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Regulation of polarised growth in Fungi

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Abstract

Polarised growth in fungi occurs through the delivery of secretory vesicles along tracks formed by cytoskeletal elements to specific sites on the cell surface where they dock with a multiprotein structure called the exocyst before fusing with the plasmamembrane. The budding yeast, *Saccharomyces cerevisiae* has provided a useful model to investigate the mechanisms involved and their control. Cortical markers, provided by bud site selection pathways during budding, the septin ring during cytokinesis or the stimulation of the pheromone response receptors during mating, act through upstream signalling pathways to localise Cdc24, the GEF for the rho family GTPase, Cdc42. Cdc42 in its GTP-bound activates a multiprotein protein complex called the polarisome which nucleates actin cables along which the secretory vesicles are transported to the cell surface. Hyphae can elongate at a rate orders of magnitude faster than the extension of a yeast bud, so understanding hyphal growth will require substantial modification of the yeast paradigm. The rapid rate of hyphal growth is driven by a structure called the Spitzenkörper, located just behind the growing tip and which is rich in secretory vesicles. It is thought that secretory vesicles are delivered to the apical region where they accumulate in the Spitzenkörper. The Spitzenkörper then acts as vesicle supply centre in which vesicles exit the Spitzenkörper in all directions, but because of its proximity, the tip receives a greater concentration of vesicles per unit area than subapical regions. There are no obvious equivalents to the bud site selection pathway to provide a spatial landmark for polarised growth in hyphae. However, an emerging model is the way that the site of polarised growth in the fission yeast, *Schizosaccharomyces pombe*, is marked by delivery of the kelch repeat protein, Tea1, along microtubules. The relationship of the Spitzenkörper to the polarisome and the mechanisms that promote its formation are key questions that form the focus of current research.

Introduction

Fungal hyphae grow almost exclusively from their tips. To do this, membranes and the raw materials and enzymes for the synthesis of new cell wall material are delivered to the tip in the form of secretory vesicles, which fuse with the plasma membrane at the tip. Research in the budding yeast *Saccharomyces cerevisiae* has served as a model for the processes involved and their regulation. Sites of polarised growth are marked by cortical markers formed by the bud site selection pathways, the site of septation in vegetative cells or the site of pheromone stimulation in mating projections. At these sites a small GTPase, Cdc42, is activated by its GEF, Cdc24p (Fig. 1). Activated Cdc42p promotes the formation of a multiprotein complex called the polarisome, which nucleates the formation of actin cables. Post-Golgi secretory vesicles are transported along these actin cables to dock with a second protein complex called the exocyst before fusion with the plasma membrane mediated by the interaction between v-SNARES on the vesicle and a t-SNARE complex on the membrane (Fig. 2). Polarised growth in *S. cerevisiae* has been the subject of an excellent recent review to which the reader is referred to for details of these processes (Park and Bi, 2007).

The yeast model serves to highlight the different stages in this process where control over polarised growth can be exerted. First, polarised growth requires the establishment of the site, which may be subject to both temporal and spatial control. Second, once the site has been established the actin or tubulin cytoskeletons must be polarised toward this site. This requires the formation of a structure such as the polarisome whose formation and activity may be controlled. Third, the formation and flow of post Golgi secretory vesicles along the cytoskeletal tracks can be regulated. Fourth, the assembly of the exocyst and the docking of secretory vesicles with the exocyst may be controlled. Small GTPases, in particular

Cdc42p and the closely related Rac GTPase, play a key role at many of these levels (Park and Bi, 2007).

This review takes the *S. cerevisiae* model as a base, but will seek to consider to what extent the yeast paradigm can be extended to fungi that grow in a hyphal rather than yeast form. Fungal hyphae can extend at a rate that is at least two orders of magnitude greater than a yeast bud or mating projection (Trinci, 1973). An indication that the budding yeast paradigm will require substantial modification is the presence of a structure called the Spitzenkörper, which is dominated by an accumulation of secretory vesicles at or just behind hyphal tips, for which there does not appear to be a counterpart in *S. cerevisiae* (Girbardt, 1969; Harris and Momany, 2004; Harris *et al.*, 2005; Virag and Harris, 2006) (Fig 3). It is thought that secretory vesicles are delivered to the Spitzenkörper along microtubules before onward delivery to the hyphal tip, possibly along microfilaments. Mathematical modelling showed that the hyphoid shape at the tip can be simply predicted by positing that the vesicles radiate from the Spitzenkörper in all directions with equal velocity and that the Spitzenkörper is maintained at a fixed distance from tip (Bartnicki-Garcia *et al.* 1989; Bartnicki-Garcia, 2002).

Some species in the order *Saccharomycetales* can grow as hyphae. *Ashbya gossypii* is closely related to *S. cerevisiae* yet grows exclusively in a hyphal form (Philippsen *et al.*, 2005). So presumably, the components that generate bud growth in *S. cerevisiae* are also capable of generating hyphae. In the case of the polymorphic pathogen *Candida albicans*, its genome encodes components that support yeast, pseudohyphal or hyphal growth, including the formation of a Spitzenkörper-like structure in hyphae (Berman and Sudbery, 2002; Sudbery *et al.*, 2004; Crampin *et al.*, 2005) (Fig. 3). Despite the ability of *A. gossypii* and *C. albicans* to form hyphae, it is likely that novel mechanisms exist that have not been

exemplified by research in *S. cerevisiae*. The recent sequencing of a number of fungal genomes and advances in molecular genetic technology in fungi such as *Neurospora crassa* and *Aspergillus nidulans* have started to reveal new insights and this review will finish with a consideration of these developments. For a more detailed consideration of these issues, see (Sudbery and Court, 2007).

