6 Hyphal Growth and Virulence in *Candida albicans*

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I. Introduction

Fungi grow either as unicellular yeasts or form elongated tubes known as hyphae that, by branching, can form large mycelia. Yeast-like growth, as seen, e.g. in the unicellular brewer's and baker's yeast *Saccharomyces cerevisiae*, includes an active step of cell separation after mitosis. Such a cytokinesis requires a partial degradation of the cell wall and the chitin-rich septum at the mother daughter cell junction to allow separation of both cells. In contrast, hyphae consist of multiple concatenated cells which are compartmentalized by septation but which are not fragmented. Dimorphic fungi, such as the human pathogen *Candida albicans* can switch growth modes between yeast and hyphal stages. This versatility allows conquering different environmental or host niches and in *C. albicans* contributes to the successful colonization and infection of its host. The pathogenicity of *C. albicans* is brought about in concert with other virulence factors such as the production of secreted aspartic proteases and lipases or the phase-specific expression of genes, as well as the reduced ability of the host to fight off infections due to a compromised immune system. In this chapter we discuss differences on the molecular level between yeast and hyphal growth by comparison of *C. albicans* with *S. cerevisiae*. From there we review the signals that induce filamentation in *C. albicans*, the signal transduction cascades used to process these signals, and the output in terms of changes at the transcriptional level that induce phase-specific gene expression.

II. Comparison of Yeast and Hyphal Growth

Cell growth at some time point requires the generation of an axis of polarity at which to direct vesicle delivery to initiate polarized growth. This initiates two distinct growth phases, namely the establishment of cell polarity and the maintenance of polarized cell growth. While yeast cells show a cell cycle-dependent stop of polarized growth at the tip of the newly formed bud to prepare for cytokinesis, filamentous fungi keep their growth polarized at the hyphal tip. Several morphological and cytological differences can be found that promote this process. Most notably, in filamentous fungi an organelle has been observed

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at the hyphal apex that can act as a vesicle supply center; and this has been termed Spitzenkörper (Girbardt 1957).

A. Yeast Growth in *Saccharomyces cerevisiae* and *Candida albicans*

In *S. cerevisiae* morphogenesis is controlled by cell cycle events. In their seminal paper Lew and Reed (1993) showed that regulation of Cdc28 by cyclins results in differential activation or inactivation of polarized cell growth. This can be monitored by following the distribution of actin cortical patches in these cells using, e.g. rhodamine-phalloidin staining. Activation of the Cdc28 (which is the homolog of the mammalian Cdc2) by the G1-cyclins triggers START and the polarization of the actin cytoskeleton to a selected site at the cell cortex. Conversely, activation of Cdc28 by the G2-cyclins results in the depolarization of the actin cytoskeleton. Thus in yeast cells the polarized growth phase at the bud tip is restricted to only a small period of the cell cycle (Fig. 6.1A). There is ample evidence that downstream of Cdc2 Rho-type GTPases control the organization of the actin cytoskeleton (Bishop and Hall 2000; Casamayor and Snyder 2002). Particularly the Cdc42-GTPase has been shown to responsible for cell polarity establishment, since inactivation of Cdc42 (or its guanine nucleotide exchange factor Cdc24) gives rise to cells that are unable to form buds in *S. cerevisiae* and *C. albicans* (Bender and Pringle 1991; Ushinsky et al. 2002; Bassilana et al. 2003). Similarly deletion of *CDC42*/*CDC24* in the filamentous ascomycete *Ashbya gossypii* resulted in round germ cells that were not able to generate germ tubes (Wendland and Philippsen 2001). In *S. cerevisiae* several mechanisms are required to ensure switching of growth modes from polar to non-polar (isotropic; Fig. 6.1B). Particularly the use of feedback loops is employed: a positive feedback loop in which activated Cdc24 leads to loading of Cdc42 with GTP, thereby activating Bem1 which in turn helps to stabilize Cdc24 at the site of polarized growth (Butty et al. 2002). In contrast, a negative feedback loop results in the down-regulation of Cdc42. This is achieved by Cdc42-GTP activating the p21-activated kinase Cla4, which then phosphorylates Cdc24, leading to the dissociation of Cdc24 from Bem1 (Gulli et al. 2000). Activation of the Cdc42-GTPase module itself may be by the

