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Morphology and development in *Aspergillus nidulans*: A complex puzzle

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ABSTRACT

Like other filamentous fungi, *Aspergillus nidulans* forms a multitude of cell types that facilitate colonization and development. The molecular basis of cellular morphogenesis in *A. nidulans* is not well understood. Here, we summarize results obtained from detailed annotation of the *A. nidulans* genome sequence for genes with predicted roles in morphogenesis, with primary focus on polarized growth, calcium signaling, and development. We draw three broad conclusions from our results. First, the components of the signal transduction pathways and morphogenetic machinery as defined in the model yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* are largely conserved in *A. nidulans*. Second, *A. nidulans* possesses many additional genes implicated in morphogenesis that are not conserved in these yeasts. Third, the number of *A. nidulans* genes involved in morphogenesis is likely to be rather large; based on our annotation, we estimate that as many as 2000 *A. nidulans* genes encode proteins that may participate at some level in morphogenesis during vegetative growth and development.

1. Introduction

Fungi exhibit two predominant modes of cellular morphogenesis; hyphal growth and yeast growth (for review; see Harris, 1997, 2006; Momany, 2002; Philippsen et al., 2005; Steinberg, 2007). Hyphae are long tubular structures that extend solely at their tips and are typically partitioned into individual cellular compartments by the formation of septa. Sustained polarized growth is a characteristic feature of hyphae that requires the prolonged maintenance of a stable polarity axis. The establishment of hyphal polarity usually occurs during the germination of a spore or the formation of a lateral branch from a pre-existing hypha. Yeasts are unicellular fungi that display diverse morphologies ranging from ovals to elongated rods. Two key features distinguish the yeast and hyphal modes of growth. First, yeast cells do not undergo sustained polarized growth; they generally cycle between periods of polarized and isotropic growth. Second, upon the formation of septa, yeast cells subsequently separate into individual cells. The two primary forms of yeast growth are budding, whereby ovoid daughter cells bud from a discrete site on the surface of their mother, and fission, whereby a rod-shaped cells splits in half to yield two progeny. Notably, many filamentous fungi exhibit both forms of cellular morphogenesis. During their vegetative phase they proliferate as hyphae, followed by a switch to yeast-like growth upon entry into asexual development (i.e., conidiation).

Aspergillus nidulans is a widely recognized model filamentous fungus with well-established genetic and genomic toolkits (Galagan et al., 2005). As a result, *A. nidulans* has been used extensively for studies of morphogenesis and development. For example, the roles of numerous genes in the establishment and maintenance of hyphal polarity have been characterized (for review, see Harris, 2006), as have the signaling components that regulate both asexual and sexual development (Yu et al., 2006; Busch and Braus, 2007). Like other filamentous fungi, *A. nidulans* exhibits both hyphal and yeast modes of growth. In particular, during asexual development (i.e., conidiation), there is a gradual transition from hyphal to yeast-like growth, resulting in the formation of phialides that undergo repeated budding to yield chains of conidiospores (Timberlake, 1991). By contrast, sexual development includes the fusion of multiple hyphae to form the walls of the fruiting body (i.e., cleistothecium) in which meiosis and ascospore formation occur (Champe and Simon, 1992). In addition, maternal nursing cells (i.e., Hülle cells) are also produced. All together, *A. nidulans*

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generates numerous cell types with distinct morphologies and/or developmental fates (e.g., vegetative hyphae, conidiophore stalks, conidiophore vesicle, phialides, conidia, ascogenous hyphae, Hülle cells, and ascospores).

Much of what is known about cellular morphogenesis in fungi is derived from detailed studies using the budding yeast *Saccharomyces cerevisiae* and the fission yeast *Schizosaccharomyces pombe* (Martin and Chang, 2005; Park and Bi, 2007). These studies have resulted in the characterization of a multitude of proteins that function in morphogenetic processes and have also begun to elucidate the networks in which these proteins operate. As discussed previously (Harris and Momany, 2004), this so-called "yeast paradigm" has proven to be a useful guide for understanding cellular morphogenesis in filamentous fungi as well. For example, by analogy to yeasts, polarized hyphal growth is presumed to require an initial symmetry breaking event that leads to generation of a stable polarity axis and the localized recruitment of the morphogenetic machinery (i.e., the components of the cytoskeleton and vesicle trafficking complexes that enable localized cell surface expansion and cell wall deposition). At the same time, although the yeast paradigm has facilitated the identification of gene products with important roles in hyphal morphogenesis, it has also become readily apparent that this paradigm is limited. In addition to the differences outlined above that distinguish hyphal growth from that of yeasts, morphogenesis and development in fungi such as *A. nidulans* is subject to regulatory inputs that are seemingly irrelevant to yeasts. These inputs, which include factors such as light, reactive oxygen species, and a variety of low molecular weight signaling molecules (e.g., Purschwitz et al., 2006; Tsitsigiannis and Keller, 2007; Semighini and Harris, 2008), presumably necessitate the presence of additional signal transduction pathways beyond those that are already known based on analogies to yeasts. One of the objectives of the *A. nidulans* morphology and development annotation group was to determine if this is indeed true.

Although hyphae appear ideally adapted for foraging and colonization, they exhibit a remarkable diversity in organization. Some fungi, such as the plant pathogen *Ustilago maydis*, form dikaryotic filaments that are largely composed of vacuoles (Steinberg and Fuchs, 2004), whereas others, such as *Neurospora crassa* and *A. niger*, generate hyphal networks that are surprisingly differentiated in terms of gene expression and physiological functions (Tey et al., 2005; Levin et al., 2007). In other words, there appears to be more than one way to make a hypha. Initial comparison of the molecular mechanisms underlying hyphal morphogenesis provides further support for this view. For example, *Ashbya gossypii* essentially possesses the same set of genes as *S. cerevisiae* (Philippsen et al., 2005), yet instead of forming budding yeast cells it proliferates solely as hyphae. On the other hand, genetic screens using *A. nidulans* and *N. crassa* have identified many genes with important roles in hyphal morphogenesis that are either absent or are poorly conserved in yeast (summarized in Harris, 2006). This leads to an intriguing question; why do fungi such as *A. nidulans* possess additional genes needed for hyphal morphogenesis if it is possible to form hyphae using the same set of genes as yeast? The answer to this question remains unknown, though it is tempting to suggest that increased gene content might correlate with more complex patterns of hyphal morphogenesis. Nevertheless, an accurate estimate of the gene content implicated in hyphal morphogenesis would emphasize the scale of this paradox (i.e., how many additional genes does *A. nidulans* possess compared to yeast/*Ashbya*?) and would identify genes that might contribute to the complexity of hyphal morphogenesis. Accordingly, this was an important second objective of the *A. nidulans* morphology and development annotation group.

