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Orchestration of Morphogenesis in Filamentous Fungi: Conserved Roles for Ras Signaling Networks

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Abstract

Filamentous fungi undergo complex developmental programs including conidial germination, polarized morphogenesis, and differentiation of sexual and asexual structures. For many fungi, the coordinated completion of development is required for pathogenicity, as specialized morphological structures must be produced by the invading fungus. Ras proteins are highly conserved GTPase signal transducers and function as major regulators of growth and development in eukaryotes. Filamentous fungi typically express two Ras homologues, comprising distinct groups of Ras1-like and Ras2-like proteins based on sequence homology. Recent evidence suggests shared roles for both Ras1 and Ras2 homologues, but also supports the existence of unique functions in the areas of stress response and virulence. This review focuses on the roles played by both Ras protein groups during growth, development, and pathogenicity of a diverse array of filamentous fungi.

Keywords

Ras; fungal development; fungal pathogenesis; fungal virulence; signal transduction; hyphal morphogenesis

1. INTRODUCTION

The Ras super-family is composed of membrane-associated GTPase proteins serving as major signal transduction elements in eukaryotic cells. Ras proteins act as molecular switches, toggling between active and inactive states, bound to either guanosine triphosphate (GTP) or guanosine diphosphate (GDP), respectively. Ras signaling is activated by interaction with guanosine nucleotide exchange factors (GEFs), a protein-protein interaction that aids Ras in the release of GDP allowing subsequent binding of the more cytoplasmically available GTP. Inactivation is accomplished by interaction of GTP-bound Ras with GTPase activator proteins (GAPs) that accelerate the slow intrinsic GTPase activity characteristic of

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Ras proteins. Within the Ras super-family is a sub-family of proteins characterized as "prototypical" Ras proteins, displaying high structural homology with mammalian H-ras (Wennerberg et al., 2005). Members of the Ras sub-family have conserved domains required for protein activation, effector interactions and membrane association. The latter is required for a series of post-translational modifications that can promote specific signaling events through compartmentalization of Ras proteins to either the endomembranes of the endoplasmic reticulum (ER) and Golgi or to the plasma membrane (Figure 1). The localization domains of Ras sub-family proteins include a classic CAAX motif necessary for prenylation, found in all Ras homologues, and typically one of two additional membrane association motifs found in the hypervariable region (HVR): 1) a conserved cysteine residue(s) utilized for palmitoylation or; 2) a stretch of polybasic residues generating a positively charged C-terminus (Konstantinopoulos et al., 2007). Prenylation of the CAAX motif can be accomplished via either farnesylation or geranylgeranylation and the composition of the C-terminus reliably predicts the specific prenylation event (Konstantinopoulos et al., 2007).

In filamentous fungi, prototypical Ras homologs can usually be divided into two sub-groups: 1) a "Ras1-like" sub-group that has a CAAX motif predicted to be farnesylated coupled with a "dual cysteine" motif for palmitoylation; or 2) a "Ras2-like" subgroup that is predicted to be geranylgeranylated and encodes an HVR with multiple basic residues (Figure 2). It is important to note that the Ras1-like and Ras2-like homologs of filamentous fungi typically encode these differing localization motifs (Figure 2), as this dichotomy suggests the potential for differential sub-cellular localizations and, therefore, potentially unique signaling partners under physiological conditions. In support of this possibility, Ras2-like homologs have been deleted in many filamentous fungi whereas deletion of Ras1-like homologs has only been achieved for *A. fumigatus*, *T. reesei* and *B. cinerea* (Fortwendel et al., 2008; Minz Dub et al., 2013; Zhang et al., 2012a). The Ras1- and Ras2-like homolog paradigm is in contrast to the *S. cerevisiae* model, where Ras1p and Ras2p are highly homologous and have identical prenylation and palmitoylation domains, or *S. pombe* where only one Ras homolog exists (Weeks and Spiegelman, 2003).

Fungal Ras protein signaling has been studied most extensively in the model yeast organisms, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and in the human pathogens, *Candida albicans* and *Cryptococcus neoformans*. Ras-mediated morphogenetic signaling has been reviewed extensively in these organisms (Fortwendel, 2012; Inglis and Sherlock, 2013; Tamanoi, 2011; Weeks and Spiegelman, 2003). In brief, the *S. cerevisiae* Ras1-like homologs, Ras1p and Ras2p, orchestrate varied cellular processes, including cell cycle, pseudohyphal growth and stress response, via signal transduction to cyclic-AMP/protein kinase A (cAMP/PKA), mitogen-activated protein kinase (MAPK), and Rho-like GTPase networks (Tamanoi, 2011; Weeks and Spiegelman, 2003). These findings are largely mirrored in studies with the Ras1-like homolog of *C. albicans*, Ras1p (Inglis and Sherlock, 2013). *C. albicans* Ras1p signals through the cAMP/PKA pathway to control yeast morphology, stress response, adhesion and mating (Inglis and Sherlock, 2013). However, *C. albicans* is not only capable of yeast and pseudohyphal growth morphologies, but can also form true hyphae in a manner similar to moulds. This yeast-to-hyphae transition