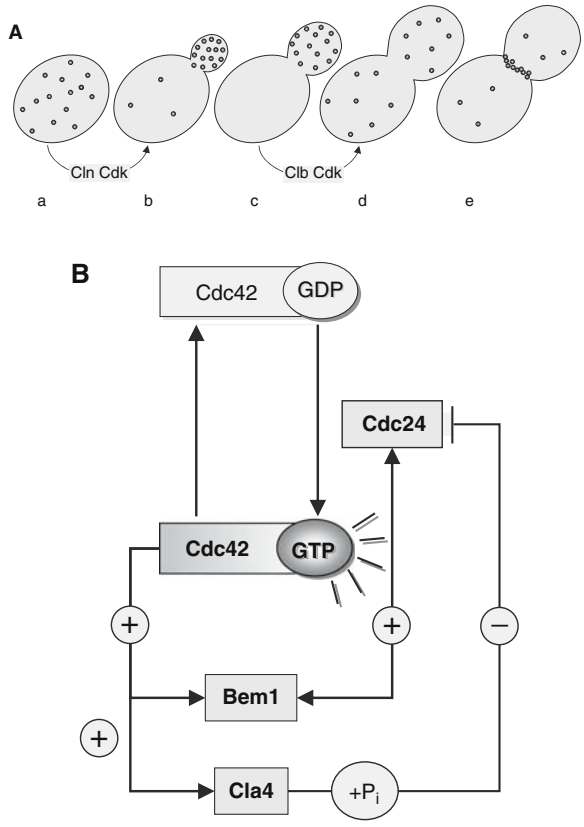

Fig. 6.1. **A** Distribution of cortical actin patches during the *Saccharomyces cerevisiae* cell cycle. In *S. cerevisiae* isotropic growth in the G1 phase of the cell cycle is characterized by a random distribution of cortical actin (*a*). Cyclin-dependent kinase (CDK) complexed with G1 cyclins (Cln) promote clustering of cortical actin patches at the incipient bud-site and in the bud at the time of bud emergence and during the initial phases of bud growth (*b*). Depolarization of the actin patches, indicating isotropic growth of the daughter cell is brought about by the G2–cyclin complexes (Clb-CDK) (*c, d*). Repolarization of the actin cytoskeleton at the end of mitosis to the bud neck region occurs prior to cytokinesis (*e*). **B** Regulatory circuits controlling Cdc42-activity. Cdc42 activity is controlled by a positive feedback loop in which Bem1 stabilizes Cdc24 at sites of polarized growth. Cdc24 acts as guanine nucleotide exchange factor on Cdc42 reinforcing this feedback loop. A negative feedback loop can be used to interrupt Cdc42 activation. Here, the Cla4-kinase phosphorylates Cdc24, which leads to a weakening of its interaction with Bem1

ras-related GTPase Bud1 or in its absence by a stochastic mechanism (Michelitch and Chant 1996; Park et al. 1997; Wedlich-Söldner et al. 2003). To actually gain control on the actin cytoskeleton Rho-GTPases activate another set of effector proteins. One class comprises the formin homologs Bni1 and Bnr1. Formins are actin nucleators

involved in the formation of actin cables which are required for directed delivery of secretory vesicles to sites of growth (Dong et al. 2003). Bni1 functions as part of a complex that is termed the polarisome and includes Spa2 and Bud6 (Ozaki-Kuroda et al. 2001; Bidlingmaier and Snyder 2004).)

In *C. albicans* homologs of the *S. cerevisiae* *BEM1*, *BNI1*, *BNR1*, *BUD1*, *BUD6*, and *SPA2* genes are present in the genome. Their molecular study has been carried out recently and phenotypes quite similar to the *S. cerevisiae* mutants could be detected. *CaBEM1* is an essential gene (Michel et al. 2002). Deletions of the polarisome components *BNI1*, *SPA2*, or *BUD6* give rise to *C. albicans* mutant cells which are enlarged and develop a widened bud neck indicating defects in polarity (Zheng et al. 2003; Crampin et al. 2005; Li et al. 2005; Martin et al. 2005; Song and Kim 2006). Deletion of *CaBNR1* resultsd in a mild cellular defect with increased cell length and defects in cell separation (Martin et al. 2005).Deletion of *CaBUD1*/*RSR1* results in defects in yeast growth, particularly in random budding (Yaar et al. 1997; Hausauer et al. 2005). This demonstrates that similar networks are used to promote polarized cell growth in *S. cerevisiae* and *C. albicans*. Nevertheless, these protein networks have evolved differently and other players may be part of the species-specific networks. An interesting example is given by the cyclins. Cyclins are subunits that interact with the Cdc28 cyclin-dependent kinase and by elaborate mechanisms control the timing of events in the cell cycle. The *C. albicans* G2-cyclins negatively regulate polarized cell growth, as in *S. cerevisiae* (Bensen et al. 2005). There are, however, differences concerning the G1-cyclins between *C. albicans* and *S. cerevisiae*. Molecular analysis of *CaCLN3* showed that this gene is essential for budding. Depletion of Cln3 resulted in unbudded enlarged cells which eventually produced filaments (Bachewich and Whiteway 2005; Chapa y Lazo et al. 2005). Surprisingly, *C. albicans* possesses a hypha-specific G1-cyclin, encoded by *HGC1*, which is specifically expressed in hyphal stages and is essential for hyphal morphogenesis (Zheng et al. 2004). This indicates that cell cycle regulation in *C. albicans* requires specific cyclin/Cdc28 complexes during different growth modes. In contrast, overexpression of *HGC1* is not sufficient to generate hyphal growth in *C. albicans*, showing that Hgc1 does not control the yeast-to-hyphal switch (see below). In summary, work on the *C. albicans* cyclins indicates that a block in cell cycle progression leads to elongated cell growth, which is reminiscent of *S. cerevisiae* cells in which, e.g. overexpression of the G1-cyclins also results in hyperpolarized growth. Similarly, a delay in cell cycle progression, for example, by a deletion of the *C. albicans* dynein heavy chain encoding gene *DYN1*, also results in elongated cell phenotypes (Martin et al. 2004).

B. Hyphal Growth in *Candida albicans*

The drastic differences between yeast and hyphal growth become apparent in *C. albicans* yeast cells induced for hyphal formation (see Section IIIA). Upon induction *C. albicans* yeast cells form germ tubes that extend by polarized hyphal growth; and after several hours they form branched mycelia as seen in other non-dimorphic filamentous fungi (Fig. 6.2). Several other morphological and cytological features distinguish hyphae from yeast cells in *C. albicans*: (a) the organization of the actin cytoskeleton, (b) the positioning of the first septum after germ tube emergence, and (c) the presence of a Spitzenkörper in the hyphal tips.

