7 Heterotrimeric G Proteins

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I. Introduction

All living organisms are exposed to a variety of environmental signals that elicit appropriate cellular responses that dictate cell survival and development. A major system used by eukaryotic organisms to sense and respond to environmental stimuli involves **heterotrimeric G-proteins** (Tesmer 2010) and their cognate membrane-bound receptors, known as **G protein-coupled receptors** or GPCRs (Bock et al. 2014) (Fig. 7.1). Heterotrimeric G proteins are composed of three different subunits, Gα, Gβ, and Gγ (Jastrzebska 2013; Tesmer 2010). The Gα subunit is bound to guanosine diphosphate (GDP) when inactive and exchanges GTP for GDP after activation (Jastrzebska 2013). In the inactive state, the heterotrimer is associated with G-protein-coupled receptors (GPCRs), on the cytoplasmic face of the plasma membrane (Bock et al. 2014; Pierce et al. 2002; Neves et al. 2002). GPCRs function as **guanine nucleotide exchange factors** (**GEFs**) and are characterized by the presence of seven transmembrane alpha helices (Shukla et al. 2014; Chini et al. 2013). Binding of the extracellular ligand to the GPCR causes exchange of GDP for GTP on the Gα subunit and dissociation of Gα-GTP from the

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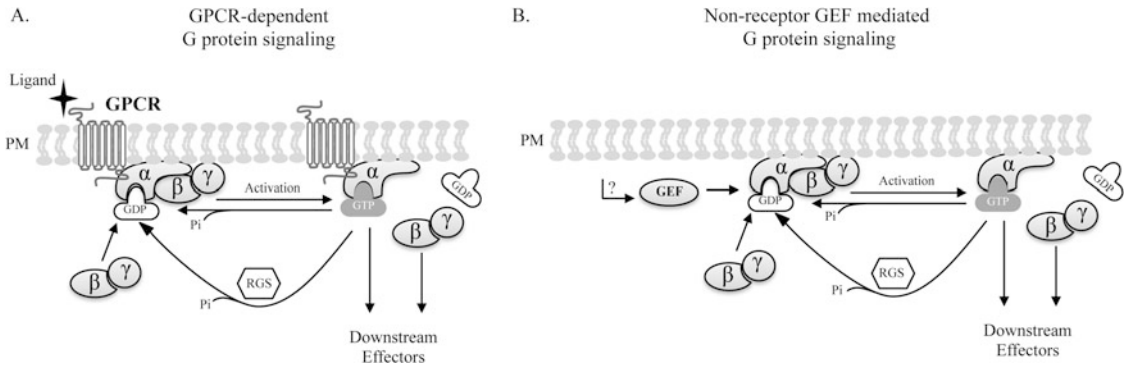

Fig. 7.1 Overview of heterotrimeric G protein pathways. Heterotrimeric G protein signaling mechanisms in fungi are regulated via GPCR-dependent and GPCR-independent, non-receptor GEF pathways. (**a**) Ligand binding to a GPCR stimulates a conformational change in the receptor which leads to GDP–GTP exchange on the Gα protein and subsequent dissociation of the Gα from the Gβγ dimer. The Gα and Gβγ units regulate downstream effectors, such as adenylyl cyclase and MAPK cascades. Hydrolysis of GTP on the Gα subunit occurs via its intrinsic GTPase activity, and this reaction can also be accelerated via RGS proteins. Reassociation of the Gβγ dimer with the GDP-bound Gα returns to the heterotrimeric G protein to the resting state in association with the GPCR. (**b**) Non-receptor GEFs such as RIC8 and ARR4/GET3 can also stimulate GDP-GTP exchange on Gα proteins. In various fungal systems, RIC8 has been shown to interact with Group I and III Gα proteins to regulate cAMP signaling. *Abbreviations*: *GPCR* G protein-coupled receptor, *PM* plasma membrane, *GDP* guanosine diphosphate, *GTP* guanosine triphosphate, *RGS* regulators of G protein signaling, *GEF* guanine nucleotide exchange factor, *Pi* phosphate

Gβγ dimer, allowing both moieties to regulate downstream effectors (Jastrzebska 2013; Chini et al. 2013; Neves et al. 2002; Mende et al. 1998). In addition to GPCRs, GDP–GTP exchange on Gα subunits can also be mediated via non-receptor GEFs, such as RIC8 and GET3 (Miller et al. 2000; Tall et al. 2003; Wilkie and Kinch 2005; Siderovski and Willard 2005; Lee and Dohlman 2008) (Fig. 7.1b). Finally, examples of downstream effectors regulated by G proteins are adenylyl cyclases, ion channels, mitogen-activated protein kinases (MAPK), phosphodiesterases, and phospholipases (Chini et al. 2013; Neves et al. 2002; Mende et al. 1998) (Figs. 7.2 and 7.3).

Termination of the signal is facilitated by hydrolysis of GTP to GDP on the Gα subunit and reassociation of Gα-GDP with the Gβγ dimer and the GPCR at the plasma membrane (Jastrzebska 2013; Chini et al. 2013; Neves et al. 2002; Mende et al. 1998) (Fig. 7.1). The Gα subunit has intrinsic GTPase activity, and this process is accelerated via certain other proteins called **Regulators of G protein Signaling** (**RGS**) or **GTPase Accelerating Proteins** (**GAPs**) (Ross and Wilkie 2000; Siderovski et al. 1996; Berman and Gilman 1998).

In this chapter, we will provide a brief summary of heterotrimeric G protein signaling components and effector pathways in fungi. The focus will then shift to select G protein signaling paradigms in several fungal species: *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Neurospora crassa*, *Aspergillus nidulans*, *Magnaporthe grisea*, *Cryptococcus neoformans*, and *Ustilago maydis*. Due to space limitations, we are unable to reference all of the work in this rapidly expanding field, and we direct the reader to several reviews (Raudaskoski and Kothe 2010; Xue et al. 2008; Yu 2006; Li et al. 2007b; Lengeler et al. 2000).

II. Components of Heterotrimeric G Protein Signaling

A. Gα, Gβ, and Gγ Proteins

Gα subunits are composed of α-helical and a GTPase domains, the latter of which imparts intrinsic GTPase activity to the Gα subunit (Bohm et al. 1997; Birnbaumer 1992). The GTPase domain consists of five α helices sur-

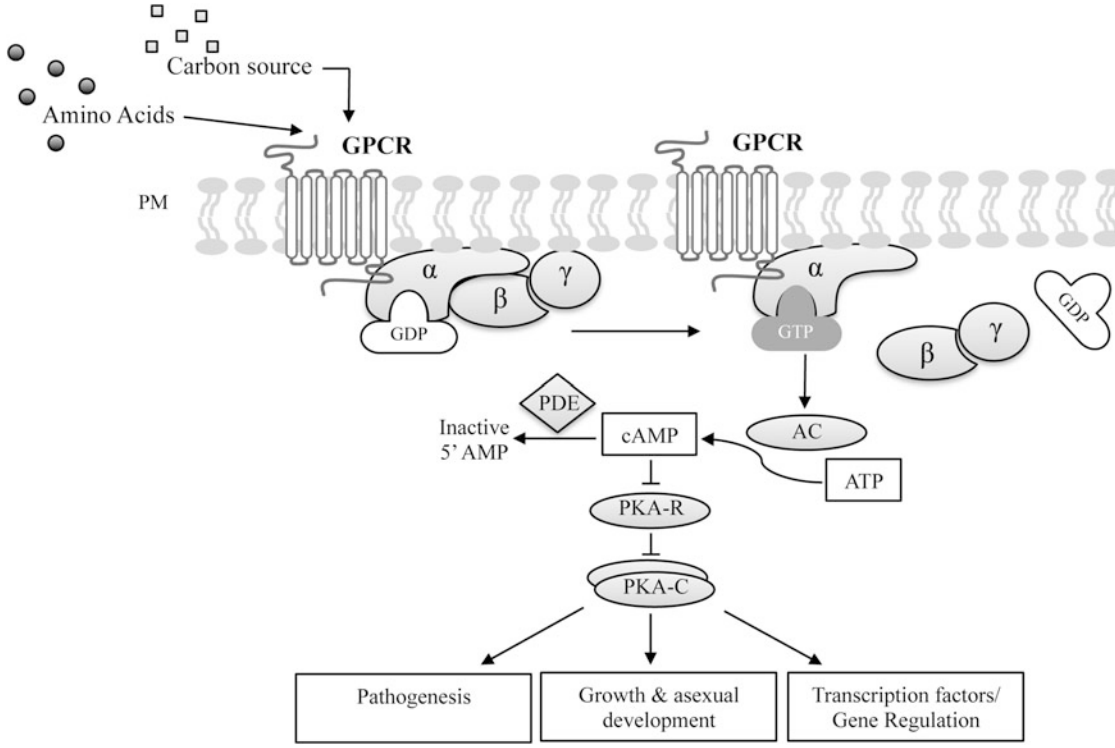

Fig. 7.2 Nutrient sensing. Heterotrimeric G protein-mediated nutrient sensing occurs mainly via the cAMP/PKA signaling pathway. Upstream GPCRs sense carbon or amino acids and couple these responses to downstream G proteins, resulting in activation of adenylyl cyclase and generation of cAMP. The PKA-regulatory subunits binds cAMP, allowing for release of the catalytic subunits that phosphorylate downstream targets, including transcription factors. Additional regulation of cAMP levels takes place via phosphodiesterases, which hydrolyze cAMP to the inactive 5′AMP. GPCRs involved in nutrient sensing in: *N. crassa*—GPR-4 (carbon-sensing), *C. neoformans*—Gpr4 (amino acid sensing), *A. nidulans*—GprC, GprD (putative carbon sensors), *S. cerevisiae*—Gpr1 (carbon-sensing), *S. pombe*—Git3 (carbon sensing). *Abbreviations*: *GPCR* G protein-coupled receptor, *PM* plasma membrane, *GDP* guanosine diphosphate, *GTP* guanosine triphosphate, *cAMP* cyclic adenosine monophosphate, *PDE* phosphodiesterases, *PKA-R* protein kinase A regulatory subunit, *PKA-C* protein kinase A catalytic subunit