Small GTPases

Small GTPases of the Ras superfamily act as molecular switches in many biological processes. They cycle between a GDP-bound and GTP-bound state. Normally the GTP-bound form is the active “on” state of the switch and the GDP-bound the inactive “off” state. In addition, the active state requires the GTPase to be attached to a membrane by prenylation at its C-terminus. However, it is becoming increasingly clear that in some cases GDP/GTP cycling is necessary for the GTPase to promote its biological function. Conversion of the GDP-bound to the GTP-bound form is mediated by a Guanine Exchange Factor (GEF) that is specific to the particular GTPase. Return to the GDP-bound form is mediated by specific GTPase activating proteins (GAPs). Together GEFs and GAPs control the activity of the GTPase. In addition, Guanine dissociation inhibitors may play a regulatory role. These proteins inhibit the dissociation of GDP from the GTPase and extract the GTPase from the membrane where they normally perform their biological function. During polarised growth a variety of GTPases mediate different aspects of polarised growth. Homologues of Ras itself play an upstream role in signal transduction of environmental signals. Rab GTPases such as Sec4 control vesicle trafficking (Walworth *et al.*, 1992). Rho GTPases control the organisation of the cytoskeleton, the formation of the exocyst and docking of secretory vesicles, and cell wall biosynthesis. In yeast, Cdc42p plays a master role in orchestrating polarised growth at different levels (Fig. 1). In filamentous fungi the closely related Rac GTPase plays overlapping and distinct roles with Cdc42p in controlling developmental processes and polarised growth in hyphae. A critical

difference between Rac and Cdc42 GTPases is that the specificity of the activating GEF is determined by the identity of the amino acid at position 56 which is phenylalanine in Cdc42 GTPases and tryptophan in Rac GTPases (Karnoub *et al.*, 2001).

Ras

In *S. cerevisiae* Ras, encoded by two redundant genes *RAS1* and *RAS2*, is essential for vegetative growth and for passage through Start. Ras is converted to its active GTP-bound form by its GEF Cdc25p and return to its GDP bound form by its GAPs Ira1p and Ira2p (Tanaka *et al.*, 1990). Ras-GTP activates adenylate cyclase, encoded by *CDC35*, to increase cAMP levels (for a review of Ras proteins in *S. cerevisiae* see (Santangelo, 2006). This results in the activation of cAMP-dependent protein kinase (PKA) as cAMP binds to the regulatory subunit Bcy1p and causing it to dissociate and so activates three catalytic subunits Tpk1p, Tpk2p and Tpk3p. Activated PKA targets transcription factors that promote transcription of genes involved in stress responses, ribosomal biogenesis and filamentous growth. Cells lacking functional Ras, Cdc25p or Cdc35p cease growing and arrest as unbudded cells in G1. As well as playing an essential role in vegetative growth and proliferation, Ras is an upstream regulator of polarised growth during pseudohyphal growth. It activates two separate signal transduction pathways. For a review, see (Pan *et al.*, 2000). First Ras acts through Cdc42p to activate a MAP kinase module. Second, it activates the cAMP-based pathway. Both of these pathways ultimately converge on the transcription factor Flo11p, which has an exceptionally extensive and complex promoter that allows it to respond to a diverse range of signals.

In *C. albicans*, Ras plays a key role in activating signal transduction pathways that mediate the transition from a yeast to a hypha (Fig. 4). As in *S. cerevisiae*, *CaRas* activates a MAP kinase pathway and a cAMP-based pathway, which target the Cph1p and Efg1p transcription factors respectively. Cells lacking Ras1p are viable but are unable to undergo

the yeast-hyphal transition; however, they are able to form pseudohyphae (Feng *et al.*, 1999; Leberer *et al.*, 2001). Similarly, cells expressing the dominant negative *RAS1*^{A16} allele show reduced hyphal growth, while cells expressing the dominant active *RAS*^{V13} allele showed enhanced hyphal growth and were able to filament on solid media in conditions that normally favour yeast growth. In the dimorphic pathogen *Penicillium marneffeii*, the Ras homologue, RasAp, is required for polarised growth of both yeast and hyphal phases (Boyce *et al.*, 2005).

As well as mediating the yeast-to-hyphal transition, activation of these pathways initiates an extensive program of hyphal-specific transcription including cell wall genes, adhesins and secreted aspartyl proteases (Fig. 4). It is thought that the cAMP pathway, which targets Efg1p, plays a more major role than the Cph1p pathway. Other pathways also operate to stimulate the yeast to hyphal transition in response to environmental cues. These include neutral pH via the transcription factor Rim101, growth when embedded in a matrix via the transcription factor Crz1p and 5% CO₂, which is sensed by adenylate cyclase. The developmental program is also negatively regulated by Rbf1p, Nrg1p-Tup1p and Rfg1p-Tup1. These pathways have been recently reviewed (Brown *et al.*, 2007).