is regulated by Ras1p signaling through MAPK and cAMP/PKA pathways (Inglis and Sherlock, 2013). Unlike both *S. cerevisiae* and *C. albicans*, the *S. pombe* Ras1 protein does not signal through the cAMP/PKA pathway. Instead, *S. pombe* Ras1 activates a MAPK pathway (Byr2-Byr1-Spk1) to control mating and pheromone response and a Cdc42 pathway, via the Cdc42 GEF Scd1, to control yeast morphology (Tamanai, 2011; Weeks and Spiegelman, 2003). Finally, in the basidiomycete yeast, *Cryptococcus neoformans*, Ras1 plays a minor role in cAMP/PKA activation but directly interacts with the duplicated Rho-like GTPases, Cdc42 and Cdc420, as well as a Rac1 homolog, to control morphogenetic development and virulence (Ballou et al., 2010; Fortwendel, 2012; Vallim et al., 2005). Filamentous fungi are also known to express Rac homologs, whereas *S. cerevisiae* and *S. pombe* do not, making the ascomycete yeast poor models for this level of Ras signaling. Study of the C-terminal protein motifs regulating Ras post-translational modification and sub-cellular localization in *S. pombe* and *C. neoformans* has also revealed that spatial segregation of Ras signals between plasma and endomembranes is conserved in the fungal kingdom (Nichols et al., 2009; Onken et al., 2006). In contrast to the well-studied signaling mechanisms of yeast organisms, only a few studies with filamentous fungi have reported specific signaling partners of Ras and even less work has been done to understand the determinants and outcomes of Ras sub-cellular localization. As such, the putative pathways controlling Ras protein localization and signal transduction, assimilated from these studies, are summarized as a general model for fungal Ras signaling in Figure 1.

Filamentous fungal organisms represent an interesting group of organisms for further study on Ras protein function. The complex developmental programs of many filamentous fungi provide the opportunity to understand signaling mechanisms regulating processes like conidial germination, polarized morphogenesis, conidiogenesis, and sexual development. Additionally, because some filamentous fungi switch between yeast-like and mould-like growth modalities and/or develop specialized infection structures, the roles of Ras pathways have been explored in these specialized developmental steps as well. This review will cover studies from multiple filamentous fungi that express both Ras1- and Ras2-like homologs, revealing overarching themes of Ras-mediated morphological development, growth control and asexual differentiation. Although important roles for Ras signaling in mating have been uncovered in the yeast *C. albicans* (Huang et al., 2010), *S. pombe* (Xu et al., 1994), and *C. neoformans* (Alspaugh et al., 2000), Ras-mediated sexual differentiation has only been explored in relatively few filamentous fungi. However, because the vast majority of signaling components are conserved between fungi, a central role for Ras in sexual differentiation and pheromone response is likely conserved.

2. The model filamentous fungi: *Aspergillus nidulans* and *Neurospora crassa*

Some of the earliest studies on the contribution of Ras activity to filamentous fungal growth and development come from work with the model fungus, *A. nidulans*. The first filamentous fungal Ras homolog to be identified in *A. nidulans* was *rasA*, a member of the Ras1-like homologues (Som and Kolaparthi, 1994). A *rasA* deletion strain could not be obtained, suggesting the RasA protein serves an essential function in *A. nidulans* growth. To