rounding a β sheet and connected to the α-helical domain by two linkers. The guanine nucleotide-binding pocket within the GTPase domain contains a consensus sequence GXGXXGKS for GTP binding (Hamm and Gilchrist 1996). Mutations in the GTPase domain of the Gα subunit lead to disruption in its GTPase activity and result in constitutive Gα-GTP signaling (Freissmuth and Gilman 1989; Graziano and Gilman 1989). Finally, many Gα subunits undergo posttranslational lipid modifications, such as palmitoylation and myristoylation, which aid in tethering the protein to the membrane to facilitate interaction with the Gβγ dimer (Resh 1996).

Most filamentous fungi possess three Gα proteins in each of three groups, I–III, but the basidiomycete *Ustilago maydis* also has a fourth Gα (Group IV) (Bolker 1998) (Table 7.1). In contrast, budding and fission yeasts contain only two Gα proteins (Bolker 1998). In general, Group I Gα proteins share high amino acid similarity and regulate sexual and asexual developmental pathways (Li et al. 2007b). The *Neurospora crassa* Group I Gα protein GNA-1 was the first Gα protein identified in filamentous fungi and found to share 55 % identity to members of the mammalian $G\alpha_i$ superfamily (Turner and Borkovich 1993). In addition, the C-terminus of GNA-1 contains the consensus

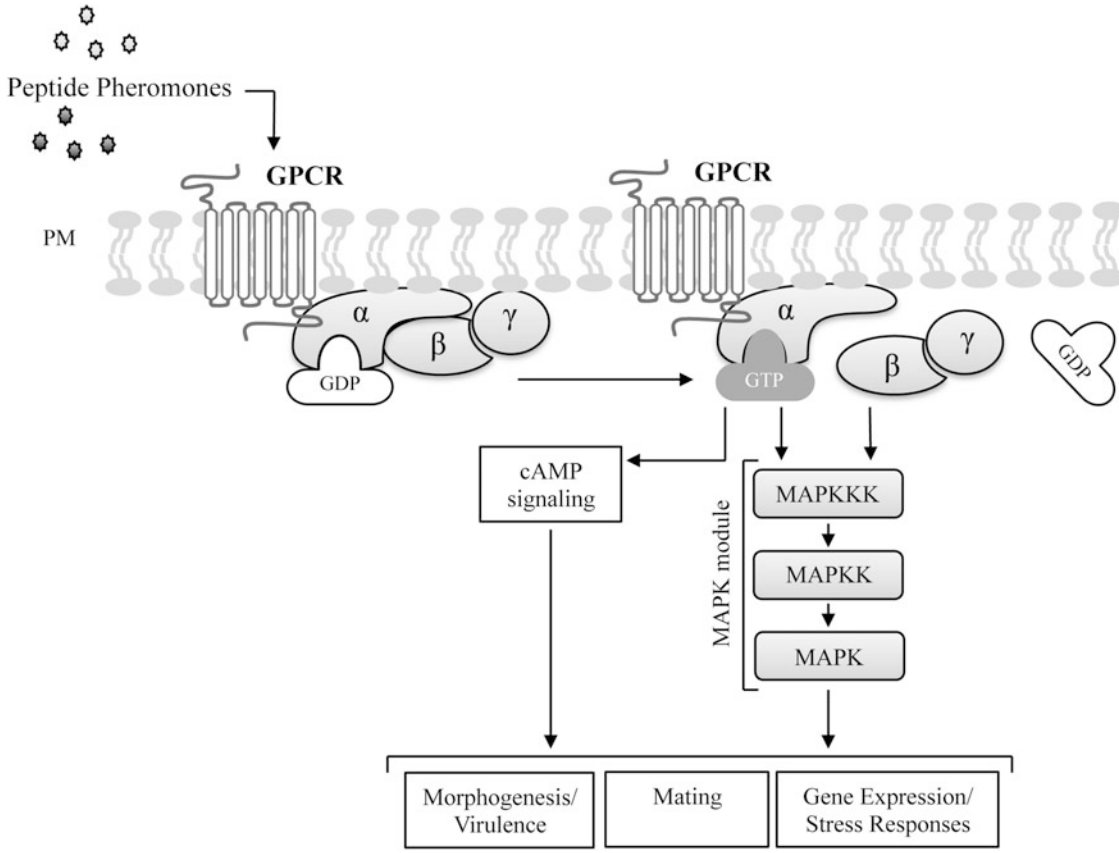

Fig. 7.3 Pheromone sensing and mating. In fungi, typically two different types of peptide pheromones (colored *gray/black*) are responsible for activation of their respective GPCRs (pheromone receptors). The GPCRs respond to the pheromones—α-factor and a-factor in *S. cereviseae*, M-factor and P-factor in *S. pombe*, MFA-1 and CCG-4 in *N. crassa*, and Mfa1-3 and Mfα1-3in *C. neoformans*. In *S. cerevisiae*, it has been demonstrated that activation of the Gα subunit leads to dissociation of the Gβγ heterodimer and subsequent activation of the MAPKKK, leading to sequential phosphorylation of MAPK module components, eventually influencing mating-specific gene expression. In *N. crassa* and other fungi, mating is regulated via both cAMP-dependent and cAMP-independent signaling mechanisms. GPCRs involved in pheromone sensing: PRE-1 and PRE-2 in *N. crassa*, Ste3α and Ste3a in *C. neoformans*, GprA and GprB in *A. nidulans*, Ste2p and Ste3p in *S. cereviseae*, Mam2 and Map3 in *S. pombe*, Pra1 and Pra2 in *U. maydis*. *Abbreviations*: *GPCR* G protein-coupled receptors, *GDP* guanosine diphosphate, *GTP* guanosine triphosphate, *cAMP* cyclic adenosine monophosphate, *MAPKKK* mitogen-activated kinase kinase kinase, *MAPKK* mitogen-activated kinase kinase, *MAPK* mitogen-activated kinase

Table 7.1 Gα, Gβ, and Gγ subunit proteins in various fungi

	Class					
	Gα protein subunits					
Organism	I	II	III	IV	Gβ protein subunit	Gγ protein subunit
Saccharomyces cereviseae	Gpa1	–	Gpa2	–	Ste4	Ste18
Schizosaccharomyces pombe	–	Gpa1	Gpa2	–	Git5, Gnr1	Git11
Neurospora crassa	GNA-1	GNA-2	GNA-3	–	GNB-1	GNG-1
Aspergillus nidulans	FadA	GanA	GanB	–	SfaD	GpgA
Cryptococcus neoformans	Cga1	Gpa3	Gpa1		Gpb1	Gpg1, Gpg2
Magnaporthe oryzae	MAGB	MAGC	MAGA	–	MGB1	MGG-10193.5
Ustilago maydis	Gpa1	Gpa2	Gpa3	Gpa4	Bpp1	UM06109.1

sequence for ADP-ribosylation by pertussis toxin (CAAX) and is modified by pertussis toxin: both properties are a hallmark of the $G\alpha_i$ super family (Turner and Borkovich 1993).