Note that additional cellular processes relevant to morphogenesis (i.e., cell wall synthesis, vesicle trafficking) have been analyzed by other annotation groups and are summarized elsewhere in this issue.

2. Annotation summary

2.1. Hyphal morphogenesis

As has already been described, the positional markers that direct bud site selection in yeast are not that well-conserved in *A. nidulans* (Harris and Momany, 2004). Homologues of Rax1 (RgaB) and Rax2 (AN6658) could be identified, as well as proteins with weak homology to Bud3 (AN0113) and Bud4 (AN6150). Notably, AN0113 possesses a RhoGEF domain, suggesting that it might be a component of a Rho GTPase module. In yeast, the Ras-like GTPase Bud1/Rsr1 and its regulators, the GEF Bud5 and the GAP Bud2, relay positional information generated by the positional markers to the Cdc42 GTPase module (Chang and Peter, 2003). Homologues of Bud1/Rsr1 (AN4685), Bud5 (AN4369), and Bud2 (AN3735) are present in *A. nidulans*. Indeed, *A. nidulans* appears to possess three distinct Ras GTPases; RasA, RasB, and AN4685. Based on their functional characterization in *A. nidulans* and *A. fumigatus* (Som and Kolaparthi, 1994; Fortwendel et al., 2005), RasA and RasB have already been implicated in the regulation of polarized hyphal growth.

As expected, the Cdc42 GTPase module is conserved in *A. nidulans* (Fig. 1). Functional characterization of Cdc42 show that it is involved in hyphal morphogenesis, particularly for the formation of lateral branches and secondary germ tubes (Virag et al., 2007). Homologues of the Cdc42 GEF Cdc24 (AN5592) and the GAPs Rga1/2 (AN1025) and Bem3 (AN5887) were readily identified but have not yet been characterized. In animals, the Rac1 GTPase is a close relative of Cdc42 that has also been implicated in several aspects of morphogenesis (e.g., de Curtis, 2008). Whereas yeast only possesses an extremely divergent version of Rac1 (i.e., Rho5)(Singh et al., 2008), *A. nidulans*, like other filamentous fungi, harbors a strongly conserved homologue (AN4743, RacA). A recent study shows that RacA and Cdc42 share at least one essential function required for hyphal morphogenesis, and also highlight roles for RacA in development (Virag et al., 2007). In addition, RacA also appears to regulate NADPH oxidase function in *A. nidulans* (Semighini and Harris, 2008) (Fig. 1). The identity of the GEFs and GAPs that regulate RacA remain unknown at this time. Although it is quite conceivable that these could be shared with Cdc42, *A. nidulans* does possess homologues of ELMO (AN8877) and DOCK180 (AN0463) (Fig. 1). In animals, ELMO and DOCK180 form a complex that functions as a GAP specific for Rac1 (Cote and Vuori, 2007).

In addition to the Cdc42 and Rac1 GTPases, *A. nidulans* possesses homologues of the other known Rho GTPases in yeast (Table 1), including Rho1 (AN5740), Rho2 (AN4953), and Rho3 (AN4782). Unlike yeast, but similar to *Schizosaccharomyces pombe* and other filamentous fungi, *A. nidulans* also possesses a homologue of Rho4 (AN2687; note that this is distinct from *S. cerevisiae* Rho4, which is an apparent orthologue of Rho3). Furthermore, in agreement with studies of the *N. crassa* Rho4 homologue (Rasmussen and Glass, 2005), *A. nidulans* Rho4 appears to be necessary for septum formation (H. Si and S. Harris; unpublished observations). Homologues of the yeast Rho GEFs Rom1/2 (AN4719), as well as the GAPs Lrg1 (AN7576) and Bem2 (AN4745) were identified in *A. nidulans*. However, two additional predicted Rho/Cdc42 GEFs were identified; AN0113 and AN10442. These predicted proteins share limited homology within their putative GEF domains to yeast Bud3 and Fus2, respectively.

Arf GTPases are members of the Ras GTPase superfamily that have been implicated in the regulation of vesicle trafficking in ani-

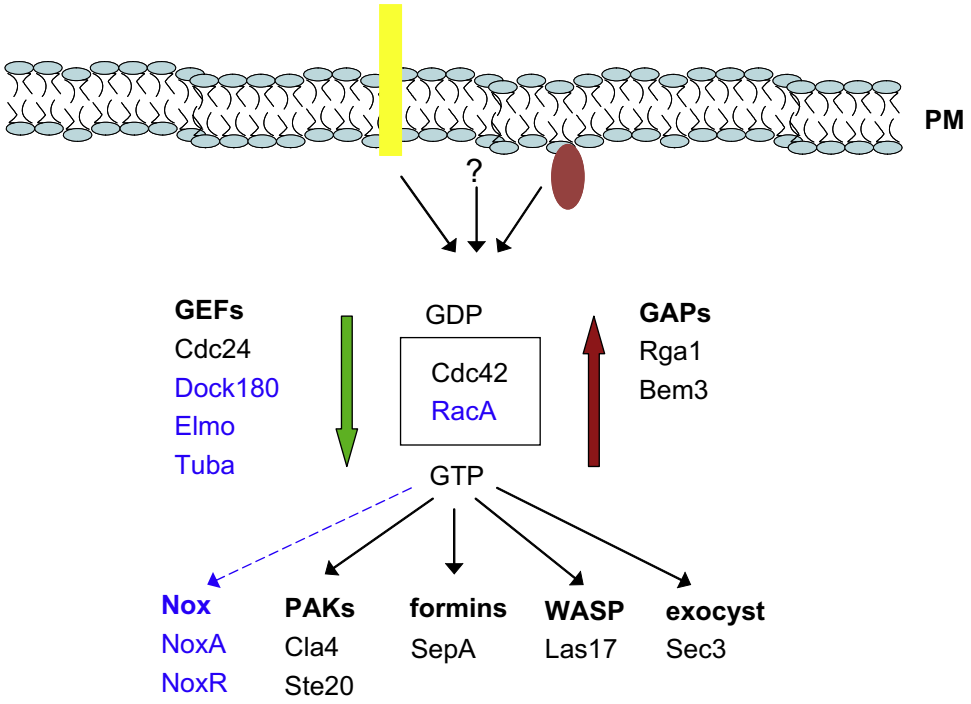

Fig. 1. Role of the Cdc42 and Rac1 GTPases in the regulation of polarized hyphal growth. Both GTPases are proposed to respond to upstream positional information, which may take the form of internal (brown oval) or external (yellow block) landmarks. Other mechanisms (i.e., spontaneous polarization) may also trigger GTPase activation (question mark). The respective GEFs and GAPs are listed, as are possible Cdc42 and RacA effectors. Genes are listed by their known *Aspergillus* designation (i.e., RacA, SepA) or by the name of the closest yeast homologue (i.e., Cdc42, Sec3). Corresponding AN designations can be found in Table 1. Genes listed in blue are either absent (e.g., NoxA, NoxR) or poorly conserved (e.g., Dock180, Tuba) in yeast. The dashed blue arrow indicates the likelihood that Nox is a specific effector of RacA. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