circumvent this, constitutively active and inactive mutants of *rasA* were expressed in the wild type genetic background to generate dominant active and dominant negative alleles, respectively. Studies with other filamentous fungi, discussed here, have relied heavily on this strategy to reveal roles for Ras proteins in fungal development. The mutations introduced to alter Ras activity in each of these species are based either on the naturally occurring mammalian Ras protein mutations leading to cellular transformation or those that have been generated in vitro to study Ras activation in yeast and mammalian systems. Over-expression of activated *rasA* (*rasA*^{G17V}) precociously induces *A. nidulans* conidial germination, but subsequently inhibits the production of the initial germ tube (Osherov and May, 2000; Som and Kolaparthi, 1994). At later stages of growth, over-expression of *rasA*^{G17V} blocks conidiophore development (Som and Kolaparthi, 1994). However, both germ tube production and conidiophore development can progress once levels of activated *rasA* are decreased (Som and Kolaparthi, 1994). These data suggested that RasA activity must be regulated temporally during development. This hypothesis is further supported by studies employing a strain deleted for *gapA*, encoding the predicted GAP inhibitor of RasA, in *A. nidulans*. The *gapA* mutant produces phenotypes remarkably similar to those associated with over-expression of *rasA*^{G17V} (Harispe et al., 2008). Because RasA was shown to play a major role in conidial germination and likely regulates pathways important for sensing the presence of a germinant, subsequent work explored a potential link between the RasA and cAMP/PKA pathways in *A. nidulans*. However, these studies showed that RasA signals to the molecular germination program independently of PKA (Fillinger et al., 2002). Although RasA does not appear to be upstream of PKA for germination, a RasA-PKA network interaction is at least partially required for the production of secondary metabolites contributing to development in *A. nidulans*. For example, expression of *rasA*^{G17V} leads to a transcriptional and post-transcriptional repression of *aflR*, a regulator of sterigmatocystin biosynthetic gene expression (Shimizu et al., 2003). The post-transcriptional repression of *aflR* is mediated, at least in part, through the PKA pathway (Shimizu et al., 2003). The *A. nidulans* genome also encodes for a second Ras homolog that is a member of the Ras2-like group, RasB. Although no mutational analyses have been performed on *rasB*, a recent study employing siRNA technology has revealed roles for RasB in morphological development (Kalleda et al., 2013). Silencing of *rasB* expression causes irregular hyphal branching, reduced radial growth rate, and aberrant conidiophore formation (Kalleda et al., 2013). Although the exact signaling mechanisms of RasA and RasB pathways in *A. nidulans* have not been delineated, it's apparent that these proteins play both shared and unique roles in growth and development.

Like *A. nidulans*, *N. crassa* also contains two Ras homologs, termed *ras-1* and *ras-2*. The RAS-2 protein was the first to be studied in *N. crassa*. Loss of *ras-2* causes reduced growth rates, altered hyphal morphology marked by increased branching, reduced conidiation and altered cell wall formation (Kana-uchi et al., 1997). These phenotypes are similar to those produced by repression of *A. nidulans* *rasB* expression. Roles for *ras-1* in *N. crassa* growth and development were not known until a recent study revealed the band mutant to be a dominant mutant allele of *ras-1* (Belden et al., 2007). The *N. crassa* band mutant is an important conidial banding mutant, used for many years as a genetic tool to study circadian rhythms (Liu and Bell-Pedersen, 2006). In this study, the band mutant was shown to be a

ras-1 (T79I) point mutation, termed *ras-1* (bd), with a modest enhancement of GDP/GTP exchange (Belden et al., 2007). The *ras-1*(bd) mutant displays increased expression of conidiation-specific genes and altered light-regulated genes, suggesting that the WHITE COLLAR-1 photoreceptor is down stream of RAS-1 (Belden et al., 2007). This was the first identified link between Ras protein signaling and circadian rhythm output in a filamentous fungus. Taken together, the studies of Ras protein function in the model filamentous fungi show that Ras1-like and Ras2-like homologues play conserved roles during important developmental stages including; germination, hyphal branching, and asexual development.

3. Human pathogenic fungi: *Aspergillus fumigatus*, *Mucor racemosus*, *Penicillium marneffe* and *Paracoccidioides brasiliensis*

Human mould pathogens typically require considerable morphologic plasticity to cause invasive disease, as most pathogenic fungi undergo either conidia-to-hyphae, hyphae-to-yeast, or yeast-to-hyphae transitions within the host tissue. Therefore, delineating the signaling pathways regulating these processes, of which Ras is a major contributor, is of major interest. Work with *A. fumigatus*, the most prevalent mold-pathogen of immunocompromised hosts, has produced morphological data with *rasA* and *rasB* mutants that largely corroborate that of *A. nidulans* (Fortwendel et al., 2008; Fortwendel et al., 2011; Fortwendel et al., 2004). The most unique advances made through the study of *A. fumigatus* Ras signaling in recent years revolve around the delineation of the determinants and outcomes of RasA localization. After post-translational modification is complete, RasA primarily localizes to the *A. fumigatus* plasma membrane (PM) (Fortwendel et al., 2012). However, mutation of either the highly conserved cysteine residues required for palmitoylation or of the CAAX-box cysteine required for prenylation, leads to mislocalization of RasA to endo-membranes (EM) and to the cytoplasm, respectively (Fortwendel et al., 2012; Norton and Fortwendel, 2014). Further study of these mutants revealed that PM-localized RasA regulates the major signaling programs for hyphal morphogenesis, cell wall integrity, asexual development and virulence (Fortwendel et al., 2012). However, EM-localized RasA supports hyphal growth to a greater extent than cytoplasmic RasA, suggesting that RasA may retain the ability to engage signaling partners from the EM (Fortwendel et al., 2012; Norton and Fortwendel, 2014). These findings require further investigation, but may suggest that filamentous fungal Ras proteins, like those of their yeast and human counterparts, are able to use multiple membranes as signaling platforms to achieve distinct outcomes.