Cdc42 and Rac GTPases

Cdc42p was first identified in budding yeast through temperature sensitive *cdc42* mutants that arrest as large multinucleate, unbudded cells with a completely depolarised actin cytoskeleton and random distribution of chitin (Hartwell *et al.*, 1974; Adams *et al.*, 1990). The continued isotropic growth of these cells suggests that Cdc42p is essential for bud formation and polarised growth, but not for isotropic growth. Cdc42p is activated by its GEF Cdc24p, which is essential for its function because temperature sensitive *cdc24* alleles also arrest as large unbudded cells (Hartwell *et al.*, 1974; Zheng *et al.*, 1994) (Fig. 1). Cdc42p GAPs are encoded by *BEM1* and *RGA1/2* (Zheng *et al.*, 1994; Stevenson *et*

al., 1995; Smith *et al.*, 2002; Caviston *et al.*, 2003). Yeast cells lacking all three GAPs show highly polarised growth suggesting that these genes act as negative Cdc42p regulators. However, simple activation of Cdc42p is not sufficient for cell viability as *CDC42*^{G12V} and *CDC42*^{G61L} alleles that lock Cdc42p in the GTP-bound state are dominant lethals. Moreover, there is accumulating evidence that at least some functions of Cdc42p require cycling between GDP-bound and GTP-bound states as exemplified by the role of Cdc42p in septin formation in *S. cerevisiae* (Gladfelter *et al.*, 2002) and in hyphal growth of *Candida albicans* (Court and Sudbery, 2007).

In vegetative, cells Cdc24p is localised to sites of polarised growth by the action of the bud site selection pathways (Fig. 1) (for reviews see (Chant, 1999; Park and Bi, 2007)).

Haploid yeast respond to the presence of their cognate mating pheromone by the formation of a mating projection that shows polarised growth against the gradient of the mating pheromone toward its source. Mating pheromone is sensed by seven-transmembrane receptors encoded by *STE2* (α -factor) and *STE3* (a-factor). These receptors are complexed to a tripartite G-protein complex that dissociates upon pheromone binding into α and $\beta\gamma$ subunits. The $\beta\gamma$ dimer localises Cdc24p to promote polarised growth at the site of the activated receptor. In the unstimulated state, Cdc24p is sequestered in the nucleus. It is exported from the nucleus in a complex with Far1p when Far1p is phosphorylated by the MAP kinase Fus3p, activated by the mating pheromone response pathway. Cdc42p orchestrates diverse processes in polarised growth and morphogenesis that can be genetically separated from each other by specific mutations in its effector domain (Fig. 1). For reviews and references see: (Sudbery and Court, 2007; Park and Bi, 2007)

- Cdc42p mediates the formation of the polarisome that nucleates the formation of actin cables for the delivery of vesicles.

- Cdc42p recruits septin subunits to the incipient bud site and by cycling between GDP and GTP mediates the maturation of these subunits into a ring that acts as scaffold for proteins that mediate diverse processes required for cytokinesis. It may also act as a diffusion barrier to corral these proteins to the site of cytokinesis.
- Together with Rho1, Cdc42p is required for the localisation of Sec3p that acts as spatial landmark for the formation of the exocyst complex.
- Together with Rho3p, Cdc42p mediates the docking of secretory vesicles with the exocyst.

Cdc42p is an evolutionarily conserved protein. In yeasts such as *C. albicans*, *Schizosaccharomyces pombe* and *Ashbya gossypii* Cdc42p is essential for polarised growth. *C. albicans* can grow in yeast, pseudohyphal and hyphal forms, and thus is a useful model to investigate the regulatory mechanisms that result in hyphal growth. In *C. albicans* a low level of *CDC42* expression from the regulatable *MET3* promoter, which was sufficient for viability, was insufficient to sustain hyphal growth, indicating that more Cdc42p is required for the polarised growth of hyphae compared to yeast (Bassilana et al. 2003). Moreover, in *C. albicans* Cdc42p and Cdc24p were shown to be required for the expression of hyphal-specific genes (Bassilana et al. 2005). A *bem3Δ/Δ rga2Δ/Δ* mutant, which lacked all Cdc42p GAPs, showed more highly polarised growth, so that cells grown under pseudohyphal-promoting conditions displayed a hyphal morphology (Court and Sudbery, 2007). Moreover, septin rings appeared within the germ tube rather than at the bud neck, a characteristic feature of hyphae. This observation suggested that high levels of Cdc42p-GTP promoted hyphal development. However, a strain conditionally expressing *CDC42*^{G12V}, in which Cdc42p is locked in the GTP-bound state, formed swollen cells when grown under pseudohyphal-promoting conditions. This suggested that Cdc42p must also be capable of GDP/GTP cycling to promote the polarised growth seen in hyphae. The difference between the phenotypes of the *bem3Δ/Δ rga2Δ/Δ* and *CDC42*^{G12V} expressing

strains is probably due to the high intrinsic GTPase activity of wild type Cdc42, which would allow some cycling to occur in the *bem3Δ/Δ rga2Δ/Δ* strain even though the lack of Cdc42p GAPs would elevate the level of the GTP-bound form. In addition to the swollen cell phenotype, expression of the *CDC42^{G12V}* prevented the maturation of septin subunits into a ring, consistent with the previous observation in *S. cerevisiae* that Cdc42pGDP/GTP cycling was necessary for assembly of subunits into a ring. The *C. albicans* genome also contains a member of the Rac family of GTPases. Mutants lacking Rac1p have a normal phenotype except they are unable to form hyphae when embedded in a matrix (Bassilana and Arkowitz, 2006). Thus, Rac1p plays a minor role compared to Cdc42p in *C. albicans*.

In the dimorphic pathogen *Penicillium marneffeii* the Cdc42p homologue is encoded by the *cfIA* gene. Attempts to delete *cfIA* were unsuccessful, suggesting that like yeasts, Cdc42p is essential for viability (Boyce *et al.*, 2001). Its role was investigated by the expression of *cfIA^{D120A}*, which is equivalent to the *S. cerevisiae CDC42^{D118A}* dominant negative allele. Colonial growth was reduced at 25°C that promotes hyphal growth. Conidia were slower to germinate and slower to become polarised to form hyphae, which were extensively curled. Yeast cells expressing *CDC42^{D118A}* failed to initiate cytokinesis. However, the dimorphic switch and asexual development were not affected. Cells lacking the closely related Rac GTPase encoded by *cfIB* failed to undergo asexual development, thus CflBp, rather than CflAp, is required for this developmental process (Boyce *et al.*, 2003). Cells expressing a dominant negative *cfIB^{D123A}* allele were unable to polarise hyphae and the cytoskeleton was disrupted. Epistasis analysis showed that RasAp acted upstream of both CflAp and CflBp to regulate polarised growth in yeasts and hyphae, and spore germination (Boyce *et al.*, 2005).