As discussed above, during yeast growth the actin cytoskeleton is polarized to the bud tip and subsequently depolarized in a cell cycle-dependent manner. During hyphal growth, however, the actin cytoskeleton consisting of actin cables and cortical actin patches are continuously polarized to the hyphal tip, thus maintaining polarized delivery of secretory vesicles to the tip (Fig. 6.3). It is not known how this switch is brought about and maintained on the molecular level. But it seems likely that a continuous positive feedback establishes Cdc42 signaling at the hyphal tip. Constitutively activating Cdc42 in *C. albicans* by mutations that keep Cdc42 in the GTP-bound form ($CDC42^{G12V}$) proved to be lethal, indicating that a cycling of Cdc42 between GTP- and GDP-bound forms is required (Ushinsky et al. 2002).

One peculiarity in germ tube formation in *C. albicans* distinguishes not only hyphal from pseudohyphal growth in *C. albicans* but also germ tube emergence in *C. albicans* from that in other filamentous ascomycetes, such as *Ashbya gossypii* and *Aspergillus nidulans*. This is concerned with the placement of the first septum after germ tube emergence in *C. albicans*. This septum is placed at the germ cell–germ tube junction in *Ash. gossypii* and *Asp. nidulans*. In *C. albicans*, however this septal site is placed within the germ tube some 10–15 µm away from the germ cell (Sudbery 2001).

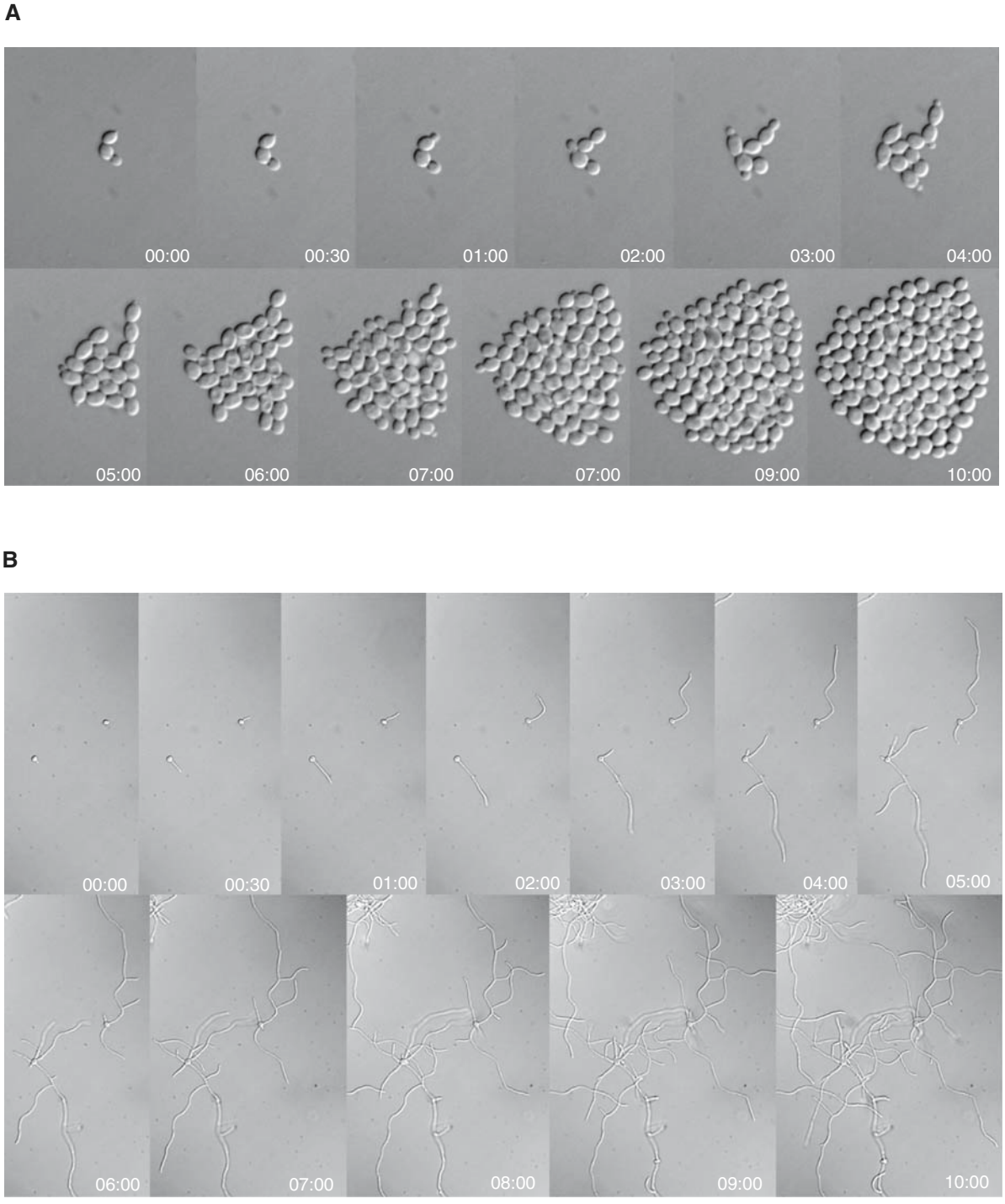

Fig. 6.2. **A** Time lapse recording of *Candida albicans* yeast growth. Images of cells grown at 30 °C on a microscope slide were acquired at the indicated time-points (*hours:minutes*). Cells proliferate by budding and separate from each other by cytokinesis **B** Time lapse analysis of *C. albicans* cells grown under hypha-inducing conditions. Images of cells grown at 37 °C on a microscope slide containing a medium supplemented with 10% serum were acquired at the indicated time-points. A *C. albicans* yeast cell is induced to form filaments that branch and form septa. After 10 h a mycelium was formed, in contrast to colonial growth of yeast cells