The zygomycete human pathogen, *M. racemosus*, encodes three Ras proteins (Casale et al., 1990). Expression of *Mras1* and *Mras3* are differentially regulated during development and expression of *Mras2* has not yet been detected (Casale et al., 1990; Roze et al., 1999). *Mras3* transcript levels are highest during sporulation whereas *Mras1* transcripts are most abundant during germination and hyphal growth, suggesting these Ras proteins may be important at different developmental stages (Roze et al., 1999). Unlike all other fungi covered in this review, *M. racemosus* MRas1 and MRas3 proteins both align with the Ras1-like homologues and no Ras2-like homologue has been reported (Figure 2). Expression of activated (*Mras3*^{G18V}) or inactivated (*Mras3*^{K22N}) *Mras3* causes reduced germination and

growth rates, whereas activated *Mras1* (*Mras1*^{G19V}) displays increased growth rate during germination (Roze et al., 1999). These data further suggest opposing actions of each Ras protein. Although no direct signaling partners of *M. racemosus* Ras proteins have been identified, *Mras3* expression has been shown to be under the control cAMP regulation, suggesting cross-talk of Ras and PKA networks (Roze et al., 1999). Further biochemical analyses also identified MRas1 in the cytoplasm and as associated with membrane fractions, whereas MRas3 was found only associated with membranes (Roze et al., 1999). Interestingly, the MRas3 amino acid sequence contains a classical "dual cysteine" palmitoylation motif, identical to those found in other filamentous fungal Ras1-like homologues. At this same position, the MRas1 protein has only one cysteine (Figure 2). It is enticing to hypothesize that the dual cysteine palmitoylation motif of filamentous fungal Ras proteins is an important factor for stable membrane association, and may explain the noted difference in protein localization for the *M. racemosus* Ras homologues. This speculation is supported by work in *A. fumigatus* showing that mutation of a single cysteine within the palmitoylation motif can alter membrane distribution of RasA (Fortwendel et al., 2012). Although morphogenesis is affected by alteration of Ras activity levels in *M. racemosus*, and each Ras protein appears to play opposing roles in growth, no virulence test has been reported.

The first dimorphic fungal pathogen to yield insights into the roles of Ras pathways was *P. marneffei*. Expression of either a constitutively activated (*rasA*^{G19V}) or inactivated (*rasA*^{D125A}) mutant of *rasA* results in a reduced growth rate and inhibition of aerial hyphae during early growth (Boyce et al., 2005). Strains expressing inactivated *rasA* precociously enter the asexual developmental program for conidiophore production, whereas the activated strains are delayed (Boyce et al., 2005). These data suggest that RasA activity directly suppresses the asexual developmental program in *P. marneffei*. Interestingly, both inactivated and activated *rasA* alleles inhibit conidial germination (Boyce et al., 2005). Furthermore, both mutants produce irregular hyphal phenotypes under carbon limitation, suggesting carbon status regulates Ras activity in *P. marneffei* (Boyce et al., 2005). In addition, both mutants produce swollen, misshaped yeast cells at 37°C (Boyce et al., 2005). Taken together, RasA regulates polarity establishment and maintenance in *P. marneffei*, and directly suppresses conidiation. The work with *P. marneffei* was also the first to suggest interactions between Ras and Rho proteins in a filamentous fungus. Expression of an activated Cdc42 homologue, *cflA*^{G14V}, in the constitutively inactive *rasA* background restores germination levels to wild type and suppresses aberrant hyphal phenotypes, suggesting Cdc42 functions downstream of RasA in *P. marneffei* (Boyce et al., 2005). The RasA-CflA pathway signals through the p21-activated kinase, pakA, and is activated, at least in part, by the heterotrimeric alpha subunit G-protein (*gasC*) to regulate conidial germination during hyphal growth at 25°C but not during yeast growth at 37°C (Boyce and Andrianopoulos, 2007; Zuber et al., 2003).