mals and yeast (D'Souza-Schorey and Chavrier, 2006). In addition, Arf6 regulates actin organization in animals through its effects on Rac1 signaling (Santy et al., 2005). Its yeast homologue, Arf3, is also involved in actin organization via mechanisms that are not fully understood (Huang et al., 2003). *A. nidulans* possesses six distinct Arf GTPases (Table 1 and Lee et al., 2008), including a likely Arf6 homologue (AN5090; ArfB). Recent analyses show that ArfB is required for the maintenance of hyphal polarity (Lee et al., 2008).

In animals and yeast, effectors of Cdc42 and Rac1 can be identified based on the presence of a p21-binding domain (PDB, also known as a CRIB domain). *S. cerevisiae* possesses five Cdc42 effectors with this domain; three p21-activated kinases (PAKs; i.e., Ste20, Cla4, and Skm1), as well as Gic1 and Gic2 (Park and Bi, 2007). By contrast, *A. nidulans* only possesses homologues of the PAKs Ste20 (AN2067) and Cla4 (AN8836)(Fig. 1). No additional proteins possessing the PBD/CRIB domain could be identified. Notably, yeast Gic1 and Gic2 appear to mediate septin organization in response to Cdc42 signals (Iwase et al., 2006). Animals harbor a similar class of PBD/CRIB proteins, the Borgs, which perform the same function (Iwase et al., 2006). The apparent absence of these proteins from *A. nidulans* suggests that Cdc42 may not affect septin organization, or, it does so through a potentially novel mechanism.

Besides the PBD/CRIB domain proteins, yeast Cdc42 interacts with additional proteins that organize the actin cytoskeleton (formins Bni1 and Bnr1; the WASP homologue Las17) and regulate vesicle trafficking (the secretion landmark Sec3)(Park and Bi, 2007). Homologues of each of these proteins and their associated regulators (i.e., the polarisome, Arp2/3 complex, the exocyst) were identified in *A. nidulans* (Table 1)(Fig. 1). Curiously, *A. nidulans* only possesses a single formin, SepA (AN6523), whereas the seemingly less complex yeasts *S. cerevisiae* and *S. pombe* posses two and three, respectively. Accordingly, unlike these yeasts, a single formin is presumably responsible for the formation of all types of unbranched actin filaments (i.e., actin cables at the hyphal tip, actin rings at septation sites) in *A. nidulans* hyphae.

Table 1 summarizes our comparative annotation of *A. nidulans* for homologues of yeast genes implicated in cellular morphogenesis. With a few notable exceptions, many of which have already been identified (or will be in the Discussion), most of the proteins involved in signal transduction, organization of the cytoskeleton, and vesicle trafficking are well-conserved in *A. nidulans*. Nevertheless, it is also apparent that *A. nidulans* possesses a sizable number of genes required for normal morphogenesis that are not shared with yeast or have undergone significant divergence (Table 1).

2.2. Calcium signaling

Although the importance of calcium for hyphal growth of *A. nidulans* has long been recognized, the machinery involved in calcium signaling and homeostasis is largely unexplored. Based on studies from other eukaryotes, it can be assumed that free cytosolic calcium is kept at a low resting level in *A. nidulans* by the activity of Ca^{2+} ATPases and Ca^{2+} transporters, which are responsible for transporting excess calcium into internal stores or outside the cell. Internal or external signals, however, cause calcium to flow down the concentration gradient via Ca^{2+} channels, resulting in a transient increase of cytosolic calcium that in turn acts as an intracellular signal (Fig. 2).

Aspergillus nidulans genes that putatively code for Ca^{2+}-ATPases, -transporters, and -channels were identified based on comparative genome analysis using known or predicted proteins from *S. cerevisiae*, *Magnaporthe grisea*, *N. crassa*, and *A. fumigatus* (Zelter et al., 2004; Calcium Signaling Proteins Database at http://129.215.156.68/links.html). As summarized in Tables 2 and 3, *A. nidulans* harbors the greatest repertoire of Ca^{2+}-ATPases, -transporters and -channels, suggesting either a more complex role of

Table 1
Predicted *A. nidulans* proteins involved in polarity establishment and maintenance

S. cerevisiae homologue	Mammalian homologue	*A. nidulans* locus name	*A. nidulans* protein	Known or predicted function in *A. nidulans*
Landmarks/membrane organization				
Afi1		AN0268	MesA	Membrane organization
No hit		AN4332	BarA	Ceramide synthase
Lag1		AN2464	LagA	Ceramide synthase
Sur2		AN0640	BasA	Sphinganine hydroxylase
Bud3		AN0113		
Bud4		AN6150		
Axl2		AN1359		
Rax1		AN3622	RgsB	Sexual development
Rax2		AN6685		
Bud8		No hit		
Bud9		No hit		
Cell surface				
Flo11		No hit		
Flo1		No hit		
Flo5		No hit		
Flo10		No hit		
Fig. 1		AN3036		Predicted membrane protein
Ldb17		AN3732		Protein of unknown function
Ecm33		AN4390		GPI-anchored protein
Msb1		AN2983		Possible GAP for Rho GTPase
Msb2		AN7041		Mucin
Sho1		AN7698		Osmosensor
Rho GTPases				
Cdc42		AN7487	ModA	Rho GTPase
Rac1		AN4743	RacA	Rho GTPase
Rho1		AN5740	RhoA	Rho GTPase
Rho2		AN4953		Rho GTPase
Rho3		AN4782		Rho GTPase
Rho4 (*S. pombe*)		AN2687		Rho GTPase
Cdc24		AN5592		Cdc42 GEF
	Tuba	AN10442		Predicted Cdc42 GEF
Rom1		AN4719		Rho GEF
Bem2		AN4745		Rho GAP
Bem3		AN5887		Cdc42 GAP
Rga1		AN1025		Cdc42 GAP
Sac7		AN7650		Rho GAP
Lrg1		AN7576		Rho GAP
Ste20		AN2067		p21-activated kinase
Cla4		AN8836		p21-activated kinase
Bem1		AN7030		Scaffold protein
	Dock180	AN0463		Rac GEF
	ELMO	AN8877		Rac GEF
Ras GTPases				
Rsr1/Bud1		AN4685		Ras GTPase
Bud2		AN3735		Ras GAP
Bud5		AN4396		Ras GEF
Ras2		AN0182	RasA	Ras GTPase
		AN5832	RasB	Ras GTPase
Arf GTPases				
	Arf6	AN5090	ArfB	Arf GTPase
	Centaurin	AN4274		Arf GAP
	Cytohesin	AN6120		Arf GEF
Arf1/5		AN1126		Arf GTPase
Arl1		AN5912		Arf GTPase
Arl3		AN0634		Arf GTPase
Polarisome				
Bni1		AN6523	SepA	Formin
Spa2		AN3815	SpaA	Scaffold protein
Bud6		AN1324	BudA	Actin monomer-binding protein
Pea2		No hit		
Msb3		AN10355		Rab GAP
Gic1		No hit		
Arp2/3				
Las17		AN11104		Actin nucleation; endocytosis
Arp2		AN0673		Actin nucleation; endocytosis
Arp3		AN0140		Actin nucleation; endocytosis
Vrp1		AN1120		Actin nucleation; endocytosis
Myo5		AN1558	MyoA	Actin nucleation; endocytosis
Exocyst				
Sec3		AN0462		Vesicle tethering; exocytosis
Sec5		AN1002		Vesicle tethering; exocytosis