Gβ and Gγ subunits are tightly associated and transduce signals downstream as a heterodimer. In higher eukaryotes, multiple isoforms of Gβ and Gγ subunits are known (Hamm 1998). In fungi, there is one canonical Gβ subunit (Li et al. 2007b) (Table 7.1), but emerging evidence suggests that RACK1 like proteins can also function as Gβ proteins in fungi (Zeller et al. 2007; Wang et al. 2014; see below). Gβ proteins are approximately 340 amino acids and are known to be the most highly conserved of the three subunits. Structurally, the Gβ subunit is composed of an amphipathic α-helix at its N-terminus that extends into seven repeating units of a WD motif of 36–46 amino acid residues with tryptophan (W) and aspartate (D) at the ends (Fong et al. 1986; Neer et al. 1994; Gautam et al. 1998; Downes and Gautam 1999). The seven repeating units form a β-sheet of four antiparallel strands that protrude away from the center such that the overall structure appears to be a seven bladed propeller, called a β-superbarrel. The opposite ends of β-superbarrel interact with Gα and Gγ subunits. In contrast to the Gβ subunit, Gγ subunits are relatively small proteins, consisting of 68–75 amino acid residues. There are numerous Gγ proteins in mammals, but most fungi possess only one canonical Gγ protein (Table 7.1). *C. neoformans* is an exception, with two predicted Gγ genes (Li et al. 2007a). The characteristic feature of Gγ subunits is the presence of a CaaX motif at the C-terminus that provides site for posttranslational lipid modifications such as farnesylation and geranylgeranylation (Lai et al. 1990; Yamane et al. 1990). These modifications allow the Gγ to bind the plasma membrane and aid the interaction with the Gα subunit (Iniguez-Lluhi et al. 1992; Gautam et al. 1998; Zhang and Casey 1996). The Gβ and Gγ proteins in different fungi are summarized in Table 7.1.

In recent years, it has become clear that other proteins may take the place of canonical Gβ and Gγ subunits during signal transduction in fungi. For example, proteins homologous to the RACK1 Gβ-like protein have been found to interact with Gα or/or Gγ subunits and to regulate signaling in various fungi (Zeller et al. 2007; Wang et al. 2014). In yeast, two Kelch repeat proteins can interact with the Gα subunit Gpa2 to activate protein kinase A without intermediate activation of adenylyl cyclase (Peeters et al. 2006).

B. Guanine Nucleotide Exchange Factors (GEFs)

1. G Protein-Coupled Receptors (GPCRs)

The typical GPCR contains a structural signature of conserved **seven-hydrophobic transmembrane** (**7-TM**) helices that are connected by intracellular and extracellular loops with the N-terminus extending to the outer environment and the C-terminus localized in the cytoplasm (Fig. 7.1a). The most variable regions of GPCRs are the C-terminus, N-terminus, and intracellular loops, with greatest diversity in the N-terminal region (Kobilka 2007; Katritch et al. 2012; Venkatakrishnan et al. 2013).

GPCRs represent the largest and most diverse group of transmembrane receptors, with well over 800 potential GPCRs in the human genome (Hill 2006). Based on sequence similarity in the 7-TM segments, mammalian GPCRs have been clustered into five families: rhodopsin (701 members), adhesion (24 members), frizzled/taste (24 members), glutamate (15 members), and secretin (15 members) (Fredriksson et al. 2003). Several GPCR classification schemes have been proposed for fungal genomes (Galagan et al. 2003; Kulkarni et al. 2005; Lafon et al. 2006; Li et al. 2007b; Zheng et al. 2010; Krishnan et al. 2012; Gruber et al. 2013). However, common to all classification systems, fungal GPCRs include pheromone receptors, cAMP-receptor-like proteins, carbon- and amino acid-sensing receptors, putative nitrogen sensors, and microbial opsin-like proteins.

2. Non-receptor GEFs

Recent studies have revealed the existence of cytosolic non-GPCR proteins that can act as GEFs to positively regulate Gα proteins (Fig. 7.1b). Two examples of such proteins in fungi are RIC8 and GET3/ARR4. RIC8 (resis-

tance to inhibitors of cholinesterase 8; Synembryn) was first identified in the nematode *Caenorhabditis elegans* as a protein that exhibits GEF activity toward Gα proteins (Miller and Rand 2000). RIC8 is found in animals and certain fungi, but absent from plants and the model yeast *Saccharomyces cerevisiae* (Wilkie and Kinch 2005; Wright et al. 2011). In animals, RIC8 has been implicated in cAMP and MAPK signaling and G protein abundance, with downstream regulation of asymmetric cell division, priming of synaptic vesicles, and hormone signaling (Tall 2013; Hampoelz et al. 2005; Schade et al. 2005; Afshar et al. 2004; Miller and Rand 2000).

RIC8 isoforms have been found to be potent GEFs for a subset of Gα proteins, including $G\alpha_q$, $G\alpha_{i1}$, and $G\alpha_o$. Ric8A preferentially interacts with GDP-bound Gα to facilitate the release of GDP and then form a stable nucleotide-free transition state complex with Gα until Gα binds to GTP (Tall et al. 2003; Thomas et al. 2011). The stable transition state complex prevents Gα ubiquitination (Nagai et al. 2010; Chishiki et al. 2013). Further studies on embryonic stem cell lines deleted for Ric8A and Ric8B revealed the functions of RIC8 as a molecular chaperone required for the initial association of nascent Gα subunits with cellular membranes (Gabay et al. 2011; Chan et al. 2013).

The role of RIC8 has been studied in the following four filamentous fungal genera: the rice pathogen *M. oryzae* (Li et al. 2010), the saprobe and model system *N. crassa* (Wright et al. 2011; Eaton et al. 2012), the opportunistic human pathogen *A. fumigatus* and the model system *A. nidulans* (Kwon et al. 2012), and the human pathogen *C. neoformans* (Gong et al. 2014). In *M. oryzae*, *Δmoric8* mutants exhibit defects in formation of infection structures (appressoria) that are necessary for rice blast disease (Li et al. 2010). The appressorial defect is partially rescued by addition of exogenous cAMP, but the induced appressoria are nonfunctional (Li et al. 2010). MoRIC8 is localized in the cytoplasm of vegetative hyphae, conidia, and appressoria, with the highest expression in appressoria, consistent with its role in infection-related morphogenesis (Li et al. 2010). In *N. crassa*, loss of *ric-8* gene leads to extreme phenotypes, including impaired growth, short aerial hyphae, inappropriate conidiation in submerged culture, and female sterility (Wright et al. 2011). These phenotypes are similar to double mutants lacking the *gna-1* and *gna-3* Gα genes (Kays and Borkovich 2004). Many defects of the Δ*ric8* mutant are suppressed by mutation of the regulatory subunit of PKA, *mcb* (Bruno et al. 1996), which is consistent with RIC8 operating upstream of cAMP signaling in *N. crassa* (Wright et al. 2011). Similar to the situation in *M. oryzae*, *N. crassa* RIC8 is localized to the cytoplasm of vegetative hyphae and mature conidia (Wright et al. 2011). Importantly, like its mammalian homolog, *N. crassa* RIC8 binds and exhibits GEF activity towards the GNA-1 and GNA-3 Gα proteins, with the greatest stimulation of GNA-3 (Wright et al. 2011). In *A. nidulans* and *A. fumigatus*, loss of RIC8 profoundly affects conidial germination, hyphal proliferation, and asexual/sexual fruiting (Kwon et al. 2012). AnRICA physically interacts with the Gα protein GanB in *A. nidulans* (Kwon et al. 2012). A role for RIC8 in capsule formation, melanin production, and virulence has been revealed in the human pathogen *C. neoformans* (Gong et al. 2014). RIC8 interacts with and stimulates GEF activity on the Gpa1 and Gpa2 Gα subunits and impacts cAMP and pheromone signaling in *C. neoformans* (Gong et al. 2014). Taken together, the data from multiple fungal species suggest that RIC8 is a cytoplasmic Gα GEF that interacts with Group I and Group III Gα proteins to modulate cAMP and other signaling pathways (Fig. 7.1).

In contrast to RIC8, GET-3/ARR4 is widely conserved in eukaryotes, including fungi (Lee and Dohlman 2008). ARR4 is so named because of homology to Arsenicals Resistance proteins in bacterial species (Shen et al. 2003). In yeast, Get3p/Arr4p is required for ATP-dependent tail-anchoring of proteins in the endoplasmic reticulum (Denic et al. 2013). Get3p has also been demonstrated to bind to, and exhibit GEF activity towards, the nucleotide-free form of the Gpa1p Ga protein (Lee and Dohlman 2008). Get3p is also required for normal MAPK signaling, gene regulation, and mating in *S. cerevisiae* (Lee and Dohlman 2008).

C. Regulator of G Protein Signaling (RGS) Proteins

RGS proteins are crucial elements in controlling the intensity and duration of G protein signaling. Most RGS proteins are considered as negative regulators of G protein signaling, either by accelerating intrinsic GTPase activity on Gα subunits (GTPase activation) or by competitively blocking the Gα-GTP interaction with downstream effectors (effector antagonism) (Ross and Wilkie 2000; Anger et al. 2004; Wang et al. 2013). All RGS proteins contain a conserved RGS core domain of ~120 to 130 amino acids that is necessary and sufficient for binding to transition state of GTP-Gα subunits for accelerating GTP hydrolysis (Hollinger and Hepler 2002). In addition to the RGS domain, RGS proteins contain additional motifs that allow integration into different G-protein signaling pathways or function as scaffold elements that link G proteins to other related signaling proteins (Wang et al. 2013). These domains include DEP (Dishevelled, Egl-10, Pleckstrin) for membrane targetting, Phosphotyrosine interaction (PTB), Ras binding (RBD), GoLoco for guanine nucleotide exchange, and GGL (Gγ subunit like) for binding Gβ subunits (De Vries et al. 2000; Chasse and Dohlman 2003; Wang et al. 2013). The presence of such diverse domains also makes RGS proteins attractive targets for drugs to modulate cell signaling.