Transcriptional profiles of the *ras1* and *ras2* genes in another dimorphic pathogen, *P. brasiliensis*, provide evidence for roles during macrophage engulfment of yeast cells, likely regulating stress responses during this process (Fernandes et al., 2008). Treatment of *P. brasiliensis* with a farnesyltransferase inhibitor, which should mislocalize and inhibit

activation of Ras proteins, increases the percentage of yeast cells undergoing the yeast-to-hyphae transition during temperature-mediated conversion (Fernandes et al., 2008). These studies suggest that Ras proteins work to negatively regulate this important morphological transition in *P. brasiliensis*. Later studies showed that stimulation of *P. brasiliensis* yeast cells with low levels of H₂O₂ or NO₂ causes increased levels of active Ras, post-transcriptionally (Haniu et al., 2013). Low levels of H₂O₂ and NO₂ are accepted proliferative signals for fungal organisms (Haniu et al., 2013). Therefore, H₂O₂ and NO₂ may act through the Ras GTPase switch to drive proliferation by directly causing activation of Ras proteins (Haniu et al., 2013). Although not yet studied in other filamentous fungi, it would be interesting to explore this phenomenon as a common mechanism of fungal Ras protein activation.

4. Plant pathogenic fungi: *Colletotrichum trifolii*, *Fusarium graminearum*, *Magnaporthe grisea*, *Botrytis cinerea*, and *Setosphaeria turcica*

In *C. trifolii*, a filamentous fungal pathogen of alfalfa, expression of activated *ct-ras1* (*ct-ras1*^{G17V}) causes development of compact colonies with loss of hyphal polarity on minimal media (Truesdell et al., 1999). Ct-Ras1 activation also causes increased accumulation of reactive oxygen species (ROS) and induction of apoptosis-like cell death, a process that is remediated by exogenous proline (Chen and Dickman, 2005; Memmott et al., 2002). This antioxidant effect appears to be caused by a proline-mediated increase in catalase activity after prolonged culture. Down-regulation of Ct-Ras1 activity via protein mislocalization, mutational inactivation and/or antisense *ct-ras1* expression reduces germination and growth rates (Ha et al., 2003). In *C. trifolii*, Ct-Ras1 appears to signal through multiple GTPase cascades, including Cdc42 and Rac. Expression of a constitutively active *ct-cdc42* (*ct-cdc42*^{G14V}) mimics the majority of phenotypes developed by *ct-ras1*^{G17V}. Furthermore, expression of either an activated or inactivated (*ct-cdc42*^{T19N}) *ct-cdc42* restores wild type phenotypes to the inactivated and activated *ct-ras1* mutants, respectively (Chen et al., 2006). Whereas these data argue that Ct-Ras1 signals directly upstream to activate Ct-Cdc42, the relationship between Ct-Ras1 and Ct-Rac1 is less clear. Expression of activated *ct-rac1* (*ct-rac1*^{G17V}) in the *ct-ras1*^{G17V} background stimulates MAPK activation and restores the wild type phenotype (Chen and Dickman, 2004). The high levels of cytoplasmic ROS generated by the *ct-ras1*^{G17V} mutant can be ameliorated by expression of a constitutively inactivate *ct-rac1* or treatment with a phospholipase A2 inhibitor (Chen and Dickman, 2004). These results argue that Rac antagonizes Ras signaling in *C. trifolii* for growth and germination by regulating MAPK cascades and that Ras and Rac cooperate to produce ROS via a cPLA₂-mediated pathway (Chen and Dickman, 2004).

In *F. graminearum*, a ubiquitous wheat, barley and maize pathogen, deletion of *RAS2* inhibits mating by blocking perithecia formation (Bluhm et al., 2007). Loss of *RAS2* also causes reduced radial outgrowth of colonies, decreased production of aerial hyphae and slower germination rates. Although *RAS2* deletion does not inhibit the ability of *F. graminearum* to colonize wheat and maize hosts, the mutant is largely unable to infect plant tissue and therefore displays a reduction in virulence (Bluhm et al., 2007). Further work revealed that the *ras2* mutant does not alter cAMP levels and addition of exogenous cAMP

does not recover mutant defects (Bluhm et al., 2007). However, phosphorylation of the MAPK protein, Gpmk1, is greatly reduced in the *ras2* mutant. In addition, expression of *FGL1*, a Gpmk1-mediated lipase required for virulence, is also down-regulated by *RAS2* deletion (Bluhm et al., 2007). Together, these findings suggest that a Ras2-Gpmk1 pathway has been adapted to control specific *F. graminearum* pathogenesis attributes.