(*continued on next page*)

Table 1 (*continued*)

S. cerevisiae homologue	Mammalian homologue	*A. nidulans* locus name	*A. nidulans* protein	Known or predicted function in *A. nidulans*
Sec6		AN1988		Vesicle tethering; exocytosis
Sec8		AN7730		Vesicle tethering; exocytosis
Sec10		AN8879		Vesicle tethering; exocytosis
Sec15		AN6493		Vesicle tethering; exocytosis
Exo70		AN6210		Vesicle tethering; exocytosis
Exo84		AN0560		Vesicle tethering; exocytosis
Sec4		AN6974	SrgA	Rab GTPase
Sec2		AN4759		Rab GEF
Scaffolds				
	Paxillin	AN7626		Scaffold protein
	Paxillin	AN3659		Scaffold protein
	Striatin	AN8071		Scaffold protein
Actin				
	α-Actinin	AN7707		Actin filament bundling
Sac6		AN5803		Fimbrin; endocytosis
Scp1		AN1935		Actin filament bundling
	Gelsolin	AN1306		Actin filament severing
	Fragmin	AN0837		Actin filament severing
Tpm1		AN5686	TpmA	Actin filament stabilization
Cap1		AN2126		Actin capping
Aip1		AN10942		Actin depolymerizing factor
Cof1		AN2317		Actin depolymerizing factor
Crn1		AN6341		Actin patch assembly
Sla1		AN1462		Actin patch assembly
Sla2		AN2756		Actin binding protein; endocytosis
Myo1		AN4706		Myosin II
Myp2 (*S. pombe*)		AN8862		Myosin II
Mlc1		AN6732		Myosin light chain
Microtubules				
Tea1 (*S. pombe*)		AN4564	TeaA	Cell end marker
Tea2 (*S. pombe*)		AN8286	KipA	Kinesin
Tea4 (*S. pombe*)		AN1099		SH3 domain protein
Mod5 (*S. pombe*)		AN4214	TeaR	Cell end marker protein kinase; cell end identity
Pom1 (*S. pombe*)		AN7678		Microtubule-binding protein
Bim1		AN2862		CAP-Gly domain protein
Tip1 (*S. pombe*)		AN1475	ClpA	Microtubule-associated protein
Alp14 (*S. pombe*)		AN5521	AlpA	CAP-Gly domain protein
Other proteins involved in polarity				
Cbk1		AN5529	CotA	Serine/threonine kinase
Vps18		AN2266	DigA	Vacuolar sorting
Vps33		AN2418	HbrA	Vacuolar sorting
		AN6720	HbrB	Likely involved in branching
Trs120		AN6533	HypA	Vesicle tethering; TRAPPII subunit
Sec7		AN6709	HypB	COPI-mediated vesicle formation
Nmt1		AN3839	SwoF	*N*-myristoyltransferase
Pmt4		AN5105	SwoA	Protein mannosyltransferase
		AN5802	SwoK	RRM RNA binding protein
Ynk1		AN8216	SwoH	Nucleoside diphosphate kinase
Pgi1		AN6037	SwoM	Phosphoglucose isomerase
Cbf5		AN8851	SwoC	rRNA pseudouridine synthase
Cdc28		AN4182	AhbA/NimX	Cyclin-dependent kinase
		AN5137	AhbB	Cytochrome P450 protein
Msf1		AN6253	PodG	Phenylalanine tRNA synthetase alpha subunit
Fcp1		AN2902	PodH	Transcription factor IIF interacting component of the CTD phosphatase

these proteins in calcium signaling and homeostasis when compared to yeast or that the function of some proteins might be redundant in *A. nidulans*. Interestingly, *A. nidulans* possesses an additional Ca^{2+}-ATPase (AN5743) for which no putative homolog was found in the genome of *S. cerevisiae*.

In *S. cerevisiae*, the Ca^{2+}/calmodulin/calcineurin/Crz1 signaling pathway responds to the spike of cytosolic calcium and up-regulates expression of diverse target genes involved among other things in ion homeostasis, pH adaptation, cell wall synthesis and glucose metabolism (e.g., Yoshimoto et al., 2002; Viladevall et al., 2004; Ruiz et al., 2008). Components of this pathway are conserved in *A. nidulans* (Table 3) and have recently been reported to be important for germination, polarity maintenance, conidiophore development, cell wall synthesis as well as for the adaptation to high external calcium concentrations and alkalinization (Wang et al., 2006; Soriani et al., 2008; Spielvogel et al., 2008). First insights into the target genes of CrzA and Crz1p, respectively, already indicate clear differences between the effector genes of the signaling pathway in *S. cerevisiae* and *A. nidulans*. Whereas Crz1p is not involved in expression of the *VCX1* gene (coding for the vacuolar transporter Vcx1p) but plays an essential role in *ENA1* expression (coding for the plasma membrane localized Na^+ ATPase Ena1p), the opposite picture has been observed for *A. nidulans*. Here, CrzA is crucial for *vcxA* regulation but has a minor if any role in *enaA* expression (Spielvogel et al., 2008). Most interestingly, the SltA transcription factor (AN2919, no homolog is present in *S. cerevisiae*) is positively involved in *enaA* but negatively in *vcxA* expression (Spielvogel et al., 2008). Consequently, SltA has to be taken into account for studies on ion stress responses; however, with respect to calcium responses, CrzA is essential whereas SltA has no apparent