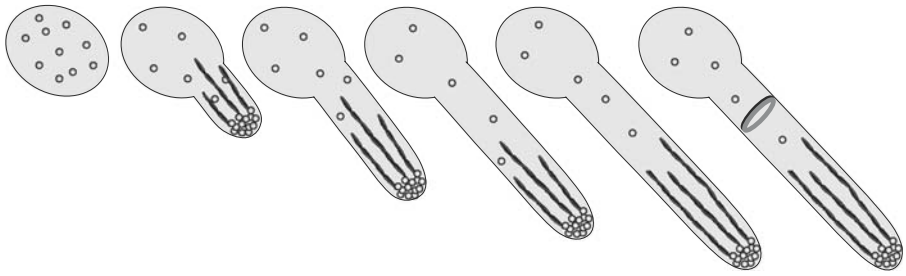

Fig. 6.3. Organization of the actin cytoskeleton during germ tube formation of *Candida albicans*. Similar events as during yeast like growth lead to the accumulation of cortical actin patches at the tip of the germ tube. At the hyphal tip actin cables are nucleated that serve as tracks for the delivery of secretory vesicles. In contrast to yeast cell growth, the organization of the actin cytoskeleton is constantly polarized in the tip during hyphal growth, thus uncoupling morphogenetic events from the cell cycle. The position of the actin ring marking the first septal site after germ tube formation is within the germ tube and not at the mother cell–germ tube neck in *C. albicans*

Furthermore, the first nuclear division after germ tube emergence takes place in the germ tube across the septal site, with subsequent transport of the mother nucleus back into the germ cell. This is different from nuclear division in *C. albicans* yeast cells, in which the mother nucleus does not leave the mother cell (Fig. 6.4). Nuclear migration required for this process is dependent on the dynein motor protein; and mycelium formation is blocked in *dyn1* mutants due to failure in the delivery of nuclei into the hyphal filaments and hyphal tips (Martin et al. 2004).

Filamentous ascomycetes such as *Asp. nidulans* and *Neurospora crassa* show a stainable structure at the hyphal apex that is termed the Spitzenkörper (Girbardt 1957; Harris et al. 2005; Harris 2006). *C. albicans* yeast cells do not generate a Spitzenkörper. However, recent evidence using *C. albicans* filaments stained by the lipophilic dye FM4-64 show Spitzenkörper at the hyphal tips (Crampin et al. 2005; Martin et al. 2005) (Fig. 6.5). A distinction between the polarisome and the Spitzenkörper can be made, suggesting that the polarisome components are positioned as a cap close to or within the hyphal tip membrane, whereas the Spitzenkörper is localized as a ball structure just subapical of the tip. This indicates the different roles for both complexes: the polarisome is a complex that establishes cell polarity, whereas the Spitzenkörper is a vesicle supply center collecting secretory vesicles and recycling vesicles to be transported into the hyphal tip.

C. Different Colony Morphologies and Biofilm Formation

The colony morphology in *C. albicans* is dependent on the growth mode of the cells and differs based on the media used. Yeast cells grow in round, shiny, and smooth colonies, while filamentous forms show wrinkled colony morphologies with protruding filaments forming a corona of hyphae at the colony edges (Fig. 6.6). Other distinct morphological stages are elongated "pseudohyphal" cells as well as "opaque" cells generated from the yeast or "white" forms which are mating-competent stages in *C. albicans* (Miller and Johnson 2002; Sudbery et al. 2004). A particular solid medium, referred to as Staib agar, is generally used to distinguish between the closely related species *C. albicans* and *C. dubliniensis*. This distinction is based on the growth of *C. albicans* as smooth colonies on Staib agar, while *C. dubliniensis* colonies are rough on this medium, indicative of filament formation (Staib and Morschhäuser 1999; Al Mosaid et al. 2001).

Yeast and filamentous cell forms are not mutually exclusive during colonial growth. One peculiar form of growth which also includes both cell forms is three-dimensional growth on solid surfaces, termed biofilm. The ability of *C. albicans* to form biofilms on medical devices such as catheters and prosthetic devices causes severe problems, particularly due to resistance of these biofilms and their cells to most of the antifungals in use (d'Enfert 2006). Therefore, any undetected biofilm forma-