Both Ras sub-family homologues of the rice blast fungus, *M. grisea*, have been examined. Expression of a constitutively activated *RAS2* allele (*RAS2*^{G23V}) causes the development of appressoria without attachment to host material (Park et al., 2006). However, deletion of *RAS1*, the Ras2-like homologue, causes reduced conidiation as the only overt phenotype (Park et al., 2006). Loss of the MAPK pathway adaptor protein, Mst50, in the *RAS2*^{G23V} background remedies the precocious formation of appressoria (Park et al., 2006). In addition, both MAPK and cAMP signaling pathways are over-activated in the *RAS2*^{G23V} mutant and loss of any component of the Mst11-Mst7-Pmk1 pathway blocks Ras2-mediated appressorium formation (Park et al., 2006; Zhao et al., 2005). Therefore, the *M. grisea* Ras1-like homologue, Ras2, appears to function upstream of both PKA and MAPK pathways to control asexual and pathogenic development.

In the gray mold, *B. cinerea*, *bcras2* deletion leads to slowed germination and greatly reduced hyphal growth (Minz Dub et al., 2013). In spite of this, the *bcras2* strain is able to infect bean leaves, albeit with a delayed disease presentation. Addition of exogenous cAMP corrects the germination defect and partially abrogates the reduced growth rate. Furthermore, the cytoplasmic cAMP concentration of the germinated *bcras2* deletion mutant is much lower than wild type (Minz Dub et al., 2013). In contrast, deletion of *bcras1* causes severe growth defects, characterized by compact colony morphology and stunted, highly branched hyphae lacking proper cell polarity. Compared to the *bcras2* strain, the *bcras1* strain is completely avirulent and displays hypersensitivity to oxidative and osmotic stresses (Minz Dub et al., 2013). Expression analyses revealed that genes downstream of the stress regulatory MAPK, BcSAK1, are not induced under oxidative stress in the *bcras1* mutant. Additionally, BcSAK1 phosphorylation is not further enhanced under stress in a *bcras1* background (Minz Dub et al., 2013). Together, these results suggest BcRas1 is the major mediator of Ras-regulated growth in *B. cinerea*, signaling upstream of MAPK pathways for stress response.

In the ascomycete, *S. turcica*, the major cause of northern leaf blight of maize, a single study has reported findings using an RNAi-mediated inhibition of *StRas2*. Transformants with the greatest amount of *StRas2* suppression display a stunted hyphal phenotype, polarity maintenance defects and reduced conidiation (Zhang et al., 2012b). Exogenous cAMP does not abrogate the aberrant hyphal phenotype but does partially resolve the conidiation defect caused by *StRas2* suppression. *StRas2* suppression is also associated with loss of appressorium development, reduced appressorial turgor pressure, and reduced pathogenicity. Exogenous cAMP again could not remediate the defects in appressorium development (Zhang et al., 2012b). Therefore, *StRas2* may signal through the cAMP/PKA pathway for asexual development but controls hyphal morphogenesis and virulence through cAMP-independent mechanisms.

5. The insect pathogen: *Beauveria bassiana*

Many biocontrol agents are filamentous fungal insect pathogens, or entomopathogenic fungi, that must colonize and penetrate their host via hyphal production. Both Ras proteins of the entomopathogenic fungus, *B. bassiana*, have been studied for their roles in these processes. *Ras1* and *Ras2* transcripts are increased upon exposure to oxidative agents, cell wall disruptive compounds, and an agricultural antifungal, carbendazim. However, under hyperosmotic stress, *Ras2* expression is upregulated and *Ras1* is suppressed (Xie et al., 2013). Expression of constitutively active (*Ras1^{G19V}*) and inactive (*Ras1^{D126A}*) mutants revealed alterations in colony size and hyphal morphology coupled with differences in conidiogenesis, together suggesting that *Ras1* is important for asexual development whereas *Ras2* is required for hyphal morphogenesis and growth (Xie et al., 2013). The *Ras2* mutant develops multiple germ tubes at early time-points, suggesting *Ras2* may also act to limit polarity establishment axes early in growth (Xie et al., 2013). Expression of *Ras1^{G19V}* did not complement this phenotype, further suggesting distinct roles for the two Ras proteins. Deregulation of *Ras1* activity (*Ras1^{G19V}*) and/or deletion of *Ras2* causes altered susceptibility profiles to oxidative, cell wall, and hyperosmolality stresses (Xie et al., 2013). These stress phenotypes are also associated with altered stress-responsive gene expression profiles. However, the stress profiles of each mutant differ, suggesting both shared and unique roles for *Ras1* and *Ras2* in stress response. To be a successful biocontrol agent, *B. bassiana* must be able to withstand the external pressures of ultraviolet (UV) and thermal stress. Although none of the mutants tested show alterations in thermotolerance, the *Ras1^{D126A}* displays increased UV resistance (Xie et al., 2013). Although only *Ras1^{D126A}* displays increased UV resistance, all mutant strains are less virulent than the wild type (Xie et al., 2013). No signaling partners have been identified for *B. bassiana* *Ras1* and *Ras2*, however a subsequent study revealed *Ras1* and *Ras2* to have altered expression levels in response to stress in a P-type calcium ATPase deletion mutant (Wang et al., 2013). This finding may suggest crosstalk between calcineurin and Ras-regulated pathways in *B. bassiana*.