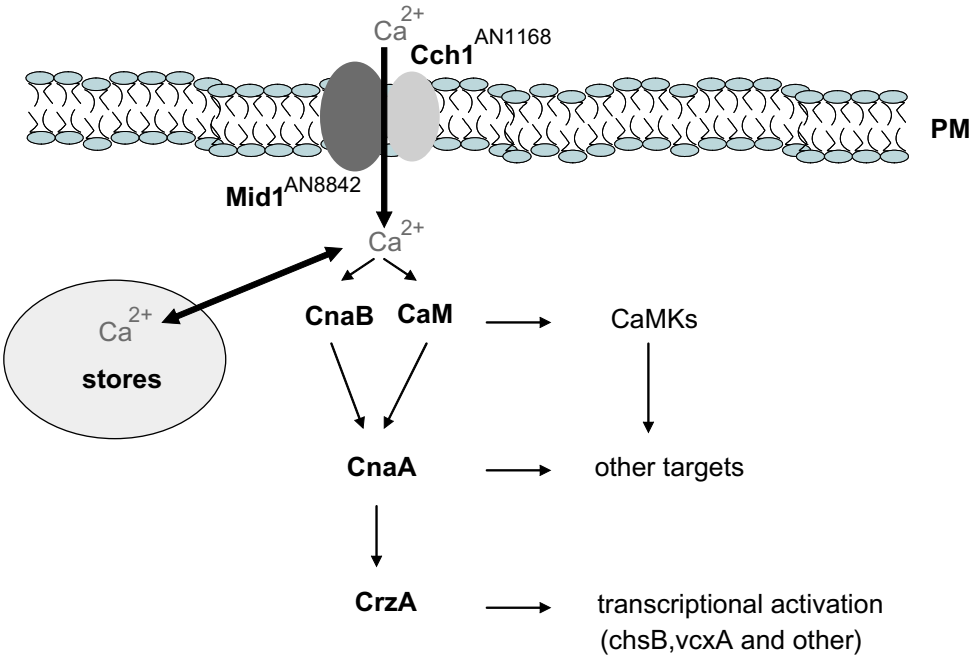

Fig. 2. A hypothetical model for calcium signaling in *A. nidulans*. Internal and external signals, such as exposure of *A. nidulans* to high external Ca^{2+} concentrations or ambient alkaline pH, presumably cause a burst of cytosolic free Ca^{2+}. This response is accomplished by the uptake of Ca^{2+} from the environment or by the release of Ca^{2+} from internal stores. Binding of Ca^{2+} to calmodulin (CaM) enables CaM to activate Ca^{2+}/CaM-dependent protein kinases (CaMKs) eliciting a specific response. Calcineurin, a phosphatase composed of a catalytic and a regulatory subunit (CnaA and CnaB) is another target activated by CaM. By analogy to yeast, activated CnaA may in turn dephosphorylate the cytosolically localized transcription factor CrzA, thereby stimulating its nuclear import and consequently increased expression of CrzA target genes. In addition, it can be hypothesized that CnaA transduces the Ca^{2+} signal to additional effector proteins necessary for cell survival. In order to restore the cytosolic level of Ca^{2+} back to its physiological level, Ca^{2+} becomes sequestered in intracellular stores such as the vacuole, endoplasmatic reticulum and Golgi. PM, plasma membrane.

Table 2
Fungal calcium transport proteins

	Ca^{2+} channels	Ca^{2+} ATPases	Ca^{2+} transporter	Total
A. nidulans	3	9	6	18
A. fumigatus[1]	3	8	5	16
N. crassa[1]	3	6	8	17
M. grisea[1]	3	8	6	17
S. cerevisiae[1]	3	4	4	12

[1] After the Calcium Signaling Proteins Database, http://129.215.156.68/links.html.

role. Based on these data, it can be assumed that CrzA regulates the expression of a specific set of genes involved in calcium responses, but that many of these targets may not be shared with Crz1p. The identification of the complete set of CrzA effector genes will thus be one interesting issue for future investigation. Another point of interest is the observation that the deletion phenotype of *cnaA* (calcineurin catalytic subunit A), which has been revised recently (Soriani et al., 2008), shows more severe morphological defects than the *crzA* knock out mutant (Spielvogel et al., 2008), suggesting that CrzA is very likely not the only protein regulated by calcineurin. Further determination of the calcineurin range of action would be of great interest, since *cnaA* is not essential for *A. nidulans*, although its deletion causes major pleiotropic effects, among others on morphology.

What is the exact role of calcium and its signaling machinery in polar growth of *A. nidulans*? Hyphal tip-growth requires a tip-high calcium gradient and calmodulin has been found to co-localize with the Spitzenkörper of *A. nidulans* (Wang et al., 2006). However, the mechanisms that direct the establishment and maintenance of this gradient are not that well understood. Furthermore, calcium homeostasis is generally essential for cellular integrity and the role of CrzA is also interconnected with other cellular processes such as pH adaptation and cell wall integrity (Spielvogel et al., 2008). Searches of the *A. nidulans* database for proteins comprising a predicted EF-hand motif (calcium-binding domain, PROSITE PS00018) or calcium/calmodulin-connected PFAM domains revealed around 250 proteins (Table 3 and data not shown), suggesting that the activity of about 2.3% of *A. nidulans* proteins is dependent on calcium. It will be a very challenging venture to uncover all calcium-dependent genes/proteins and to establish if and how their function is involved in the morphogenetic program of *A. nidulans*.

2.3. Development

A well-characterized transcription factor cascade (BrlA → AbaA → WetA) regulates asexual development in *A. nidulans* (Timberlake, 1990). Components of this cascade and associated modifiers were readily identified upon annotation of the genome sequence (Table 4). Whereas BrlA is not found in other filamentous fungi or in yeast, homologues of AbaA are well known in other ascomycetes (i.e.,ATTS DNA binding proteins). These include *S. cerevisiae* Tec1, which is required for pseudohyphal growth in yeast (Gavrias et al., 1996). StuA and MedA are developmental modifiers that spatially and temporally regulate the central transcription factor cascade (e.g., Miller et al., 1992). Homologues of StuA (i.e., APSES transcription factors) have been identified in other ascomycetes and implicated in regulation of morphogenesis (Ramirez-Zavala and Dominguez, 2008). On the other hand, MedA is conserved is ascomycetes, basidiomycetes, and zygomycetes, but is not found in yeast. The *A. nidulans* "fluffy" (i.e., *flb*) genes define a signaling pathway that acts upstream of BrlA to regulate development (Yu et al., 2006). Many of these genes are conserved in ascomycetes. Notably, FlbA is also found in basidiomycetes and zygomycetes.