RGS proteins have been discovered in large number of eukaryotes, from yeast to *C. elegans* to humans, and >30 RGS-like proteins have been identified in the human proteome (Chasse and Dohlman 2003; Wang et al. 2013; Hollinger and Hepler 2002). The first RGS protein, Sst2p, was discovered in *S. cerevisiae* through a genetic screen for negative regulators of the pheromone response (Chan and Otte 1982). Sst2p serves as a critical regulator of mating pheromone signaling by directly binding to the C-terminal tail of the pheromone receptor Ste2p via its DEP domain (Chasse et al. 2006). Sst2p also genetically and physically interacts with the Gα protein Gpa1p and accelerates its GTPase activity (Dohlman et al. 1996; Apanovitch et al. 1998). Another *S. cerevisiae* RGS protein, Rgs2p, interacts with the Gα Gpa2p and functions as a multicopy suppressor for Gpa2p-dependent loss of heat shock resistance (Versele et al. 1999).

The functions of RGS and RGS-like proteins have been extensively studied in several filamentous fungi (Liu et al. 2007; Zhang et al. 2011; Shen et al. 2008; Fang et al. 2008; Mukherjee et al. 2011). The first characterized RGS protein in filamentous fungi was *A. nidulans* FlbA (Lee and Adams 1994). FlbA interacts with the FadA Gα protein and is required for regulation of hyphal proliferation, development, and biosynthesis of secondary metabolites (Yu et al. 1996). In addition to FlbA, *A. nidulans* possesses several other RGS proteins, RgsA, RgsB, RgsC, and GprK. RgsA negatively regulates the GanB Gα signaling during stress responses and asexual sporulation (Han et al. 2004). GprK is unique in that it contains both 7-TM and RGS domains, similar to AtRGS1, which negatively regulates the Gpa1 Gα during cellular proliferation in the model plant *A. thaliana* (Lafon et al. 2006; Chen et al. 2003).

D. Effector Pathways

cAMP-dependent protein kinase (PKA) (Fig. 7.2) and MAPK (Fig. 7.3) cascades are two major effector pathways in fungi that act downstream of the activated GTP binding G-proteins to elicit cellular responses such as growth, mating, cell division, cell–cell fusion, morphogenesis, toxicognesis, chemotaxis, and pathogenic development (Bolker 1998; Lengeler et al. 2000).

1. cAMP-Dependent Protein Kinase (PKA)

The key components of the cAMP-PKA pathway include the enzyme adenylyl cyclase and the regulatory and catalytic subunits of PKA (Fig. 7.2). Fungal adenylyl cyclases are cytoplasmic peripheral membrane proteins (Matsumoto et al. 1984; Gold et al. 1994; Adachi and Hamer 1998) and contain multiple domains (from N- to C-terminus), including Gα binding, Ras-association (RA), leucine-rich repeat (LRR), protein phosphatase 2C (PP2C), cyclase catalytic, and cyclase-associated protein 1 (Cap1) binding domains (Wang 2013). The

activation of adenylyl cyclase by GTP-bound Gα proteins catalyzes the conversion of adenosine triphospate (ATP) to adenosine-3′5′-cyclic monophosphate (cAMP). The cAMP molecules act as secondary messengers to activate protein kinase A. In the absence of cAMP, the PKA holoenzyme exists as an inactive heterotetramer composed of two regulatory (R) and two catalytic (C) subunits (Robison et al. 1968; Krebs 1989). The R subunits have a conserved domain structure, consisting of an N-terminal dimerization domain, an inhibitory domain, cAMP-binding domain A, and cAMP-binding domain B (Robison et al. 1968; Krebs 1989). R subunits interact with C subunits primarily through the inhibitory site (Francis and Corbin 1999). Binding of four cAMP molecules to the cAMP-binding domains on the two R subunits releases the active catalytic subunits as dimers that can phosphorylate serine and threonine residues on downstream proteins, including transcription factors (Krebs 1989). The intensity of cAMP signaling also depends on the balance between cAMP production and its degradation. 3′5′-cyclic nucleotide phosphodiesterases (PDEs) control the level of cAMP by converting active cAMP to inactive 5′-AMP, thus terminating PKA activation (Fig. 7.2). Nutrient sensing, perhaps the most extensively studied function of the cAMP signaling pathway in yeast and filamentous fungi, is described later in this chapter.

2. Mitogen-Activated Protein Kinase (MAPK)

MAPK signaling pathways are evolutionary-conserved signaling mechanisms that regulate diverse physiological processes, ranging from cell proliferation and differentiation to cell death in eukaryotes (Gustin et al. 1998; Widmann et al. 1999; Qi and Elion 2005; Keshet and Seger 2010). In fungi, MAPKs are involved in regulating mating, morphogenesis, virulence, and stress responses (Kronstad et al. 1998; Lopez-Ilasaca 1998; Mehrabi et al. 2012; Raudaskoski and Kothe 2010). The key components of this pathway are the MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK (Qi and Elion 2005) (Fig. 7.3). These proteins operate as a three-tiered module to sequentially phosphorylate downstream proteins such as transcription factors, cytoskeletal proteins, kinases, and other enzymes, thereby greatly influencing gene expression, metabolism, cell division, cell morphology, and cell survival (Qi and Elion 2005). The first kinase of the MAPK unit is the MAPKKK. MAPKKKs have an N-terminal autoinhibitory domain, a C-terminal catalytic domain, and a large regulatory motif outside the catalytic domain. The regulatory motifs consists of Pleckstrin homology (PH) and Src homology 3 (SH3) domains, binding sites for GTP-binding proteins, leucine-zipper dimerization sequences, and phosphorylation sites for tyrosine and serine/threonine kinases (Garrington and Johnson 1999). The presence of different kinds of regulatory motifs in MAPKKKs reflects its selective regulation by a variety of upstream inputs, and its flexibility and specificity in responding to a wide range of cellular stimuli through activation of specific MAPKKs and MAPKs of three-tiered modules (Garrington and Johnson 1999). MAPKKs are dually phosphorylated by MAPKKKs at serine and threonine residues within the activation loop of the catalytic domain. They are highly specific for their cognate MAPK and activate it by phosphorylating the MAPK at conserved threonine and tyrosine residues (T–X–Y) within the activation loop (Payne et al. 1991; Ferrell and Bhatt 1997). Depending on the fungal system and environmental stimuli, all or some of the MAPK cascade components may shuttle in and out of the nucleus (Qi and Elion 2005; Nguyen et al. 2002; Bayram et al. 2012). MAPKs generally contain multiple docking domains for binding to substrates, MAPKKs, and scaffolding proteins, allowing high affinity protein–protein and protein–substrate interactions (Tanoue and Nishida 2003). Scaffolding proteins associated with the MAPKs facilitate efficient phosphorylation, preventing cross-talk between different signaling pathways, thus maintaining the integrity of the initiating stimulus (Burack and Shaw 2000). The activated MAPK phosphorylates target proteins, in many cases transcription factors, at serine and threonine residues (Wang et al. 2011; Bardwell 2006). The activated tran-

scription factor is then able to facilitate changes in gene expression in response to the initial stimulus (Fig. 7.3).

There are three conserved MAPKs in fungi (Posas et al. 1998; Hamel et al. 2012). In *S. cerevisiae*, they are required for the pheromone response and mating (Fus3p/Kss1p; Dohlman and Slessareva 2006), cell wall integrity (Mpk1p; Fuchs and Mylonakis 2009), and osmosensing (Hog1p; Saito and Posas 2012). The MAPK cascade required for the pheromone response and mating has been extensively studied in several fungi and will be described later in this chapter. For a detailed review on fungal MAPK pathways, see also chapter "Fungal MAP-Kinase-Mediated Regulatory Pathways" in this volume.

IV. Cellular Functions of G Protein Signaling Components in Yeast and Filamentous Fungi

A. Nutrient Sensing

Nutrients are the source of energy and building blocks for the cell. They act as regulators of metabolism, growth, and development. The key process for the survival of any organism depends on their ability to adapt to changing nutritional conditions. The plasma membrane of the cell is the first boundary that detects the signaling molecules via membrane receptors and transfer nutrients from the external environment to the intracellular environment via its transport system. Certain GPCRs in yeasts and filamentous fungi are known to detect nutrients and activate signal transduction in association with Gα proteins (Holsbeeks et al. 2004). Several examples of such nutrient-sensing pathways are discussed in the following sections, and a generic model diagram is presented in Fig. 7.2.