6. The cellulolytic filamentous fungus: *Trichoderma reesei*

The industrially and commercially important cellulolytic fungus, *T. reesei*, expresses two Ras proteins, TrRas1 and TrRas2. Loss of *TrRas1* causes greatly reduced colony size, lack of conidiation and highly branched, stunted hyphae reminiscent of the *A. fumigatus* *rasA* mutant. Despite this loss on polarity, the *TrRas1* strain maintains the ability to degrade cellulose (Zhang et al., 2012a). The *T. reesei* *TrRas2* strain also displays phenotypes of reduced colony size, increased branching and reduced aerial hyphae, albeit less severe than *TrRas1* (Zhang et al., 2012a). As is the case for most filamentous fungi, these data suggest that the *Ras1*-like homologue is the major regulator of fungal development in *T. reesei* and may have shared roles with the *Ras2*-like homologue, TrRas2. Interestingly, TrRas2 is important for cellulase activity, as cellobiohydrolase activity and cellulase protein accumulation are negatively affected by loss of *TrRas2* (Zhang et al., 2012a). Furthermore, expression of the major cellulase genes is controlled by TrRas2 in a manner dependent on Xyr1, a major transcriptional regulator of cellulase expression in *T. reesei*. Further study revealed that steady-state levels of cAMP are decreased in the *TrRas1* mutant and

upregulated in a strain expressing activated TrRas2 (*TrRas2^{G16V}*), suggesting overlapping roles for TrRas1/PKA and TrRas2/PKA signaling in *T. reesei* growth (Zhang et al., 2012a). Although some TrRas1 and TrRas2 regulatory mechanisms may overlap, TrRas2 appears to play a unique role in control of overall cellulase activity for *T. reesei*.

7. Mushroom-forming fungi: *Laccaria bicolor* and *Schizophyllum commune*

A final, and exciting, group of filamentous fungi lending insight into the roles of Ras proteins are the developmentally complex mushroom forming fungi. Ras proteins of the ectomycorrhizal fungus, *L. bicolor*, and the model mushroom, *S. commune*, have both been examined. In *L. bicolor*, expression of the Ras1-like homologue, *LbRas*, is dependent on the symbiotic relationship with plant roots (Sundaram et al., 2001). Free living *L. bicolor* colonies do not contain detectable levels of *LbRas* transcript by northern blot analysis, whereas *LbRas* transcript is upregulated during symbiotic growth with red pine root (Sundaram et al., 2001). Additionally, immunolocalization analysis shows that *LbRas* is localized primarily to the inner surface of the cell wall, presumptively at the plasma membrane (Sundaram et al., 2001). These findings fit with the dual cysteine palmitoylation motif found in the *LbRas* HVR (Figure 2). No further studies on *LbRas* interacting partners, or on the roles of the *L. bicolor* Ras2-like homolog, have been reported.

S. commune has two Ras proteins, Ras1 and Ras2, which have been the focus of more in-depth study. Due to their order of discovery, the Ras1 protein of *S. commune* actually aligns with the Ras2-like group whereas Ras2 aligns with the Ras1-like group (Figure 2). Expression of a constitutively active *ras1* (*ras1^{G12V}*) leads to compact colony morphology, meandering apical growth of hyphae and increased hyphal branching (Knabe et al., 2013). However, an *S. commune* strain expressing constitutively inactive *ras1* (*ras1^{G15N}*) could not be obtained, suggesting loss Ras1 activity is lethal (Knabe et al., 2013). This is potentially an interesting distinction between *S. commune* and the other filamentous fungi reviewed here, as the Ras2-like homolog is not typically essential in these other organisms. A test for mating revealed that constitutively active Ras1 strains cannot accept nuclei from their mating partners and, therefore, cannot form fruiting bodies (Knabe et al., 2013). In contrast, earlier studies revealed that deletion of a Ras GAP, *gap1*, produced a strain that cannot fully undergo clamp closure, forms increased numbers of fruitbody primordia, develops fruitbodies with aberrant gills and does not produce spores (Knabe et al., 2013; Schubert et al., 2006). Exploring molecular mechanisms of *S. commune* Ras signaling again led to the study of PKA and Rho-GTPase pathways. No increase of cytoplasmic cAMP could be identified in the *gap1/ gap1* or constitutively active Ras1 mutants, suggesting that Ras may not signal upstream of the PKA pathway via cAMP production (Knabe et al., 2013; Schubert et al., 2006). However, PKA protein activity is significantly increased for both of these mutants, suggesting Ras-PKA pathway interactions (Knabe et al., 2013). Microarray expression analyses also show that a number of genes are co-regulated by *Gap1*, Ras1 and Cdc42, suggesting a common pathway for these three proteins (Knabe et al., 2013). Genes co-regulated by this hypothetical pathway include predicted regulators of mating, transcription, autophagy, and ubiquitination.