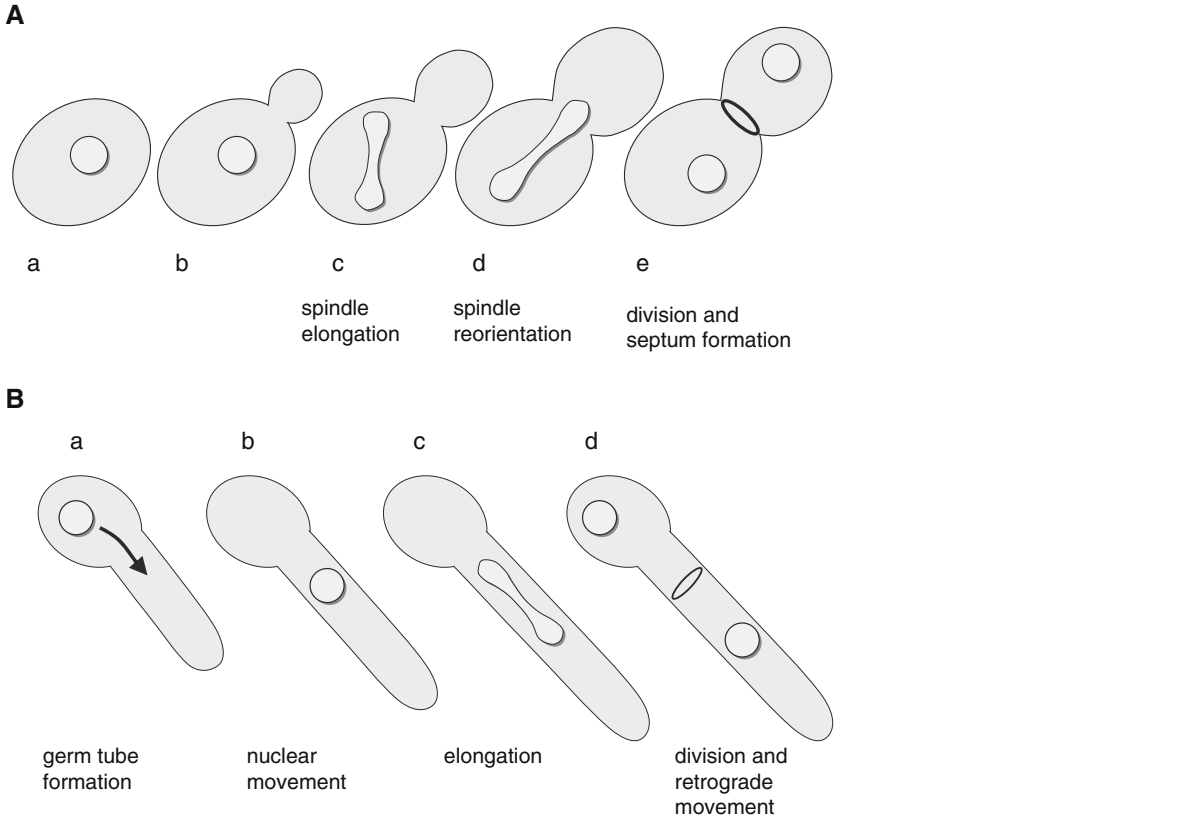

Fig. 6.4. Nuclear migration in *Candida albicans* yeast and hyphal stages. **A** Nuclear dynamics during the cell cycle of a yeast cell (*a*–*e*) include elongation of the spindle (*c*), realignment of the elongated spindle with the mother–bud axis (*d*), and migration of the daughter nucleus into the bud which is followed by septum formation and cell separation (*e*). **B** During germ tube formation (*a*) the mother nucleus moves into the germ tube (*b*) where the spindle elongates (*c*) and divides across a predefined septal site (*d*). This is followed by nuclear migration of the mother nucleus back into the germ cell

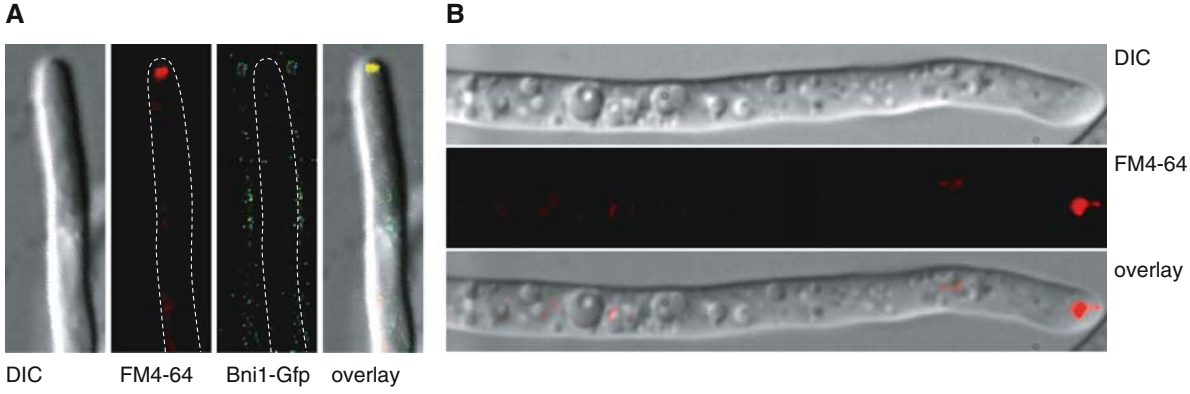

Fig. 6.5. Filamentous fungi possess a Spitzenkörper at the hyphal tip. **A** In *Candida albicans* hyphae a Spitzenkörper can be stained by FM4-64. The forming Bni1 co-localizes with this structure. **B** In the filamentous ascomycete *Ashbya gossypii* FM4-64 stains a similar structure at the hyphal apex. Since FM4-64 is used to visualize endocytic vesicles, the Spitzenkörper therefore contains apparently both endocytic and exocytotic vesicles