The extensive conservation of MAP kinase signaling pathways in fungi has been previously described (Bahn et al., 2007). Thus,

Table 3
Predicted *A. nidulans* proteins involved in calcium signaling and homeostasis

S. cerevisiae homologue	*A. nidulans* locus name	*A. nidulans* protein	Known or predicted function in *A. nidulans*
Ca^{2+} channels			
Cch1	AN1168		Plasma membrane-located Ca^{2+} channel
Mid1	AN8842		Plasma membrane and/or ER-located Ca^{2+} channel
Yvc1	AN3155		Vacuolar membrane-located Ca^{2+} channel
Ca^{2+}-ATPases			
Pmc1	AN1189		Vacuolar membrane-located Ca^{2+} P-type ATPase
Pmc1	AN2827		Vacuolar membrane-located Ca^{2+} P-type ATPase
Pmc1	AN5088		Vacuolar membrane-located Ca^{2+} P-type ATPase
Pmc1	AN4920		Vacuolar membrane-located Ca^{2+} P-type ATPase
Pmc1	AN8399		Vacuolar membrane-located Ca^{2+} P-type ATPase
Pmr1	AN7464		Golgi membrane-located Ca^{2+}/Mn^{2+} P-type ATPase
Spf1	AN10367		ER-located ion P-type ATPase
Neo1	AN6614		flippase, ER- and Golgi-located phospholipid P-type ATPase
no hit	AN5743		Ca^{2+} P-type ATPase
Na^{+}-ATPases			
Ena1	AN6642	EnaA	Plasma membrane-located Na+ P-type ATPase
Ena2	AN1628	EnaB	Plasma membrane-located Na+ P-type ATPase
Ena5	AN10982	EnaC	Plasma membrane-located Na+ P-type ATPase
Undefined cation ATPases			
YOR291W	AN8864		P-type ATPase
Ca^{2+} transporter			
Vcx1	AN0471	VcxA	Vacuolar membrane-located Ca^{2+}/H+ exchanger
Vcx1	AN7510		Vacuolar membrane-located Ca^{2+}/H+ exchanger
Vcx1	AN5821		Vacuolar membrane-located Ca^{2+}/H+ exchanger
Vcx1	AN7173		Vacuolar membrane-located Ca^{2+}/H+ exchanger
YNL321W	AN6986		Ca^{2+}/H+ exchanger
Ecm27	AN4266		Ca^{2+}/Na+ exchanger
YDL206W	no hit		
Crz1 signaling pathway			
Cmd1	AN2047	CaM	Calmodulin
Cna1	AN8820	CnaA	Calcineurin catalytic subunit A
Cna2	AN8820	CnaA	Calcineurin catalytic subunit A
Cnb1	AN6566		Calcineurin regulatory subunit B
Rcn1	no hit		Calcineurin inhibitor
Crz1	AN5726	CrzA	C2H2-type zinc-finger transcription factor
Other Ca^{2+}/calmodulin-dependent proteins			
Cmk1	AN2412	CmkA	Ca^{2+}/calmodulin-dependent protein kinase A
Cmk2	AN3065	CmkB	Ca^{2+}/calmodulin-dependent protein kinase B
Pak1	AN8827	CmkC	Ca^{2+}/calmodulin-dependent protein kinase C
Rck1	AN4483		Ca^{2+}/calmodulin-dependent protein kinase
Rck2	AN4483		Ca^{2+}/calmodulin-dependent protein kinase
Kin4	AN0822		Protein kinase involved in mitotic exit delay
no hit	AN6211	PlaA	Phospholipase A2
Plc1	AN0664		Phospholipase C
Plc1	AN2947		Phospholipase C
Spo14	AN6712	PldA	Phospholipase D
Spo14	AN2586		Phospholipase D
no hit	AN1606		Annexin
no hit	AN2427		Annexin
Myo5	AN1558	MyoA	Myosin I
Mlc1	AN6732		Myosin light chain 1
none	AN7707	AcnA	α-Actinin
Frq1	AN5341		Frequenin, putative transcription factor
Sal1	AN2173		Ca^{2+}-dependent mitochondrial carrier protein
Agc1	AN8785		Ca^{2+}-dependent mitochondrial carrier protein
Nde1	AN1094	NdiF	Mitochondrial NADH dehydrogenase
Cdc24	AN5592		Guanine nucleotide exchange factor
Cdc31	AN5618	An-Cdc31	Spindle pole body protein
Spc110	AN3062		Spindle-pole body protein
Cam1	AN6563		Elongation factor 1-gamma
Cne1	AN3592		Calnexin (calreticulin)
Kex2	AN3583	KpcA	Kex2p-like endoprotease
Nth1	AN5635	TreB	Neutral trehalase
Inm1	AN1136	QutG	Quinate catabolism cluster phosphatase
YDR287W	AN1136	QutG	Quinate catabolism cluster phosphatase
Yat1	AN1059	FacC	Cytosolic carnitine acetyl transferase
End3	AN1023	SagA	Sensitivity to alkylating agents
Zrt2	AN3799		Plasma membrane-located zinc transporter
Ccc1	AN3681		Vacuolar membrane-located Fe^{2+}/Mn^{2+} transporter
Ccc1	AN4990		Vacuolar membrane-located Fe^{2+}/Mn^{2+} transporter
Cdc43	No hit		
Rcn1	No hit		
Csg2	No hit		

Table 3 (*continued*)

S. cerevisiae homologue	*A. nidulans* locus name	*A. nidulans* protein	Known or predicted function in *A. nidulans*
Pde1	No hit		
Pmp3	No hit		
Hkr1	No hit		
Num1	No hit		
Ste12	No hit		

it is not particularly surprising that components of these pathways were identified in *A. nidulans* (Table 4). Notably, based on functional analysis of the *A. nidulans* homologues of yeast Ste7 (SteC; AN2269) and Ste12 (SteA; AN2290), the pheromone response pathway plays a central role in regulation of sexual development (Vallim et al., 2000; Wei et al., 2003). In addition, the *A. nidulans* homologue of the MAP kinase Hog1 has been characterized. Besides its expected role in stress signaling, SakA (AN1017) also controls sexual development (Kawasaki et al., 2002).