In the dimorphic pathogen *Ustilago maydis*, cells in which either *cdc42* or *rac1* had been separately deleted were viable but showed distinct phenotypes (Mahlert *et al.*, 2006). *Cdc42* Δ mutants showed no gross morphological abnormalities but fail to separate after cytokinesis. *Rac1D* mutants were swollen and mishappen in the yeast phase and divided through a single, centrally located septum in the mother cell rather than two septa at the bud neck. Furthermore, *rac1D* cells were unable to generate dikaryotic filaments in response to expression of the heterodimeric transcription factor encoded by the *bW* and *bE* mating type genes. However, induction of ectopic expression of *rac1* resulted in unscheduled filament formation. Thus, Rac1p is necessary and sufficient for hyphal formation. While Rac1p and Cdc42p mediate separate processes, they share a common essential function because depletion of both GTPases is lethal.

Aspergillus nidulans contains homologues of Cdc42 and Rac called ModA and RacA respectively (Virag *et al.*, 2007). Deletion of ModA is not lethal, but results in a delay of germinating spores to initiate polarised growth and the resulting germ tubes grow more slowly and show morphological abnormalities. Moreover, conidiation is reduced. In the absence Cdc42, polarisome components localise and a Spitzenkörper forms normally, however microtubules become essential for polarised growth. RacA deletion mutants only show a defect in conidiation; however a strain lacking both ModA and RacA was apparently inviable because no double mutants were recovered when the two single mutants were crossed together. A *GFP-ModA* allele showed impaired functionality; a *GFP-ModA racA* Δ strain could be generated but was very sick. Thus, in *A. nidulans* ModA, the Cdc42 homologue, and RacA also share an essential overlapping function. A tentative picture that emerges is that in two ascomycetes (*P. marneffeii*, *A. nidulans*) and a basidiomycete (*Ustilago maydis*) the functions of Cdc42p in *S. cerevisiae* are divided

between Cdc42p and Rac1p (Boyce *et al.*, 2001; Boyce *et al.*, 2003; Boyce *et al.*, 2005; Mahlert *et al.*, 2006; Virag *et al.*, 2007).

Rho-type GTPases

Other Rho-type GTPases also play essential roles in polarised growth. In *S. cerevisiae*, Rho1p plays multiple roles in cell integrity and polarised growth including:

- the activation of Pkc1p, the upstream activator of the cell-integrity MAP kinase pathway (Nonaka *et al.* 1995),
- acting through Pkc1p to re-polarise actin after a shift to 37°C (Nonaka *et al.* 1995; Kohno, 1996; Schmidt *et al.* 1997; Drgonova *et al.* 1999),
- the activity of β (1-3)-D-glucanase, which synthesises a major component of the cell wall (Qadota *et al.* 1996; Drgonova *et al.* 1996),
- together with Cdc42, Rho1p localises the exocyst landmark protein, Sec3p (Zhang *et al.* 2001; Guo *et al.* 2001).

In *S. cerevisiae*, Rho3p promotes the localisation of the exocyst component Exo70p and the fusion of secretory vesicles with the exocyst, a role it shares with Cdc42p (Adamo *et al.*, 1999; Adamo *et al.*, 2001). Rho3p, together with Rho4p also acts on the formins to polarise the actin cytoskeleton (Dong *et al.* 2003). In *A. gossypii*, loss of the Rho3p homologue resulted in a severe defect of polarised growth in which growth of the hyphal tips became isotropic resulting in swollen tips. Polarised growth would often re-initiate at these tips, but with a different axis from that of the initial hypha (Wendland and Philippsen 2001). In *C. albicans* Rho3p is required for polarised growth so that when cells are depleted of Rho3p hyphal tips become swollen (Dunkler and Wendland, 2007), suggesting that, as in *S. cerevisiae*, Rho3p is required for the docking and fusion of secretory vesicles with the plasma membrane (see below). Rho4p is required for normal cytokinesis (Dunkler and Wendland, 2007).

Control of vesicle traffic

Secretory vesicles originate in the Golgi and in *S. cerevisiae* travel along actin cables to sites of polarised growth. The motive power for their transport is provided by the type V myosin encoded by Myo2p together with its regulatory light chain Mlc1p. The Rab GTPase Sec4p and its GEF Sec2p play a key role in regulating the exit of vesicles from the Golgi and their fusion with the exocyst (Boyd *et al.*, 2004; Medkova *et al.*, 2006; Novick *et al.*, 2007). The current model is described in Fig. 2. Sec2p is recruited to nascent vesicles in the Trans Golgi network by an upstream pair of redundant GTPases Ypt31p and Ypt32p. Sec2p recruits Sec4p and converts it to the activated GTP-bound form. Once activated Ypt31/32p is displaced from Sec2p by the exocyst component Sec15p and the vesicle can now exit the Golgi network and travel along actin cables toward the site of polarised growth. Other members of the exocyst complex are also transported to the exocyst complex on secretory vesicles, with the exception of Sec3p and Exo70p: Sec3p is localised independently of the secretory pathway (Finger and Novick, 1997); Exo70p forms two pools: one is transported to sites of polarised growth on secretory vesicles while the other localises independently of the secretory pathway in a Rho3-dependent fashion (Adamo *et al.*, 1999; Boyd *et al.*, 2004). On arrival at the site of polarised growth, Sec4p-GTP is required for fusion with the plasma membrane. The Sec4p GAPs, Msb3p and Msb4p are also required for vesicle docking, so it is possible that Sec4p GDP/GTP cycling is important for fusion. Msb3p and Msb4p also physically interact with the polarisome component Spa2p providing a physical bridge between the polarisome and vesicle traffic (Fig. 2) (Tcheperegine *et al.*, 2005). Msb3p and Msb4p also physically bind Cdc42p, but in its GDP bound form. It has been suggested that this may maintain a pool of Cdc42p-GDP ready to be activated by Cdc24p (Tcheperegine *et al.*, 2005). The exocyst component Sec15p also interacts with the Cdc42p GAP Bem1p, providing a further physical link between vesicle traffic and the establishment of polarity (France *et al.*, 2006). It is not clear