1. Glucose Sensing in *Saccharomyces cerevisiae* and *Schizoaccharomyces pombe*

In *S. cerevisiae*, Gpr1p is a sugar-sensing GPCR that interacts with the C-terminal end of the Gpa2p Gα protein (Yun et al. 1997). Gpr1p is very effective in sensing glucose and sucrose, while the structurally similar sugars fructose and galactose had no effect and mannose is antagonistic (Lemaire et al. 2004; Rubio-Texeira et al. 2010). Results from cysteine scanning mutagenesis of residues in transmembrane domain VI of *GPR1* support a model in which glucose and sucrose are ligands for Gpr1p (Lemaire et al. 2004). Glucose-mediated Gpr1p signaling in *S. cerevisiae* results in low levels of the storage carbohydrates trehalose and glycogen and reduced expression of stress resistance genes (Tamaki 2007; Rubio-Texeira et al. 2010). The current model is that glucose binding to Gpr1p directs the formation of the GTP-bound active form of Gpa2p, which then stimulates adenylyl cyclase to transiently increase cAMP levels, setting in motion a PKA-mediated protein phosphorylation cascade (Kraakman et al. 1999). In this scenario, the RGS protein Rgs2p negatively controls the activity of Gpa2p to terminate signaling (Versele et al. 1999).

Yeast cells grown in glucose medium are larger in size than those cultured with non-fermentable carbon sources, such as ethanol (Johnston et al. 1979; Busti et al. 2010). *gpr1* and *gpa2* mutant cells are smaller than wild type in glucose medium, leading to the suggestion that Gpr1p–Gpa2p signaling regulates cell size and longevity in *S. cerevisiae* (Tamaki et al. 2005; Tamaki 2007). Signaling through Gpr1p and Gpa2p is also essential for pseudohyphal filamentous growth in *S. cerevisiae* (Lorenz et al. 2000). For instance, diploid *Δgpr1* or *Δgpa2* mutants cannot undergo the yeast to pseudohyphal transition under certain environmental conditions (Lorenz and Heitman 1997; Lorenz et al. 2000).

The fission yeast *S. pombe* primarily responds to glucose via the cAMP-signaling pathway. The components of this pathway include the GPCR Git3, the Gα Gpa2, the Gβ Git5, the Gγ Git11 and the adenylyl cyclase Git2/Cyr1 (Landry and Hoffman 2001; Landry et al. 2000; Hoffman 2005a). Loss of *git3*, *gpa2* or *git2* results in slow cell growth, low intracellular cAMP levels and mating and sporulation even in the absence of nitrogen, supporting the action of these three genes in the same pathway.

Gpa2 binds to and directly activates adenylyl cyclase (Ivey and Hoffman 2005), and mutation of residues important for GTPase activity in *gpa2* leads to a sharp increase in cAMP levels in response to glucose stimulation (Isshiki et al. 1992; Welton and Hoffman 2000). Elevated cAMP levels lead to activation of PKA by binding to the regulatory subunit, Cgs1 (Maeda et al. 1994).

The Git5 Gβ subunit is unique in *S. pombe* as it lacks the N-terminal coiled-coil region which is required for Gγ binding and proper folding of the Gβ subunit (Sondek et al. 1996). However, co-immunoprecipitation studies with Git5 and Git11 support their physical association (Landry and Hoffman 2001). Git11 is required for localization of Git5, and not for its folding, which is supported by the suppression of the *Δgit11* mutation by fusing the Git11 CAAX box to the C-terminus of Git5 (Landry and Hoffman 2001).

The *fbp1* gene encodes the gluconeogenic enzyme fructose-1,6-bisphosphatase (Hoffman and Winston 1990, 1991). Glucose represses transcription of the *fbp1* gene via PKA activation. Strains with the *Δgit3* or *Δgpa2* mutations are derepressed for transcription of *fpb1* on high glucose, while those carrying a constitutively activated *gpa2* allele ($gpa2^{R176H}$) have reduced transcription of *fbp1* (Roux et al. 2009). Simultaneous loss of *gpa2* and *git3* has an additive effect on *fbp1* expression (Welton and Hoffman 2000), suggesting some functions for Git3 independent of Gpa2.

2. Carbon-Sensing Pathways in *Neurospora crassa and Aspergillus nidulans*

Glycerol is one of the significant nutrients utilized by plant pathogenic fungi (Wei et al. 2004), and GPCR-mediated signaling is required for the growth of filamentous fungi in poor carbon sources such as glycerol, arabinose, mannitol, and sorbose (Li and Borkovich 2006). The *N. crassa* carbon-sensing machinery includes a putative GPCR, GPR-4, which is homologous to Gpr1p and Git3 of *S. cerevisiae* and *S. pombe*, respectively (Li and Borkovich 2006). Δ*gpr-4* mutants accumulate much less biomass than wild type in glycerol medium and lack the transient cAMP increase after addition of glucose to glycerol-grown cultures (Li and Borkovich 2006). In addition to glycerol, Δ*gpr-4* mutants exhibit mass defects with mannitol and arabinose as carbon sources (Li and Borkovich 2006). Results from genetic epistasis analysis and the yeast two hybrid assay indicate that the C-terminus of GPR-4 interacts with GNA-1 and that GPR-4 requires GNA-1 to transduce signals downstream (Li and Borkovich 2006).

In *A. nidulans*, the heterotrimeric G protein GanB (Gα subunit)/SfaD(Gβ)/GpgA(Gγ) activates the cAMP-PKA pathway in response to glucose (Seo et al. 2004; Lafon et al. 2005) and controls conidial germination. During the early period of conidial germination, GanB mediates a rapid and transient activation of cAMP synthesis in response to glucose and subsequent stimulation of PKA activity (Lafon et al. 2005). *ΔganB* has altered germination kinetics and defects in trehalose mobilization. Addition to this, germination of *ΔganB* conidia is even impaired in the presence of glycerol, ethanol, galactose, and fructose, indicating the role of GanB in sensing carbon sources other than glucose. Although a carbon-sensing GPCR has not been reported for *A. nidulans*, GprC and GprD are putative GPCRs with homology to *S. cerevisiae* Gpr1p (Affeldt et al. 2014) in the related species *A. flavus*. *A. flavus* mutants lacking *gprD* have reduced growth on galactose, xylose, and corn oil (Affeldt et al. 2014).

3. Methionine and Glucose Sensing in *Cryptococcus neoformans*

The GPCR Gpr4 identified in *C. neoformans* is known to sense and respond to amino acids in the environment via cAMP signaling pathway (Xue et al. 2006). Gpr4 shares amino acid similarity with the Gpr1 of *S. cerevisiae* and *N. crassa* GPR-4. In spite of its homology to carbon-sensing GPCRs, *C. neoformans* Gpr4 is not required for the carbon-regulated cAMP pathway. In contrast, evidence suggests that Gpr4 senses methionine, as exposure to this amino acid leads to internalization of fluorescently tagged Gpr4 and a transient increase in cAMP levels in wild type, but not *Δgpr4* cells (Xue et al. 2006). Methionine induces mating filamentation in wild-type, but not in *Δgpr4* or *Δgpa1* mutants, and *gpr4* mutants are defective in capsule production and mating, similar to

Δgpa1 mutants, supporting their action in the same pathways. However, Gpa1, but not Gpr4, is required for synthesis of the virulence factor melanin in response to glucose (Alspaugh et al. 1997, 2002). This suggests involvement of other receptors or an alternative mechanism for regulation of Gpa1 functions (Xue et al. 2006). The requirement for Gpr4 and Gpa1 functions in the amino acid-cAMP signaling pathway, as well as Gpa1 for the glucose-cAMP sensing mechanism, presents an example of a nutrient coincidence detection system that remains to be understood in other fungi (Xue et al. 2006, 2008).

B. Mating and Pheromone Response

1. Ste2p/α-Factor and Ste3p/a-Factor in *S. cerevisiae*

S. cerevisiae is a heterothallic yeast, with two mating types, *MATα* and *MATa* (Kurjan 1992). The two known GPCR receptors in yeast, Ste2p and Ste3p, are activated by pheromones α-factor and a-factor, respectively (Kurjan 1992). *MATα* cells produce the 13-residue peptide [WHWLQLKPGQPMY] α-factor, which activates the Ste2p receptor on the *MATa* cell (Burkholder and Hartwell 1985; Nakayama et al. 1985). The *MATa* cell generates the 12-residue lipopeptide [YIIKGVFWDPAC(farnesyl)OCH_3] a-factor that targets the Ste3p receptor (Nakayama et al. 1985; Hagen et al. 1986). Pheromone binding stimulates GDP to GTP exchange on the Gpa1p Gα subunit, allowing release of the Ste4p/Ste18p Gβγ heterodimer to activate downstream signaling (Kurjan 1992); Fig. 7.3). The Ste4p/Ste18p heterodimer recruits a scaffold protein, Ste5p, to the plasma membrane, forming the site of mating projection ("shmoo") formation (Elion 2000, 2001; Merlini et al. 2013). Through its different domains, Ste5p recruits members of a MAPK cascade: the MAPKKK Ste11p, the MAPKK Ste7p, and the MAPK Fus3p (Cairns et al. 1992; Marcus et al. 1994; Whiteway et al. 1995). Ste11p is phosphorylated by the p21-activated kinase (PAK) Ste20p that is in turn activated by the GTP-bound form of Cdc42p (Burbelo et al. 1995; Cabib et al. 1998). Ste11p binds to the adaptor protein Ste50p that also binds to Cdc42p, thereby, bridging Ste11p and active Ste20p (Ramezani-Rad 2003; Wu et al. 1999). Upon phosphorylation by Ste20p, Ste11p catalyzes the sequential phosphorylation and activation of Ste7p and Fus3p (Gartner et al. 1992; Good et al. 2009; Neiman and Herskowitz 1994). Fus3p then phosphorylates substrates in the cytoplasm to induce cell cycle arrest and polarized growth (Elion et al. 1993). Fus3p also phosphorylates two functionally redundant transcription repressors, Dig1p and Dig2p, and the transcription activator, Ste12p, to induce expression of pheromone-responsive genes (Chou et al. 2006; Tedford et al. 1997; Colman-Lerner et al. 2005). Thus, precise quantitative information regarding pheromone bound at the cell surface receptor reaches the nucleus within minutes. A generic model of a fungal pheromone sensing pathway is provided in Fig. 7.3.

