induced by glucose, implying at least one method through which fungal GAPs may control Ras activity in response to nutritional changes [121,122]. Deletion of the *A. nidulans* Ras GAP, GapA, causes many phenotypes that mimic the activated RasA phenotypes of *A. fumigatus,* showing conservation of Ras GAP function between species [89,90,123]. Although these findings support conserved control mechanisms for fungal Ras proteins, we still do not have a strong understanding of how Ras protein interactions may contribute to the regulation of Ras activity in the setting of the host tissue.

For any approach to be fungal-specific, we will need a better understanding of how Ras structural biology and Ras pathway interactions differs from that of humans. This will undoubtedly strengthen our ability to specifically target fungal Ras-pathways, with minimal effect on the human host. In addition, a better understanding of the spatiotemporal control of Ras activity during cellular morphogenesis, stress response, and virulence will provide invaluable information with which to build even better models of fungal growth.

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