how this model may be applied to hyphal growth (Walworth *et al.*, 1992; Gao *et al.*, 2003). In *Aspergillus niger*, the Sec4p homologue is dispensable for growth (Punt *et al.*, 2001). It is not yet clear whether an unidentified protein can perform the function of Sec4p or whether the mechanism controlling vesicle transport is fundamentally different in filamentous fungi.

In *C. albicans* hyphae Mlc1p, the regulatory light chain of Myo2p that provides the motive force for secretory vesicle transport, accumulates in a Spitzenkörper-like structure at the tip (Crampin *et al.*, 2005). This suggests that the arrival of vesicles at the tip is more rapid than the rate of onward transport from the Spitzenkörper to the cell surface. Both microtubules and microfilaments appear to be required for polarised growth in *C. albicans* (Akashi *et al.*, 1994; Crampin *et al.*, 2005). However, they have different roles. The microtubular inhibitor, MBC prevents hyphal elongation and results in Mlc1-YFP being localised in a surface crescent characteristic of a polarisome. Cytochalasin A, which disrupts actin cables, results in tip swelling due to a switch from polarised to isometric growth. A model to explain these observations is that long distance transport is mediated by microtubules, whereas the short distance distribution from the Spitzenkörper to the hyphal tip mediated by actin cables.

Recently, an interesting interaction between the exocyst landmark Sec3p and the septin Cdc11p has been uncovered during the growth of *C. albicans* hyphae (Li *et al.*, 2007). Mutants in which *SEC3* has been deleted are viable at 30°C but are temperature sensitive for growth at 37°C – a similar phenotype to *S. cerevisiae* *sec3Δ* mutants. When challenged to make hyphae by growth at 37°C plus serum, *C. albicans* *sec3Δ/Δ* initially produce apparently normal hyphal germ tubes. However, at the time that the first septin ring appears in the germ tube growth becomes isotropic and the tips become swollen. The

swollen tip phenotype, but not the growth defect, is rescued by deletion of either of the *CDC10* or *CDC11* genes, which encode septin subunits. Furthermore, Cdc11p and Sec3p have been shown to physically associate by co-immunoprecipitation.

Cells lacking either of the cyclins Cln3p or Ccn1p and induced to form hyphae also show the phenotype where germ tubes initially form normally but subsequently become swollen at the tip (Loeb *et al.*, 1999b; Chapa y Lazo *et al.*, 2005; Bachewich and Whiteway, 2005). Ccn1p-Cdc28p has been shown to phosphorylate Cdc11p at residue 394 (Sinha *et al.*, 2007). This phosphorylation is dependent on prior phosphorylation at position 395 by the Gin4p kinase. A *Cdc11* S394D S395D phosphomimetic allele rescues the swollen-tip phenotype of a *ccn1D/ccn1D* allele. Thus, the only Ccn1p function required for hyphal growth is to enable phosphorylation of Cdc11p by Cdc28p. A model to explain these observations is that after the septin ring forms, Cdc11 competes with the hyphal tip for localisation of the exocyst landmark Sec3. Phosphorylation of Cdc11 by Ccn1-Cdc28 weakens the affinity of Cdc11 for Sec3, allowing tip localisation to predominate and polarised growth to continue (Sudbery, 2007).

Protein kinases

PAK kinases

Cdc42p interacts with and activates a pair of related kinases called Ste20p and Cla4p which are members of the p21 activated (PAK) kinase family (Fig. 1). Both kinases localise to sites of polarised growth in a Cdc42p-dependent fashion (Cvrckova and Nasmyth, 1993; Peter *et al.*, 1996). They each have separate identifiable functions, but a *cla4Δ ste20Δ* mutant is inviable implying that they are redundant for an essential function. Both proteins have a kinase domain in the C-terminal part of the protein. In the N-terminal extension to the kinase domain is a conserved domain CRIB domain (Cdc42/Rac Interactive Binding)

that interacts with Cdc42p The CRIB of Ste20p is essential for polarised localisation and for the essential function shared with Cla4p (Peter *et al.*, 1996; Leberer *et al.*, 1997a). Ste20p functions in the pheromone response signalling pathway activating the Ste11p, which is a MAP kinase, kinase, kinase (MAP = Mitogen Activated Protein). Elements of the signalling pathway, including Ste20p are also required for pseudohyphal formation and invasive growth of haploids (Liu *et al.*, 1993). The kinase domain is required for its role in signalling. The CRIB domain is autoinhibitory and its negative effect on kinase activity is relieved by Cdc42-GTP binding.