2. Map3/M-Factor and Mam2/P-Factor in *S. pombe*

Mating is triggered by nutrient starvation in *S. pombe* (Egel 1971). The mating pheromones are P-factor and M-factor, with P-factor sensed by the Mam2 GPCR expressed on h^- cells and M-factor sensed by the Map3 GPCR expressed on h^+ cells (Kitamura and Shimoda 1991; Tanaka et al. 1993). Mam2 and Map3 are homologous to *S. cerevisiae* Ste3p and Ste2p pheromone receptors, respectively (Tanaka et al. 1993; Kitamura and Shimoda 1991; Hoffman 2005b). In *S. pombe*, pheromone signaling and downstream regulation of the MAPK cascade are activated by the Gα subunit Gpa1, in contrast to *S. cerevisiae*, where the Gβγ heterodimer plays this role (Obara et al. 1991; Hoffman 2005a). The MAPK cascade consists of the MAPKKK Byr2, MAPKK Byr1, and MAPK Spk1, which are homologs of Ste11p, Ste7p, and Fus3p from *S. cerevisiae* (Nadin-Davis and Nasim 1990; Wang et al. 1991; Xu et al. 1994). No homologue of the Ste5p scaffolding protein is found in *S. pombe*, and the mechanism whereby Byr2 is activated by Gpa1 is

unknown. However, the mating-specific protein Ste4, which is homologous to *S. cerevisiae* Ste50p, is known to interact with Byr2 and promote its activation (Okazaki et al. 1991; Barr et al. 1996; Ramachander et al. 2002). The small GTPase Ras1, which positively regulates mating and sexual sporulation in *S. pombe*, is located on the plasma membrane and activated by the Ste6 GEF and inactivated by the Gap1 GAP (Hughes et al. 1994). Ras1 activation by Ste6 recruits Byr2 to the plasma membrane and relieves its autoinhibition, thereby causing Byr2 to drive sequential phosphorylation of Byr1 and Spk1. Phosphorylated Spk1 activates Ste11, a homologue of *S. cerevisiae* Ste12p, that acts as a developmental switch from cellular proliferation to sexual differentiation (Sugimoto et al. 1991; Mata and Bahler 2006).

Constitutive expression of Ste11 causes starvation-independent sexual differentiation (Sugimoto et al. 1991), and Ste6 expression is under the direct regulation of Ste11. Once expressed, Ste11 drives its own transcription by binding to the TR box, a ten base motif (5′-TTCTTTTGTTY-3′) in its upstream region (Sugimoto et al. 1991; Kunitomo et al. 2000).

3. PRE-1/MFA-2 and PRE-2/CCG-4 in *N. crassa*

N. crassa is a heterothallic fungus with two mating types, *mat A* and *mat a* (Raju 1992). The sexual cycle in *N. crassa* is initiated by nitrogen starvation, light, and low temperature, leading to the formation of the female reproductive structures, protoperithecia (Raju 1992). Specialized hyphae (trichogynes) originating from protoperithecia of one mating type grow towards a conidium of the opposite mating through chemotropic attraction (Bistis 1981, 1983). Trichogyne attraction is regulated by the GPCRs PRE-1 and PRE-2 that recognize their respective pheromone ligands, MFA-1 and CCG-4, respectively (Kim et al. 2002, 2012; Bobrowicz et al. 2002; Kim and Borkovich 2004, 2006). PRE-1 and PRE-2 are homologous to the Ste2p and Ste3p pheromone receptors of *S. cerevisiae* (Galagan et al. 2003; Borkovich et al. 2004). *mat A* strains express high levels of *pre-1* and *ccg-4* mRNA, while expression of *pre-2* and *mfa-1* is elevated in *mat a* strains (Bobrowicz et al. 2002; Kim and Borkovich 2004, 2006). Mating-type-specific gene expression causes *Δpre-1 mat A* trichogynes to be blind and to fail to fuse with *mat a* conidia, while *Δpre-2 mat a* trichogynes are blind to *mat A* conidia (Kim and Borkovich 2004; Kim et al. 2012). Furthermore, mutants lacking the pheromone genes *ccg-1* and *mfa-1* are unable to sense trichogynes are male sterile in the *mat A* or *mat a* background, respectively (Kim and Borkovich 2006). Male fertility in *N. crassa* is due to lack of pheromones, and no other G protein signaling component is required for male fertility (Kim and Borkovich 2006). Finally, as mentioned earlier, the RIC8 cytosolic GEF is required for female fertility in *N. crassa*, but since mutants are blocked in production of female structures, it is not clear whether RIC8 is also essential for the pheromone response pathway (Wright et al. 2011).

The Gα protein GNA-1 is required for chemotropism in both mating types, suggesting a role downstream of both pheromone/ligand systems (Kim and Borkovich 2004). Similar to GNA-1, GNB-1 (Gβ), and GNG-1 (Gγ) are essential for proper trichogyne attraction, since *Δgnb-1* and *Δgng-1* trichogynes are also blind (Krystofova and Borkovich 2005). However, the low levels of GNA-1 protein in *Δgnb-1* and *Δgng-1* mutants may point to an indirect role for GNB-1 and GNG-1 in trichogyne chemotropism (Krystofova and Borkovich 2005). The adenylyl cyclase protein, CR-1, is known to act downstream of GNA-1 (and GNA-3) in *N. crassa* (Ivey et al. 1999; Kays and Borkovich 2004). However, the *cr-1* mutant is female-fertile and only delayed in postfertilization development (Ivey et al. 1996). Exogenous cAMP does not rescue the female-sterile phenotype of the *Δgna-1* mutant, suggesting the existence of CR-1/cAMP-independent signaling pathways during perithecial development (Ivey et al. 2002). Furthermore, the fertility defects of *Δgna-3* and *Δgnb-1* mutants are not corrected by exogenous cAMP (Kays et al. 2000; Yang et al. 2002). Taken together, the data suggest that mating and sexual sporulation requires both cAMP-dependent and cAMP-independent signaling pathways in *N. crassa*.

4. Mating and Cleistothecia Formation in *Aspergillus*

The mating-specific genes have also been identified in *Aspergillus* species, including both sexual and presumed asexual species (Dyer et al.

2003; Paoletti et al. 2005; Pel et al. 2007; Ramirez-Prado et al. 2008). The mating type genes *matA* and *matB* were first described in the homothallic (self-fertile) *A. nidulans*. Deletion of either gene results in production of fewer and smaller sexual fruiting bodies (cleistothecia) and sterile ascospores (Paoletti et al. 2007). Simultaneous overexpression of *matA* and *matB* resulted in vegetative growth arrest and inappropriate development of cleistothecia, further confirming their key roles in sexual development (Paoletti et al. 2007). The predicted GPCRs GprA and GprB are similar to *S. cerevisiae* Ste2p and Ste3p, respectively, and function during self-fertilization to produce the fruiting body, called a cleistothecium (Seo et al. 2004). The *ΔgprA* and *ΔgprB* single mutants produce fewer and smaller cleistothecia, while *ΔgprA ΔgprB* double mutants failed to produce cleistothecia, indicating a role for both receptors in cleistothecia formation (Seo et al. 2004). Genetic evidence supports the action of GprA and GprB upstream of a heterotrimeric G protein consisting of FadA (Gα), SfaD (Gβ), and GpgA (Gγ) (Rosen et al. 1999; Seo et al. 2005). Deletion of any of these genes results in failure of cleistothecia formation. The RGS for FadA, FlbA, is also required for the development of cleistothecia (Yu et al. 1996).

A conserved MAPK cascade acts downstream of G protein signaling during sexual development in *A. nidulans*. The components are Ste20 (PAK), SteC (MAPKKK), Ste7 (MAPKK), and MpkB (MAPK) that activate a final homeodomain protein SteA, which then triggers the further stages of sexual development (Vallim et al. 2000; Wei et al. 2003; Paoletti et al. 2007). Deletion of individual genes in the pathway results in sterility, with failure to form cleistothecia.