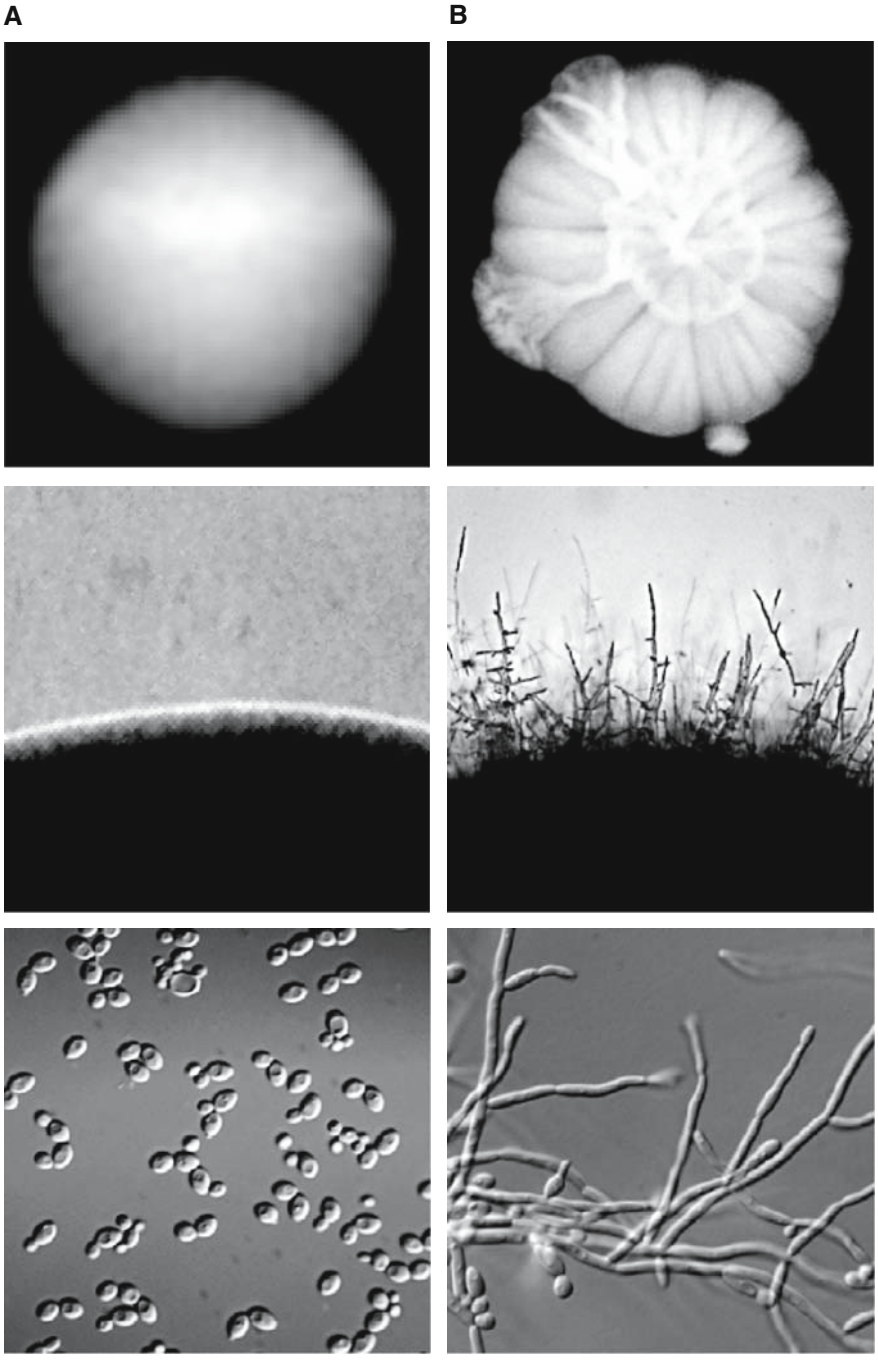

Fig. 6.6. Colony morphology in *Candida albicans*. **A** *C. albicans* cells grown at 30 °C form smooth, shiny colonies (*upper panel*). The colony edge shows a rim of yeast cells (*middle panel*). Microscopic observation of a colony sample shows only yeast cells (*lower panel*). **B** *C. albicans* cells grown at 37 °C in the presence of serum form wrinkled colonies (*upper panel*). The colony edge of such a colony shows a corona of hyphae protruding into the surrounding medium (*middle panel*). Microscopy of a sample of such a colony shows abundant hyphal filaments and also yeast cells (*lower panel*)

tion on such medical devices present a threat of severe systemic infection of patient. Biofilms are not only composed of different cell types, but these cell types are embedded in an extracellular polymeric matrix. The generation of a biofilm (which may vary in size between 25 µm and 500 µm) may

undergo the successive steps of attachment of yeast cells to a surface, initial growth that generates a yeast colony which at in the next stage also includes hyphae (Ramage et al. 2005; Nobile and Mitchell 2006). And, finally after about 24–48 h, an established biofilm can be generated, embedded in its matrix and relatively shielded from external assaults such as drug treatments (Kumamoto and Vinces 2005). The genetics of biofilm formation include several of the components required for the yeast-to-hyphal switch which will be described below. In particular, mutants defective in hyphal morphogenesis generate only poor biofilms, consisting, e.g. only of few yeast cell layers (Ramage et al. 2002). The sensing of cell–cell contacts seems to be important for biofilm formation. The conserved cell wall integrity MAP kinase cascade serves as a signal transduction cascade for biofilm formation. Upon biofilm formation the respective MAP kinase cascade Mkc1 (a homolog of the *S. cerevisiae* Mpk1/Slt2) is activated and, conversely, deletion of *MKC1* leads to mutant cells that are defective in biofilm formation (Kumamoto 2005). Another feature that was revealed using expression profiling is the upregulated expression of genes involved in amino acid biosynthesis. Deletion of a key regulatory gene, *GCN4*, led to a reduction in biofilm biomass production (Garcia-Sanchez et al. 2004). Transcriptional regulation of biofilm formation relies on the transcription factor genes *TEC1*, which is involved in hyphal formation, and *BCR1* (biofilm and cell wall regulator 1), which regulates cell adhesion properties, e.g. via *ALS3* (Nobile and Mitchell 2005; Nobile et al. 2006).

III. Signal Transduction Pathways Leading to Hyphal Growth

In this chapter we discuss the molecular biology of events that ultimately lead to hyphal growth in *C. albicans*. Previously, we noted that intrinsic cues may lead to cell polarization in *S. cerevisiae* and *C. albicans*, specifically any delays in the cell cycle. However, hyphal growth in *C. albicans* relies on a variety of extracellular signals that trigger signal transduction pathways leading to filamentation and biofilm formation (Brown and Gow 1999). While a lot of progress has been made in recent years to understand the signaling network required for hyphal growth promotion, we are still only at the beginning of understanding how these signals are perceived and how *C. albicans* manages to generate a specific response adapted to the environmental stresses or topologies.