Cla4p phosphorylates septins (Versele and Thorner, 2004). In the absence of Cla4p, septin bars form rather than a true ring, a phenotype that is enhanced when a *cla4Δ* mutation is combined with *gin4Δ* and/or *nap1Δ* alleles (Longtine *et al.*, 2000). Cla4p also regulates polarised growth during the cell cycle of *S. cerevisiae*, however, there is disagreement about whether it acts in a positive or negative way. There is agreement that just after bud emergence Cla4p phosphorylates Cdc24p and that this phosphorylation depends on Cdc42-GTP and the scaffold protein Bem1. According to one report, this causes the dissociation of Bem1p from Cdc24p so that Cla4p is part of negative feedback loop that leads to the cessation of polarised growth (Gulli *et al.*, 2000). However, a second report disagreed with this conclusion and argued that Cla4p formed part of a positive feedback loop to promote polarised growth (Bose *et al.*, 2001). The essential functions shared between Cla4p and Ste20p may involve polarisation of both actin cortical patches and cables. Phosphorylation of Myo3/Myo5p is required for the formation of actin cortical patches (Wu *et al.*, 1997). Polarisome function and activation of Bni1p is also dependent on phosphorylation by one of the PAK kinases (Goehring *et al.*, 2003). The role of Cla4p homologues in other fungi has only been investigated in *C. albicans* and *A.gossypii*

(Leberer *et al.*, 1997b; Ayad-Durieux *et al.*, 2000; Li *et al.*, 2007). Interestingly, in both organisms *cla4*Δ mutants can establish but not maintain hyphal growth

Cdk1

As well as spatial regulation, polarised growth requires temporal regulation. Polarised growth in the cell cycle of *S. cerevisiae*, and the budding yeast form of *C. albicans*, is restricted to the first part of the cell cycle when the bud is small (Kron and Gow, 1995). In the hyphal stage of *C. albicans* and during the growth of other hyphal fungi, polarised growth is continuous (Soll *et al.*, 1985; Crampin *et al.*, 2005). The temporal regulation of the growth pattern in *S. cerevisiae* is controlled by the cyclin-dependent kinase Cdk1, which controls progress through the cell cycle (for a review see (Mendenhall and Hodge, 1998). Association of Cdk1p with Cln1p or Cln2p promotes hyphal growth, whereas the Cdk1-Clb1/2p kinase promotes isotropic growth (Loeb *et al.*, 1999a). During a normal cell cycle Cln1/2p levels decline while Clb1/2p levels increase resulting in the switch to isotropic growth in late G2. The morphogenesis checkpoint, which delays mitosis and prevents the switch to isotropic growth, results in the stabilisation of Cln1/2p levels and a decrease in Clb2p levels (Lew and Reed, 1995; McCusker *et al.*, 2007). In *C. albicans*, the homologue of *CLN1/2* is called *HGC1* (Zheng *et al.*, 2004). *HGC1* is only expressed in hyphae and is required for hyphal growth. Yeast growth in *C. albicans* requires the *CLN3* homologue (Chapa y Lazo *et al.*, 2005; Bachewich and Whiteway, 2005). *CLN3* is essential for viability, but cells can be depleted of Cln3p using expression from the highly regulatable *MET3* promoter. When cells are depleted of Cln3p in this way, they spontaneously form hyphae in the absence of the normal hypha-inducing signals. So Cln3p is required for yeast growth but not hyphal growth, and Hgc1p is required for hyphal growth but not yeast growth. Thus Cdk1p takes on specialist roles in hyphal and yeast phases which are determined by the particular cyclin with which it associates.

NDR kinases

NDR (Nuclear DBF2-Related) kinases are a group that are conserved from yeast to man and act in mechanisms involved in polarised growth and morphogenesis (Tamaskovic *et al.*, 2003; Hergovich *et al.*, 2006). Dbf2p, the eponymous founding member of the group, was characterised in *S. cerevisiae* where it forms the target of the Mitotic Exit Network (MEN) that permits exit from mitosis after anaphase has been successfully completed (for a review see (McCollum and Gould, 2001)). It physically associates with its partner Mob1p, which allows it to be phosphorylated and activated by an upstream kinase, Cdc15p.

Cdc15p is related to Ste20p kinase family, but is a member of a subfamily called Germinal Centre kinases that lack the Cdc42p interacting domain of Ste20 kinases. This regulation of Dbf2p is emerging as a standard pattern for NDR kinases: they are part of a network that includes a Cdc15-related kinase and a Mob binding partner. However, although Cdc15p has been directly shown to bind and activate Dbf2, direct activation has so far not been demonstrated for any other of the related kinases that are part of NDR kinase networks. Dbf2p targets Net1p a protein which retains a phosphatase, Cdc14p, in the nucleolus. Once released from the nucleolus Cdc14p dephosphorylates and stabilises the Clb-Cdk1 kinase inhibitor, Sic1p. Cdc14p also promotes the destruction of G2 cyclins necessary for mitotic exit by dephosphorylating and stabilising Cdh1p that targets Clb cyclins to the Anaphase Promoting Complex (APC).

In diverse fungi, NDR kinases are required for both polarised growth and cell separation. In *S. cerevisiae*, Cbk1p, closely related to Dbf2p, is required for polarised growth and was uncovered in a screen for defects in polarised growth of mating projections. It is also required for cell separation after cytokinesis (Racki *et al.*, 2000; Bidlingmaier *et al.*, 2001; Colman-Lerner *et al.*, 2001). Cbk1p is part of a physically interacting network of proteins called the RAM network (Regulation of Ace2 activity and morphogenesis) (Nelson *et al.*,

2003). The RAM network targets the transcription factor Ace2p to the daughter nucleus where it programs the expression of *CTS1* and *SCW1*, which encode proteins that will degrade the primary septum and allow cell separation. The RAM network includes a number of proteins that are commonly found elsewhere to be associated with NDR kinases. Mob2p is an NDR-activating partner closely related to Mob1p. Kic1p is related to Cdc15p and is assumed to be an upstream activating kinase although this has not been directly demonstrated. Tao3p may be a scaffold protein. Ssd1p is a protein whose function is obscure but allelic polymorphisms have been found to modify the phenotypic effects of mutations affecting a wide variety of cellular processes.