5. Mating in *Cryptococcus neoformans*

There are two mating type loci, *MATα* and *MATa*, in the heterothallic basidiomycete fungus *Cryptococcus neoformans* (Kwon-Chung 1975, 1976). The mating type loci are quite large in *C. neoformans*, encoding more than 20 genes (Lengeler et al. 2002; Kozubowski and Heitman 2012). The *MATα* locus contains the peptide pheromone genes *MFα1-3*, which share sequence similarity with other fungal and yeast pheromones and contains a C-terminal CAAX box for lipid modification (Moore and Edman 1993). The *MATa* locus encodes the Mfa1-3 pheromones, that each possess a farnesylation motif (McClelland et al. 2002). Under nutrient starvation, opposite mating type cells of *C. neoformans* form conjugation tubes that fuse to form dikaryotic filaments (Kozubowski and Heitman 2012). *MATα* cells are unique in that they can produce filaments and sporulate (haploid fruiting) in response to nitrogen starvation, even in the absence of *MATa* cells (Kozubowski and Heitman 2012).

The *C. neoformans* mating type loci also encode the pheromone receptors Ste3α and Ste3a (Lengeler et al. 2002) that recognize the Mfa and Mfα type pheromones, respectively (Stanton et al. 2010). The Gpa2 Gα protein is required for mating and has been shown to interact with the pheromone receptors, Gβ subunit Gpb1 and RGS protein Crg1 (Wang et al. 2000; Li et al. 2007a). The two Gγ subunits Gpg1 and Gpg2 interact with Gpb1 and regulate sexual development (Li et al. 2007a). Gpb1 operates upstream of a MAPK cascade homologous to the pheromone response MAPK pathway in *S. cerevisiae* that is required for mating in *C. neoformans* (Wang et al. 2000). Interestingly, in addition to the two Ste3-like GPCRs, the *C. neoformans* genome contains another pheromone receptor-like gene, *CPR2*, that is not encoded by the mating type locus (Hsueh et al. 2009). Evidence suggests that Cpr2 is a constitutively activated GPCR that couples to the same downstream signaling components as the two pheromone receptors, but regulates mating in the absence of pheromone in *C. neoformans* (Hsueh et al. 2009).

C. Pathogenesis and Virulence

1. Appressorium Formation and Pathogenicity in *Magnaporthe oryzae*

M. oryzae causes rice blast disease by forming a specialized infection structure called an appres-

sorium (Li et al. 2012; Liu et al. 2010; Dean et al. 2005). The mature appressorium generates enormous internal turgor pressure that is translated into strong mechanical forces exerted on the penetration peg, allowing the pathogen to penetrate the plant cuticle and cell wall. Following penetration, the pathogen proliferates and fills up the plant cell lumen, resulting in necrotic lesions on the leaf surface.

The first step in the infection-related process is for *M. oryzae* to recognize the plant leaf. Hydrophobicity and the hard surface of the leaf are two major factors that induce appressoria formation (Li et al. 2012; Liu et al. 2010). The predicted GPCR, Pth11, is required for sensing surface hydrophobicity of leaves (DeZwaan et al. 1999). Similar to other filamentous fungi, *M. oryzae* has three Gα (MagA, MagB, and MagC), one Gβ (Mgb1), and one Gγ (Mgg1) proteins (Liu and Dean 1997; Nishimura et al. 2003; Liang et al. 2006). Loss of the *MAGB* Gα gene significantly reduces, but does not completely block, appressorium formation (Liu and Dean 1997). However, exogenous cAMP or 1,16-hexadecanediol restored appressorium formation in Δ*magB* mutants, reflecting a role for intracellular cAMP in initiating appressorium formation on leaf surfaces (Liu and Dean 1997). As mentioned earlier, mutants lacking the *RIC8* GEF gene exhibit a defect in appressorium development that can be partially remediated by exogenous cAMP, but the resulting appressoria are not infectious (Li et al. 2010). Additional support for a role for cAMP during early steps in infection is provided by the demonstration that mutants lacking adenylyl cyclase (*MAC1*) are unable to produce appressoria (Choi and Dean 1997). Furthermore, deletion of the gene encoding the catalytic subunit of PKA (*CPK1*) blocks appressorium formation, even in the presence of exogenous cAMP (Mitchell and Dean 1995; Fernandez and Wilson 2014). Taken together, the findings are consistent with a mechanism in which elevated cAMP activates PKA (Cpk1), initiating appressorial formation. For a generic model of pathogenicity in filamentous fungi, refer to Fig. 7.4.

Deletion of the RGS gene *rgs1* leads to a significant increase in cAMP levels and conidiation, as well as appressoria formation even on the noninductive hydrophilic surfaces (Liu et al. 2007; Zhang et al. 2011). In addition, Rgs1 interacts with all three Gα proteins in *M. oryzae* (Zhang et al. 2011), consistent with a role in regulating GTP occupancy on MagB and downstream appressorium development. In contrast to *MAGB*, *MGB1* and *MGG1* are essential for infection, as deletion of either gene leads to a complete failure to form appressoria (Nishimura et al. 2003; Liang et al. 2006). Exogenous cAMP induces appressorium development in *mgb1* mutants, but these appressoria are defective in shape and cannot initiate infection, suggesting the involvement of other infection-related pathways mediated by Mgb1 and Mgg1 (Nishimura et al. 2003; Liang et al. 2006).

Deletion of genes encoding the MoSom1 and MoCdtf1 transcription factors results in defects in appressorium formation and virulence, and these two proteins interact with CPK1, consistent with roles as downstream targets of CPK1 for initiation of infection (Yan et al. 2011). Taken together, the data suggest that the upstream pathway for initiating infection involves stimulation of the Pth11 GPCR by the leaf surface, which in turn activates MagB, releasing the Mgb1–Mgg1 heterodimer, leading to stimulation of adenylyl cyclase (Mac1), increased cAMP synthesis, and activation of PKA. Of interest, it has recently been shown that MagA, Rgs1, adenylyl cyclase, and the Pth11 GPCR are sequestered on late endosomes during early pathogenic development, presumably to bring these components together to facilitate efficient signal transduction (Ramanujam et al. 2013).

The later stages of appressorial formation and penetration into the leaf surface require two MAPKs, Pmk1 and Mps1, similar to *S. cerevisiae* Fus3p/Kss1p and Slt2p, respectively (Xu and Hamer 1996; Xu et al. 1998). *Δpmk1* mutants recognize leaf surface hydrophobicity, respond to cAMP, and germinate following adhesion, but are unable to form appressoria and invasive hyphae and are thus avirulent (Xu and Hamer 1996). The other components of the Pmk1 pathway are Mst11 (MAPKKK) and Mst7 (MAPKK), forming a Mst11–Mst7–Pmk1 three-tiered MAPK cascade (Zhao et al. 2005). Like the *Δpmk1* mutant, *Δmst11* and *Δmst7* mutants

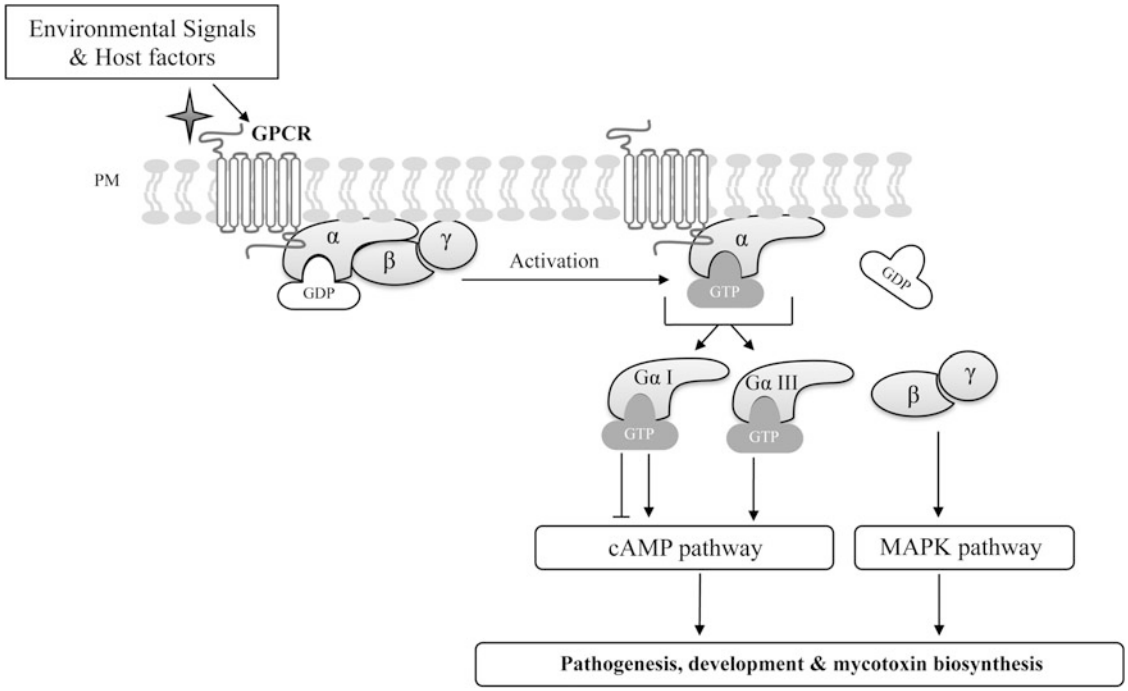

Fig. 7.4 Pathogenicity. Involvement of Group I and III Gα proteins in pathogenicity responses has been demonstrated in a wide variety of filamentous fungi. Appressorium formation in *Magnaporthe oryzae* involves the predicted GPCR Pth11 and the Gα MagB, as well as intracellular cAMP and protein kinase A (Cpk1). In *U. maydis,* the Group III Gα protein Gpa3 is involved in pathogenicity, while in *C. neoformans* Gpa1 is important for virulence and capsule formation. These Gα proteins and the Gβγ dimer impact regulation of PKA and MAPK signaling cascades to control fungal pathogenesis. *Abbreviations*: *GPCR* G protein-coupled receptors, *GDP* guanosine diphosphate, *GTP* guanosine triphosphate, *cAMP* cyclic adenosine monophosphate, *PKA* protein kinase A, *MAPK* mitogen-activated kinase