Light sensing is a key regulatory input that influences developmental decisions in filamentous fungi (Bahn et al., 2007). As shown in Table 4, components of light sensing pathways were identified in *A. nidulans*. These include blue light receptors (i.e., White Collar orthologues LreA and LreB; AN3426 and AN3607, respectively) and the phytochrome FphA (AN9008), as well as the nuclear protein VeA (AN1052)(Purschwitz et al., 2006, 2008; Stinnett et al., 2007). Recent results demonstrate that FphA, LreA, LreB, and VeA form a large protein complex in the nucleus that functions in a complex manner to regulate developmental fate (i.e., asexual vs. sexual) in response to red and blue light (Purschwitz et al., 2008). In general, *A. nidulans* genes implicated in light sensing are reasonably well-conserved in ascomycetes, partially conserved in basidiomycetes and zygomycetes, and are not found at all in *S. cerevisiae*.

3. Discussion

Our analysis of the predicted *A. nidulans* gene content devoted to morphogenesis and development has revealed three general themes. First, the components of the signal transduction pathways and morphogenetic machinery as defined in yeasts are largely conserved in *A. nidulans* (as well as other filamentous fungi). Limited functional studies suggest that many of these components have the same broad function in *A. nidulans* as they do in yeasts. Examples include the roles of Cdc42 signaling and the polarisome in polarized morphogenesis (Virag and Harris, 2006; Virag et al., 2007), as well the yeast pheromone response pathway in the regulation of development (Vallim et al., 2000; Wei et al., 2003). However, other components such as the Hog1 MAP kinase have additional functions beyond those characterized in yeast (Kawasaki et al., 2002). In addition, conserved gene families such as those involved in calcium signaling (i.e., Ca^{2+}-ATPases and Ca^{2+}-transporters) are larger in *A. nidulans* compared to yeasts. Nevertheless, these conserved components likely define a set of core pathways involved in morphogenesis and development in all fungi. Second, *A. nidulans* possesses many additional components implicated in morphogenesis and development that are not conserved in yeasts. Some of these components, such as BrlA, the central regulator of conidiation, are not well-conserved outside of the *Aspergilli* or related *Penicllium* species. Others, such proteins involved in light sensing (i.e., VeA) or hyphal morphogenesis (i.e., MesA) appear to be fungal-specific proteins that were either lost in yeasts or underwent significant divergence. Third, the number of genes functionally implicated in morphogenesis and development in *A. nidulans* is rather large. In addition to the homologues of genes from other species that are known to affect morphology, genetic screens have yielded several genes with no obvious connection to morphogenesis (*podG*, *podH*, *hbrB* etc.) based on their sequence identities. Thus, annotations based on homology alone may only represent the "tip of the iceberg", and *A. nidulans* could conceivably possess as many as 2000 genes that function in some aspect of morphogenesis and development.

One of the objectives of our annotation effort was to determine whether *A. nidulans* possesses additional genes implicated in morphogenesis compared to the known yeast models. As outlined above and in Tables 1–3, this clearly seems to be the case. Besides the increased number of genes with predicted roles in calcium signaling, we also identified homologues of several animal proteins that function in cell migration and appear to be absent from the genome of *S. cerevisiae*. Notably, many of these proteins facilitate organization of the actin cytoskeleton (i.e., α-actinin, gelsolin, and fragmin) or mediate vesicle trafficking (i.e., cytohesin ARF-GEF, centaurin ARF-GAP, and striatin). This suggests that the morphogenetic machinery that drives hyphal extension may indeed be more complex than the analogous machinery in yeast cells. Preliminary genetic analysis further supports this view. For example, *S. cerevisiae* possesses only two proteins with calponin homology (CH) domains; fimbrin (Sac6) and transgelin (Scp1). These proteins appear to function together to mediate the formation of bundled actin filaments that help drive the internalization of endocytic vesicles (Goodman et al., 2003). Notably, severe growth and morphological defects are observed only when both *SAC6* and *SCP1* are both deleted. Although *A. nidulans* contains homologues of fimbrin (AN5803; FimA) and transgelin (AN1935), it also possesses a third CH domain protein, alpha-actinin (AN7707; AcnA). Moreover, severe defects in hyphal morphogenesis are triggered when either *fimA* or *acnA* is deleted (Upadhyay and Shaw, 2008; A. Virag and S. Harris, unpublished results). Analysis of the respective deletion mutants suggests that FimA organizes actin filaments required for endocytosis, whereas AcnA stabilizes actin filaments involved in exocytosis and cytokinesis. At first glance, these observations imply that hyphal morphogenesis requires more complex control of actin filament organization than is needed during yeast budding. Whether this is a general feature of the morphogenetic machinery in filamentous fungi compared to yeasts awaits further study.

A second objective of our annotation effort was to determine if the increased morphological complexity characteristic of *A. nidulans* requires input from additional regulatory pathways beyond those involved in yeast morphogenesis. It has become increasingly clear that polarized hyphal extension is regulated by a variety of signals that appear to have a relatively minor role if any in yeast growth, such as calcium and reactive oxygen species (Nguyen et al., 2008; Semighini and Harris, 2008). Accordingly, it seems reasonable to predict that the regulatory pathways that transduce these signals might have undergone an expansion in the *A. nidulans* genome. However, as exemplified by the data presented in Table 1, this does not appear to be the case. Monomeric Rho GTPases are universally conserved in eukaryotes and play key signaling roles during morphogenesis and development. Notably, despite the increase in morphological complexity relative to yeast, *A. nidulans* possesses only one additional Rho GTPase not found in *S. cerevisiae*; Rho4 (note that *S. cerevisiae* Rho4 is a paralogue of Rho3; in addition, *S. cerevisiae* Rho5 is likely a divergent homologue of Rac1). The number of guanine nucleotide exchange factors (GEFs) and