In *S. pombe*, NDR kinases are encoded by *orb6⁺* and *sid2⁺*. As their name suggests *orb6⁻* mutants are spherical rather than rod-shaped and fail to polarise the actin cytoskeleton (Verde *et al.*, 1995). Orb6p interacts with Mob2p which is required for its activation by the Cdc15-related kinase Nak1p/Orb3p (Leonhard and Nurse, 2005) (Hou *et al.*, 2003). Sid2p is part of the Septum Initiation Network (SIN) *Sid2⁻* mutants are defective in septum formation. Sid2p interacts with Mob1p and this interaction is required both for localisation of Sid2p to a medial ring where the septum will form and for Sid2p catalytic activity (Hou *et al.*, 2004). Sid2p may promote septum formation by activating the *S. pombe* homologue of Ace2p, but the precise mechanism of how it does this remains unclear (Jin *et al.*, 2006). A Cdc15-related kinase, Cdc7p, is also required for septum formation; however direct activation of Sid2p by Cdc7p has not been demonstrated (Mehta and Gould, 2006).

In *N. crassa*, *cot-1*, which encodes an NDR kinase (Yarden *et al.*, 1992) has been recognised for many years at a genetic level as temperature sensitive *cot-1⁻* alleles cause hyper-branching and a more compact “colonial” colony morphology (Collinge *et al.*, 1978). At the restrictive temperature *cot-1⁻* mutants are defective in tip elongation and like other

similar such colonial mutants show excessive hyphal branching. *cot-1* mutants are suppressed by culture and stress conditions that lower cAMP levels and by an inhibitor, KT5720, that inhibits cAMP-dependent protein kinase (PKA) (Gorovits and Yarden, 2003). Thus, it seems likely that cAMP signalling acts in opposition to COT1 in regulating hyphal growth. Interestingly, an allele of *GUL1*, which encodes an Ssd1p homologue, also suppresses the *cot-1* mutation, suggesting that Ssd1p is part of a complex with cot1, as it is in *S. cerevisiae*. In wild type cells, COT1 is localised in a punctate fashion along the hyphal length, but excluding the Spitzenkörper region at the tip. *Cot-1* mutations are also suppressed by *ropy* mutations affecting the dynein/dynactin minus-end directed microtubular motor complex. In these mutants COT1 becomes concentrated at the tips (Seiler *et al.*, 2006). An interpretation of this result is that COT1 is transported along microtubules to the hyphal tip by plus-end directed microtubule motors and away from the tip along microtubules by the dynein/dynactin complex. Suppression of the *cot-1* mutation by *ropy* mutations could arise by the increased concentration of the defective COT1 at the tips. COT1 physically and genetically interacts with pod6, a Cdc15p-related kinase. Temperature sensitive *pod-6* mutants are suppressed by the same mutations and environmental stresses as *cot-1*. Moreover, a *pod-6 cot-1* double mutant shows the same phenotype as each single mutant (Seiler *et al.*, 2006). Taken together these observations suggest that COT1 and POD6 act in the same pathway. However, neither the kinase activity nor the localisation of either kinase was dependent on the function of the other; nor does overexpression of one kinase rescue the phenotype of temperature sensitive alleles of the other kinase. Thus, it seems unlikely that POD6 directly phosphorylates and activates cot1.

Interestingly, in dimorphic human pathogen *Cryptococcus neoformans* the Cbk1p homologue appears to negatively regulate polarised growth (Walton *et al.*, 2006). This

fungus grows in a filamentous form at 25°C and as yeast at 37°C. A screen for colony morphology mutants recovered mutant alleles affecting homologues of the *S. cerevisiae* RAM pathway, including Cbk1p. Surprisingly, when grown at 37°C, which normally favours yeast form growth, these mutants were elongated and the actin cytoskeleton hyperpolarised.

Beyond the yeast paradigm

The dramatic differences in morphology between the hyphae of filamentous fungi and budding yeast such as *S. cerevisiae* make it seem unlikely that hyphal growth only utilises the same components as bud growth. The sequencing of many different fungal genomes, including species such as *A. nidulans* and *N. crassa*, which have well developed and tractable genetic systems, provide the opportunity to search for mechanisms that have not so far been exemplified in yeast. One such mechanism in *A. nidulans* was uncovered by a screen for mutations that enhanced the phenotype of cells depleted for the formin SepAp. One of the mutants recovered, *mesA*⁻, showed polarity defects and tip splitting typical of colonial mutants such as *cot-1* (Pearson *et al.*, 2004). In *mesA*⁻ mutants, SepAp localised to a crescent at the hyphal tip but not to a spot, in contrast to wild type cells where SepAp localises to a spot and crescent at the hyphal tip. Furthermore, formation of actin cables and polarised sterol rich membrane domains (lipid rafts) was disrupted. These observations suggested that MesAp acts to facilitate the localisation of SepAp at the hyphal tip possibly by promoting the formation of the sterol-rich domains. *MesA* homologues are present in the genomes of other filamentous fungi and the fission yeast *S. pombe*, but the only possible homologue in *S. cerevisiae* shows very limited homology. However, both the clear homologue in *S. pombe* and the weak *S. cerevisiae* homologue have been shown to physically interact with components of the spindle pole body. One

possible rationalisation of this observation and the role of MesAp in hyphal polarity, is that MesAp is a microtubule-interacting protein.