A. Extracellular Signals that Promote Morphogenetic Events in *C. albicans*

C. albicans has quite a variety of host niches that force it to adapt to these locations, most likely through the regulated expression of a set of niche-specific genes. For example, different entry pathways into the human body present different environments and also different other microorganisms with which *C. albicans* has to cope. In the laboratory environment different cues are used with which *C. albicans* can be induced to form filaments. While yeast growth is generally observed at low temperatures (~30 °C) hyphal growth is most often triggered at elevated temperatures (>34 °C, usually 37 °C). Temperature as such is not sufficient to induce hyphal morphogenesis but at least another condition needs to be favorable. Among the most potent inducers is serum. However, the relevant component in serum that acts as the inducing substance has not been identified. Recently, it was suggested that glucose is one of two active substances (Hudson et al. 2004). Glucose sensing via the Hgt4 protein (which is an ortholog of the *S. cerevisiae* Rgt2 and Snf3 sensors) is important for growth decisions. A *hgt4* mutant strain is slightly attenuated in virulence and less filamentous than the wild type, whereas a constitutive signaling mutant is hyperfilamenting (Brown et al. 2006). Solid media used for monitoring hyphal growth are Spider medium and Lee's medium (Lee et al. 1975; Liu et al. 1994). Spider medium contains mannitol as a carbon source while Lee's medium uses amino acids as inducing substances (e.g. proline or arginine). Interestingly, both media contain large amounts of potassium. Potassium (and also proline) can act as a compatible solute and may be accumulated upon osmotic shock (Kempf and Bremer 1998). Furthermore, a connection between hyphal morphogenesis and intracellular potassium concentration was suggested in a study which showed that valinomycin and miconazole promoted potassium leakage from the cells and inhibited hyphal growth (Watanabe et al. 2006). Another carbon source, *n*-acetyl glucosamine, was also shown to be a filament-inducing substance (Mattia et al. 1982).

Filamentation of *C. albicans* cells was shown to be inhibited at low pH values, e.g. pH4, whereas

at alkaline pH hyphal formation can occur. Such behavior may be critically linked with the ability to cause infections (Davis et al. 2000a). Indeed it was shown that mutants in the pH-responsive gene PHR1, which is expressed at pH >5.5 are avirulent in a systemic infection model (the pH of blood is neutral to alkaline) but readily caused vaginal infections (acidic environment). Conversely, mutants in *PHR2* which is expressed at acidic pH were virulent in systemic infections but avirulent in vaginal infection assays (de Bernardis et al. 1998). This indicates that *C. albicans* is able to respond to pH (and also to changes in pH) with a morphogenetic response that may enhance its virulence (see below).

Two other hyphal inducing conditions have been described that trigger filamentation in *C. albicans* cells, namely growth under embedded conditions and growth in a CO_2-rich atmosphere. *C. albicans* cells embedded in an agar matrix rapidly induce filamentation at the even lower temperature of 25 °C (Brown et al. 1999). Hyphal growth induction under embedded conditions was, however, not dependent on the matrix itself nor on the media composition. These embedded conditions may, in part, resemble conditions *C. albicans* cells find themselves in during the generation of a biofilm. A zinc-finger transcription factor, encoded by *CZF1* (*Candida* zinc finger protein 1), was isolated in a forward genetic screen monitoring-enhanced filamentation under embedded conditions (Brown et al. 1999). Czf1 appears to function rather specifically as a transcription factor inducing morphogenesis under these special conditions but is not sufficient to trigger filamentation in standard solid- or liquid-phase assays.

Signaling via CO_2 came into the limelight of research efforts due to the inadvertent discovery that *C. albicans* (but none of the other *Candida* species tested) was able to filament under an atmosphere containing 6% CO_2 at 37 °C (Sheth et al. 2005). This may be very useful for the routine clinical determination of a pathogen in order to give early directions for a specific treatment.

These experiments reveal that *C. albicans* is able to integrate a variety of extracellular signals resulting into filamentation. This versatility may be key to survival in the different host niches and to the success of *C. albicans* as a pathogen. However, there are also mechanisms that inhibit filamentation in *C. albicans*. Phospholipase D, which hydrolyses membrane phospholipids, has been implicated in various processes such as endocytosis and motility in eukaryotic cells. Butan-1-ol has been shown to be a specific inhibitor of phospholoipase D in *Dictyostelium discoideum* resulting in strong defects in the organization of the actin cytoskeleton (Zouwail et al. 2005). In *C. albicans* propanolol inhibits germ tube formation without affecting yeast growth; and cells lacking phospholipase D, via deletion of *PLD1*, are more sensitive to propanolol (McLain and Dolan 1997; Baker et al. 2002).

Interestingly, *C. albicans* itself produces a substance that regulates morphogenesis by inhibiting germ tube emergence at high cell densities. This quorum sensing is brought about by the production and secretion of farnesol (Hornby et al. 2001). Farnesol also inhibits the formation of a biofilm in a dose-dependent manner (Ramage et al. 2002). Another quorum-sensing molecule in *C. albicans* is tyrosol. Tyrosol allows the shortening of the lag phase of cells when inoculated at lower densities into fresh medium. Furthermore, under conditions that induce filament formation, tyrosol accelerates the morphogenetic switch in low-density cultures without being itself an inducer of polarized morphogenesis (Chen et al. 2004).