Table 4
A. nidulans proteins involved in (a)sexual development

S. cerevisiae homologue	*A. nidulans* locus name	*A. nidulans* protein	Known or predicted function in *A. nidulans*
Asexual development			
YER130C	AN0973	BrlA	C2H2-type zinc-finger transcription factor
Tec1	AN0422	AbaA	TEA/ATTS domain transcription factor
No hit	AN1937	WetA	Regulator of spore-specific genes
Sok2	AN5836	StuA	APSES domain transcription factor
No hit	AN6230	MedA	Developmental regulator
Sst2	AN5893	FlbA	Regulator of G protein signaling
No hit	AN7542	FlbB	Leucine zipper transcription factor
No hit	AN2421	FlbC	C2H2-type zinc-finger transcription factor
No hit	AN0279	FlbD	Transcription factor
No hit	AN0279	FlbE	Unknown function
No hit	AN4819	FluG	Glutamine synthase homology; developmental activator
No hit	AN8129	SfgA	Zn2Cys6-type transcription factor
Gpa2	AN0651	FadA	G protein α-subunit
Gpa2	AN1016	GanB	G protein α-subunit
Ste4	AN0081	SfaD	G protein α-subunit
Tpk2	AN6305	PkaA	cAMP-dependent protein kinase, catalytic subunit
No hit	AN0055	TmpA	Putative transmembrane protein
No hit	AN3623	AcoB	Pre-induction sporulation protein
No hit	AN8209	WA	Polyketide synthase
Fet3	AN6635	YA	Laccase I precursor
No hit	AN8803	RodA	Rodlet protein
Pcl1	AN0453	PclA	G1 cyclin
Hym1	AN3095	HymA	Mo25 protein family
Num1	AN7757	ApsA	Coiled-coil protein involved in nuclear movement
No hit	AN3437	ApsB	Coiled-coil protein involved in nuclear movement
Mub1	AN0078	SamB	MYND zinc-finger protein
Sexual development			
Hcm1	AN8858		Forkhead transcription factor
Ndt80	AN6015		Transcription factor
Spo11	AN8259		Endonuclease
Nam8	AN9090		RNA binding protein
Ste13	AN2946		Dipeptidyl aminopeptidase
Ste6	AN2300		ABC transporter
Ste12	AN2290	SteA	STE-like transcription factor
Ume6	AN5170	RosA	Zn2Cys6-type transcription factor
Ume6	AN1848	NosA	Zn2Cys6-type transcription factor
Gat2	AN3152	NsdD	GATA-type zinc finger transcription factor
No hit	AN7349	MutA	Mutanase
Dop1	AN2094	DopA	Leucine zipper transcription factor
Kar5	AN1771		Tht1-like nuclear fusion protein
Ime2	AN6243		Serine/threonine protein kinase
No hit	AN6494		RNA binding protein
Dit1	AN2705		Pyoverdine/dityrosine biosynthesis protein
Msh4	AN5006		Missmatch repair ATPase
No hit	AN1052	VeA	Nuclear protein
Asc1	AN4163	CpcB	Gβ like protein, mammalian RACK1 homologue
Ccp1	AN7388	CpeA	Catalase-peroxidase
Snf3	AN6923	HxtA	High-affinity hexose transporter
No hit	AN1967	PpoA	Fatty acid oxygenase
Ste2	AN2520	GprA, PreB	G protein-coupled receptor
Ste3	AN7743	GprB, PreA	G protein-coupled receptor
No hit	AN3387	GprD	G protein-coupled receptor
Ssp31	AN2269	SteC	MAPKK kinase
Hog1	AN1017	SakA	MAP kinase
No hit	AN1539	CsnD	Subunit 4 of the COP9 signalosome
Rri1p	AN2129	CsnE	Subunit 5 of the COP9 signalosome
Trp5	AN6231	TrpB	Tryptophane synthase
His3	AN6536	HisB	Imidazoleglycerol-phosphate dehydratase
Tub1	AN7570	TubB	α-Tubulin
Rad51	AN1237	UvsC	DANN repair protein
No hit	AN2330	LsdA	Late sexual development protein
Pho85	AN8261	PhoA	Cyclin-dependent kinase
Pho80	AN5156	An-Pho80	PHO80-like cyclin
Fre5	AN5457	NoxA	NADPH oxidase
No hit	AN1037	OdeA	Oleate-Δ12-desaturase
Light sensing			
No hit	AN9008	FphA	Phytochrome
No hit	AN3436	LreA	Photoreceptor, zinc-finger domain protein
No hit	AN3607	LreB	Photoreceptor, zinc-finger domain protein
No hit	AN1052	VeA	Nuclear protein
No hit	AN3361		Opsin

GTPase activating proteins (GAPs) found in *A. nidulans* is also comparable to that of yeast. Only two additional candidate Rho GEFs are found in *A. nidulans*; AN0113 and AN10442. Perhaps most surprisingly, there appear to be fewer recognizable Rho GTPase effectors in *A. nidulans* relative to yeast. For example, *S. cerevisiae* possesses five distinct classes of Cdc42 effectors; PAK kinases, other PBD-containing proteins (i.e., Gic1 and Gic2), FH2-containing formins, a WASP homologue, and Sec3 (Park and Bi, 2007). Whereas formin, WASP, and Sec3 homologues were found in the *A. nidulans* genome sequence, there are fewer PAK kinases and no obvious Gic homologues (Fig. 1). A similar pattern is seen upon comparison of other regulatory pathways between *A. nidulans* and *S. cerevisiae* (i.e., *A. nidulans* possesses two fewer MAP kinase pathways than yeast). These observations clearly run counter to the expectation that additional regulatory pathways would be required to support the increased morphological complexity of *A. nidulans*.

How, then, are morphogenesis and development regulated in *A. nidulans*? At this time, it is obviously premature to suggest an answer to this question. Nevertheless, annotation of the *A. nidulans* genome sequence coupled with results from published functional studies provides some clues. First, it seems likely that some *A. nidulans* orthologs perform additional morphogenetic functions that have been lost in yeast. For example, *A. nidulans* AtmA shares functions in the DNA damage response with its yeast orthologue Tel1, but also has additional roles in the establishment and maintenance of hyphal polarity that are not conserved in yeast (Malavazi et al., 2006). Furthermore, this idea can likely be extended to entire pathways in *A. nidulans*. In animal cells, a complex pathway that includes the Arf GEF ARNO, the Arf GTPase Arf6, and the bipartite Rac GEF ELMO/Dock180 mediates the localized activation of Rac1 in migratory cells (Santy et al., 2005). Although these components are found in both *A. nidulans* (AN6120, AN5020, and AN8877/AN0463, respectively) and yeast, the yeast homologues have diverged to such an extent that the conservation of function seems unlikely, whereas the *A. nidulans* homologues are sufficiently similar to support a predicted role in the regulation of RacA during morphogenesis and development. Second, *A. nidulans* undoubtedly possesses regulatory components and pathways that are unique to filamentous fungi. Insights into some of these pathways have already been obtained (i.e., the complex pathways that regulation conidiation; Yu et al., 2006), whereas others await discovery as individual genes or sets of genes are subjected to genetic analysis. Assembling these pathways and understanding their regulation is an important future challenge that will significantly enhance our understanding of fungal morphogenesis and development.

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