In *N. crassa*, a large scale screen has been carried out for mutants showing abnormal hyphal growth (Seiler and Plamann, 2003). A total of 900 mutants were recovered that were assigned by genetic analysis to 100 complementation groups. The availability of an ordered cosmid library of the *N. crassa* genome then allowed the rapid identification of the genes affected in 45 of the mutants. Reassuringly the screen recovered genes already known to be centrally involved in polarised growth such as *cdc-24*, *cdc-42*, *cot-1* and genes encoding proteins of the secretory pathway. However, the screen also identified genes for which there are no obvious homologues in the ascomycete yeasts. This provides a fruitful resource to characterise mechanisms and pathways for which there are no obvious counterparts in the yeasts.

A central aspect of the *S. cerevisiae* paradigm is that sites of polarised growth are determined by physical markers – cortical bud site proteins in vegetative cells, pheromone receptors in haploid cells responding to mating pheromone and sites of cytokinesis. In *A. gossypii* and *C. albicans*, Rsr1p has been shown to be necessary to stabilise the axis of hyphal growth. Proteins of the bud site selection pathways of *S. cerevisiae* are poorly conserved in filamentous fungi (Harris and Momany, 2004). What is responsible for the spatial localisation of polarised growth to the hyphal tip? There are two possibilities. First, in *S. cerevisiae* Cdc42p is localised evenly around the cell cortex in the absence of polarity landmarks. However, localised stochastic variations in Cdc42p activity become amplified because Cdc42p itself is delivered to the cortex along the actin cables that are promoted by activated Cdc42p (Irazoqui *et al.*, 2003; Wedlich-Soldner *et al.*, 2003; Wedlich-Soldner *et al.*, 2004). This results in a self-sustaining feedback loop that allows buds in random

pattern when the bud site selection pathway is disrupted. A self-sustaining feedback loop of this sort could be responsible for the polarised growth at the hyphal tip. A second possibility is provided by the way in which sites of polarised growth are marked in *S. pombe*. The kelch repeat protein Tea1p is transported to cell ends along microtubules by the kinesin motor protein Tea2p (Mata and Nurse, 1997; Behrens and Nurse, 2002). Once delivered to the cell ends Tea1p initiates polarised growth by stimulating Cdc42p. The *A. nidulans* homologue of Tea2p is encoded by *kipA*. A strain in which *kipA* has been deleted grows in a conspicuously wavy shape, microtubules failed to converge at the tip and the Spitzenkörper moves from side to side within the hyphal tip (Konzack *et al.*, 2005). Thus, *A. nidulans* hyphae may also rely on cortical markers to stabilise the axis of growth. In addition, microtubules may fix the Spitzenkörper in its position in the centre of the hyphal tip.

Figure Legends

Fig. 1. Cdc42p orchestrates morphogenesis in *S. cerevisiae*. Cdc42p is activated by its GEF Cdc24p and returned to its GDP-bound state by its GAPs Rga1/2p and Bem3p. Cdc24p is activated by upstream bud site selection pathways and by the $\gamma\beta$ G-protein dimer released from the tripartite G-protein complex when the cognate mating pheromone interacts with the mating pheromone receptors. Acting through its immediate effectors, activated Cdc42p stimulates polarised growth, activation of the pheromone response pathway, pseudohyphal growth and cytokinesis.

Fig. 2. Polarised secretion in *Saccharomyces cerevisiae*. Secretory vesicles bleb off from the late Golgi compartments and travel along actin cables, nucleated by the polarisome, to fuse with the plasma membrane at sites of polarised growth. The process is controlled by the Rab GTPase Sec4p which is activated by its GEF Sec2p. Numerals enclosed in circles refers to exocyst components encoded by Sec genes (e.g. Sec15p, Sec6p etc). Encircled 70 and 84 refers to Exo70 and Exo84. For details see text.

Fig. 3. The Spitzenkörper. A: The *Neurospora crassa* Spitzenkörper revealed by brief FM4-64 staining which reveals secretory vesicles accumulating in a sub-apical spot (Fischer-Parton *et al.*, 2000). B: Spa 2 accumulating in the Spitzenkörper of *Ashbya gossypii* hyphae (Knechtle *et al.*, 2003). C – E Mlc1-YFP localising to the Spitzenkörper of *Candida albicans* (Crampin *et al.*, 2005). C: Mlc1-YFP in a subapical spot and the cytokinetic ring. D: Mlc1-YFP localising to the tip where the apical membrane is stained with filipin. E: Computer modelling using the information in the Z-stack from panel D, shows that Mlc1 is a 3-dimensional sphere located within the hypha rather than a two-dimensional surface crescent. F: The vesicle supply centre model of Bartnicki-Garcia *et al.* (1989): Secretory vesicles are delivered along microtubules (not to scale!). They accumulate in the Spitzenkörper before radiating in all directions. A greater concentration per unit area arrives at the tip compared to subapical areas, which drives tip growth. The shape is determined by the distance of the Spitzenkörper centre from the tip as the distance increases so also does hyphal width.

Fig. 4. Signal transduction pathways regulated hyphal development in *Candida albicans*. External environmental cues stimulate multiple pathways that target downstream transcription factors. Note the central role played by AC (adenylate cyclase) and the transcription factor Efg1p. Cst1, Hst1, Cek1 and Cph1 are the homologues of *S. cerevisiae* proteins Ste20, Ste7, Fus3/Kss1 and Ste12 respectively that form the MAPK module mediates the pheromone response and promotes pseudohyphal growth.

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