fail to form appressoria and are nonpathogenic (Zhao et al. 2005). The *M. oryzae* homolog of yeast Ste50p, Mst50, interacts with Mst11 and Mst 7, and *Δmst50* mutants are defective in appressorium formation (Park et al. 2006). Finally, mutants lacking the *MPS1* MAPK trigger early plant-cell defense responses, but are unable to penetrate the plant surface and cause disease (Xu et al. 1998).

2. Pathogenesis in *Ustilago maydis*

U. maydis is a basidiomycete fungus that causes corn smut disease (Brefort et al. 2009). The fungus propagates as haploid budding yeast-like cells and becomes infectious only in the dikaryotic stage that is generated after mating (Brefort et al. 2009; Vollmeister et al. 2012). The dikaryotic stage is characterized by filamentous growth of the fungus, by which it penetrates the meristematic tissue of the plant, culminating in the production of diploid teliospores within tumors on the plant (Brefort et al. 2009). The transition from yeast-like to constitutively filamentous growth requires involvement of G-protein-mediated cAMP signaling and a MAPK cascade (Brefort et al. 2009; Vollmeister et al. 2012). Cell recognition and fusion are regulated by genes at the *a* mating type locus that encodes a pheromone precursor, Mfa1, and pheromone receptors Pra1 and Pra2 (Bakkeren and Kronstad 1996). Following fusion, the dikaryotic stage is maintained by the multiallelic *b* mating type locus which encodes bE and bW homeodomain proteins (Banuett 1995). The pathogenicity of the organism depends on

the active formation of bE-bW heterodimers (Banuett 1995; Kahmann and Bolker 1996; Kahmann and Kamper 2004).

Four heterotrimeric G protein Gα subunits (Gpa1, Gpa2, Gpa3 and Gpa4) have been identified in *U. maydis*, with Gpa3 playing an active role in the pheromone response and pathogenic development (Regenfelder et al. 1997). *Δgpa3* mutants are nonpathogenic, fail to respond to pheromone, and are unable to mate. Introduction of a GTPase-deficient allele of *gpa3* ($gpa3^{Q206L}$) causes *U. maydis* cells to mate even in the absence of pheromone signals (Regenfelder et al. 1997). The *Δbpp1* mutant grows filamentously and has reduced expression of pheromone genes, but still causes tumors on corn plants (Muller et al. 2004). Expression of the $gpa3^{Q206L}$ allele suppresses the *Δbpp1* mutant phenotypes (Muller et al. 2004). The nonpathogenic nature of Δ*gpa3* mutants in contrast to *Δbpp1* strains suggests that Gpa3 regulates pathogenic development independently of Bpp1 (Muller et al. 2004). Gpa3 acts upstream of adenylyl cyclase (Uac1) for synthesis of cAMP (Kruger et al. 1998). The finding that mutants lacking either *uac1* or *ubc1* (encoding the regulatory subunit of PKA) are nonpathogenic supports a role for cAMP signaling in pathogenesis (Gold et al. 1994, 1997).

The High Mobility Group (HMG)-domain transcription factor Prf1 (Hartmann et al. 1996) induces the expression of *a* and *b* mating type genes and is phosphorylated by the catalytic subunit of PKA, Adr1 (Kaffarnik et al. 2003). Prf1 is also phosphorylated by the MAPK Kpp2 to trigger expression of *b* mating type genes to undergo dikaryon formation (Kaffarnik et al. 2003). Taken together, the results indicate that cAMP-PKA and pheromone-responsive MAPK pathways regulate mating, filamentous growth, and pathogenesis in *U. maydis*.

3. Melanin and Capsule Formation in *Cryptococcus neoformans*

In the human pathogenic fungus *C. neoformans,* G-protein signaling is an integral part of the virulence pathway. The Gpa1(Gα)/Cac1(adenylyl cyclase)/Pka1(catalytic subunit of PKA) cAMP signaling pathway controls growth, differentiation, and virulence (Alspaugh et al. 1997, 2002). Mutations in the components of this pathway lead to attenuated cAMP signaling, as well as alterations in capsule size and melanin formation. For example, *Δgpa1* mutants fail to produce melanin and capsule and are avirulent (Alspaugh et al. 1997). The RACK1 homolog Gib2 appears to be an essential gene in *C. neoformans*, and overexpression suppresses the melanization and capsule defects of a *gpa1* mutant (Palmer et al. 2006). Gib2 forms heterotrimeric complex with Gpa1 and Gpg1 or Gpg2 (Palmer et al. 2006), further supporting a role in signaling with Gpa1 to control virulence.

4. Quorum Sensing in *Aspergillus*

Quorum sensing is a system of stimulus and response correlated to population density (Garg et al. 2014). Quorum sensing was once thought to exist only in bacteria, but is now well established in fungi (Albuquerque and Casadevall 2012), including *Aspergillus* species. *A. flavus* is a soil-borne pathogen that infects a variety of food and feed crops including maize, peanuts, cottonseed, and tree nuts and also famous for aflatoxin (AF) production, the most potent natural carcinogen known (Squire 1981). In contrast, *A. nidulans* does not produce AF, but terminates the pathway at the penultimate precursor, sterigmatocystin (Alkhayyat and Yu 2014).

One of the primary signals for conidiation and aflatoxin formation in *Aspergillus* species are **oxylipins**, oxygenated polyunsaturated fatty acids (Horowitz Brown et al. 2008; Brown et al. 2009). Oxylipins are synthesized by oxygenases that incorporate oxygen into a fatty acid backbone. At low inoculation density, oxylipin production is low, conidiation is reduced, and there is increased production of aflatoxin in *A. flavus*. Conversely, at high inoculum density, oxylipin levels are high, resulting in increased conidiation with a reduction in aflatoxin production (Herrero-Garcia et al. 2011; Affeldt et al. 2012). Recent work has identified two putative GPCRs (GprC and GprD) in *A. flavus*

and GprA and GprD in *A. nidulans*—that are involved in oxylipin-mediated density-dependent shift (Affeldt et al. 2012, 2014). High density cultures of *A. flavus ΔgprC* and *ΔgprD* mutants produce low amounts of conidia and elevated aflatoxin levels that mimic the wild-type culture at low density. Furthermore, the spent medium from wild-type cells cultured at high density failed to induce conidiation in *ΔgprC* and *ΔgprD* strains, locking the fungus in the low density state. Evidence supports *A. nidulans* GprD as receptor that senses oxylipin ligands and initiates cAMP-PKA signaling (Affeldt et al. 2012).

IV. Conclusions

G protein-controlled pathways are used to sense pheromones and nutrients in several fungal species. These two classes of physically diverse signals elicit morphological changes and often synergize to regulate development. Heterotrimeric G proteins are also required for virulence in both animal and plant fungal pathogens.

The pheromone response pathway is perhaps the most conserved G protein pathway in fungi, with similar GPCRs, ligands, G proteins, and MAPK components utilized in diverse species. In many species, the pheromone response/mating and virulence pathways share the same heterotrimeric G protein and MAPK signaling components. In contrast, the GPCR/G protein wiring for nutrient sensing appears to be more variable, although downstream cAMP signaling is a conserved element.

Further analysis will reveal whether the use of alternative binding partners for heterotrimeric G proteins, such as RACK1 proteins, is widespread in fungi and other eukaryotes. It is also anticipated that additional novel upstream regulators, similar to RIC8 and GET3, will continue to be identified in filamentous fungi. Studies on shuttling of signaling proteins between the nucleus and cytoplasm/plasma membrane will undoubtedly reveal mechanistic details underlying both activation and down-regulation of G protein signaling.

It is clear that there are many similarities between G protein signaling pathways found in fungi and higher organisms. Future prospects for determining the mechanisms used in filamentous fungal systems are promising, since many pathway components have been identified in several species. Evolutionary comparisons between mammalian and fungal G protein pathways will continue to be a powerful tool for elucidating the molecular interactions that occur during signal transduction in eukaryotes.

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