8. Conclusions

In summary, the signaling networks controlled by Ras proteins in filamentous fungi contribute heavily to completion of normal developmental programs, fungal growth and to stress responses combating various external insults (Figure 3). An obvious paradigm exists in filamentous fungi where two Ras proteins are expressed, with the Ras1-like homologue acting as the dominant Ras protein for regulation of fungal growth. Available data suggest that the Ras2-like homologues typically share some functions with the Ras-like homologues, but can also play unique roles in growth and virulence. The unique roles played Ras1-like and Ras2-like homologues may be due in part to differential post-translational lipidation events. This may, in turn, place each Ras homologue in the proper context for interaction with activating proteins and downstream effectors. Pathways controlled by Ras continue to be an important topic for study as they are necessary for development of invasive structures in pathogens of humans, plant and insects alike. The continued study of Ras networks in filamentous fungi will undoubtedly uncover novel insights into Ras-mediated signaling mechanisms, the role of post-translational modifications in the localization and activation of Ras, and crucial developmental biology processes regulating virulence in pathogenic fungi.

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Figure 1. Putative pathways for Ras localization and signaling in fungal organisms

Nascent Ras proteins contain C-terminal “CAAX” motif (C = cysteine, A = aliphatic amino acid, X = any amino acid) that serves as a recognition sequence for a cytoplasmic farnesyltransferase (Ram1/Ram2 in *S. cerevisiae*). **Step 1:** The farnesyltransferase enzyme modifies the CAAX motif by addition of a farnesyl lipid moiety to the cysteine residue. **Step 2:** Ras is then further processed by the activity of Ras converting enzyme (Rce1 in *S. cerevisiae*), which catalyzes the proteolytic cleavage of the “-AAX” residues from the farnesylated CAAX motif. **Step 3:** The farnesylated cysteine is then carboxymethylated by action of an Isoprenylcysteine carboxymethyltransferase (ICMT, Ste14 in *S. cerevisiae*). For Ras1-like proteins to complete transit via the exocytosis machinery of the ER/Golgi complexes and become associated with the plasma membrane (PM), another ER resident protein, a palmitoyltransferase (PAT), must modify the Ras protein through addition of a palmitoyl lipid moiety. **Step 4:** The palmitoyl modification occurs at a conserved cysteine residue located just upstream of the CAAX motif, within the hypervariable domain. **Step 5:** In higher eukaryotes, Ras palmitoylation is reversible and PM localized Ras has been shown to cycle back to the ER/golgi membranes through the activity of a palmitoyl-protein thioesterase (PPT) at the PM. Reversible palmitoylation of Ras has not yet been shown in fungal organisms. Once properly localized, Ras proteins transmit signals via PKA, MAPK, and Rho-GTPase pathways to control growth, development and stress response. Pathways shown are those known for the model yeast organisms, *Sacharomyces cerevisiae* and *Schizosaccharomyces pombe*, as well as the human pathogen, *Cryptococcus neoformans*, and are discussed in the Introduction.

Figure 2. Filamentous fungal Ras homologues comprise two distinct groups
Ras proteins of the filamentous fungi studied thus far split into "Ras1-like" and "Ras2-like" subgroups. The exception to this paradigm is *M. racemosus*, which encodes only Ras1-like homologues. Members of the "Ras1-like" group are mainly predicted to be prenylated and palmitoylated, whereas the Ras2-like group members are only prenylated. Pa = palmitoylation domain, Pr = prenylation domain.

Figure 3. Ras proteins of filamentous fungi control multiple cellular processes

Ras proteins contribute to fungal differentiation, morphogenesis, and stress response.