B. The Cyclic AMP Pathway

The conserved cAMP pathway plays a major role in hyphal morphogenesis in *C. albicans*. The pathway integrates most of the extracellular inducing signals via the central Ras-GTPase Ras1; for example, nitrogen starvation triggers its activation via the ammonium transporter Mep2 (Biswas and Morschhäuser 2005). Activation of Ras1 by its guanine nucleotide exchange factors (Cdc25/Scd25) triggers activation of the adenylate cyclase Cdc35 (Lengeler et al. 2000). This is achieved by direct interaction of Ras1 with Cdc35 via the Ras association domain of Cdc35. Deletion of this domain abolishes this interaction and results in defective hyphal morphogenesis as does deletion of either the *RAS1* or *CDC35* genes (Feng et al. 1999; Rocha et al. 2001; Fang and Wang 2006). Adenylate cyclase catalyzes the production of cAMP. Cyclic AMP activates the protein kinase A. Protein kinase A consists of regulatory and catalytic subunits, encoded by *BCY1* and *TPK1/TPK2*, respectively (Sonneborn et al. 2000; Bockmühl et al. 2001). The catalytic subunits of PKA are activated by dissociation from Bcy1 which is triggered by cAMP-

binding to Bcy1 (Lengeler et al. 2000). Deletion of *BCY1* in *Candida* could not be achieved, suggesting that constitutive signaling via this pathway is lethal (Cassola et al. 2004). Conversely, overexpression of the Tpk2 catalytic subunit leads to hyphal development under non-inducing conditions, demonstrating a positive role of PKA in filamentation (Sonneborn et al. 2000). In contrast, deletion of the phosphodiesterase *PDE2* results in abnormal hyphal development, elevated cAMP levels, and a reduced *EFG1* transcription (Jung and Stateva 2003). *EFG1* is the major transcriptional regulator required for hyphal morphogenesis in *C. albicans*. Overexpression of *EFG1* (enhancer of filamentous growth 1) results in enhanced pseudohyphal/hyphal growth (Stoldt et al. 1997). Expression of *EFG1* also has phenotypic consequences on white-opaque switching. The "white" cells are the standard yeast form, whereas "opaque" cells are elongated (Slutsky et al. 1987). Cells in the opaque phase are the mating-competent form of *C. albicans* (Miller and Johnson 2002). *EFG1* expression occurs in white cells but not in opaque cells and, if induced in opaque cells, promotes switching to the white phase (Sonneborn et al. 1999; Srikantha et al. 2000). Conversely, a recently identified regulator of white–opaque switching, Wor1, is highly expressed in opaque cells, but not expressed in white cells. Deletion of *WOR1* blocks opaque cell formation and ectopic expression of *WOR1* induces opaque cell formation (Huang et al. 2006; Zordan et al. 2006). Evidence that Efg1 is a downstream target of PKA was indicated by the identification of a putative PKA phosphorylation site at position T206. Mutations of T206, T206A, and T206E led to a block of hyphal formation or caused hyperfilamentation, respectively (Bockmühl and Ernst 2001). Interestingly, Czf1 (see above) is required to mediate relief of Efg1-mediated repression of genes to allow invasive hyphal growth at low temperatures under embedded conditions while expression of *CZF1* itself requires Efg1 (Giusani et al. 2002; Vinces et al. 2006). Efg1 is a transcriptional regulator that binds to the promoters of regulated genes (E-box = 5 – CAnnTG–3 ; Leng et al. 2001). Efg1 participates in a negative feedback loop in which Efg1 activated via PKA downregulates *EFG1*-transcription (Tebarth et al. 2003). In fact, Efg1 was shown to be a repressor while a related protein Efh1, belonging to the same class of bHLH-APSES-domain containing proteins, was shown to be an activator that modulates the function of Efg1 (Doedt et al. 2004). However, *EFH1*, e.g. via overexpression, cannot suppress the block in filamentation in *efg1* mutants. One of the Efg1-regulated genes is Hgc1 (the hyphal-specific cyclin; see above). Efg1 controls hyphal specific genes together with another transcriptional regulator, encoded by *FLO8*. Consistent with this Efg1 and Flo8 were shown to interact with each other (Cao et al. 2006).

C. The Pheromone Response MAP-Kinase Cascade

Detailed knowledge of the *S. cerevisiae* pheromone response MAP-kinase cascade helped in shaping the understanding of the homologous pathway in *C. albicans* and may have been one reason that attracted researchers to start their studies with *C. albicans*. In the classic screen for non-mating mutants done by Vivian MacKay and Thomas Manney (1974a, b) sterile mutants of yeast were isolated. A conserved MAP-kinase cascade is used to relay the pheromone response to a transcription factor encoded by *STE12* in *S. cerevisiae* (Lengeler et al. 2000). The cascade is conserved in *C. albicans*, although the components have not all been analyzed to date (e.g. the *STE11* homolog; Fig. 6.7). The discovery of mating-type loci in *C. albicans* and the demonstration of mating in suitable strains triggered the analysis of the involvement of the pheromone response MAP-kinase cascade in *Candida* mating (Hull and Johnson 1999; Hull et al. 2000; Magee and Magee 2000; Johnson 2003). Disruptions in the *C. albicans STE7* and *STE12* homologs, encoded by *HST7* and *CPH1*, respectively, blocked mating in both cell types while mutations in the *STE20* and *FUS3/KSS1* homologs, encoded by *CST20* and *CEK1*, respectively, reduced mating efficiency; and in case of the *CEK1*/*CEK2* double deletion abolished mating (Chen et al. 2002; Magee et al. 2002). Deletion of *CPH1* suppressed hyphal morphogenesis, at least under some conditions on solid medium, but allowed filamentation in liquid culture and upon serum induction (Liu et al. 1994). The *efg1*/*cph1* double mutant of *C. albicans* is defective in filamentous growth even when stimulated via serum, and, furthermore, this mutant strain is avirulent in a mouse model (Lo et al. 1997). Activation of Cek1 through the MAP-kinase cascade results in activation of downstream effectors, e.g. Cph1, via phosphorylation. To terminate such activation, dephosphorylation via a protein phosphatase may

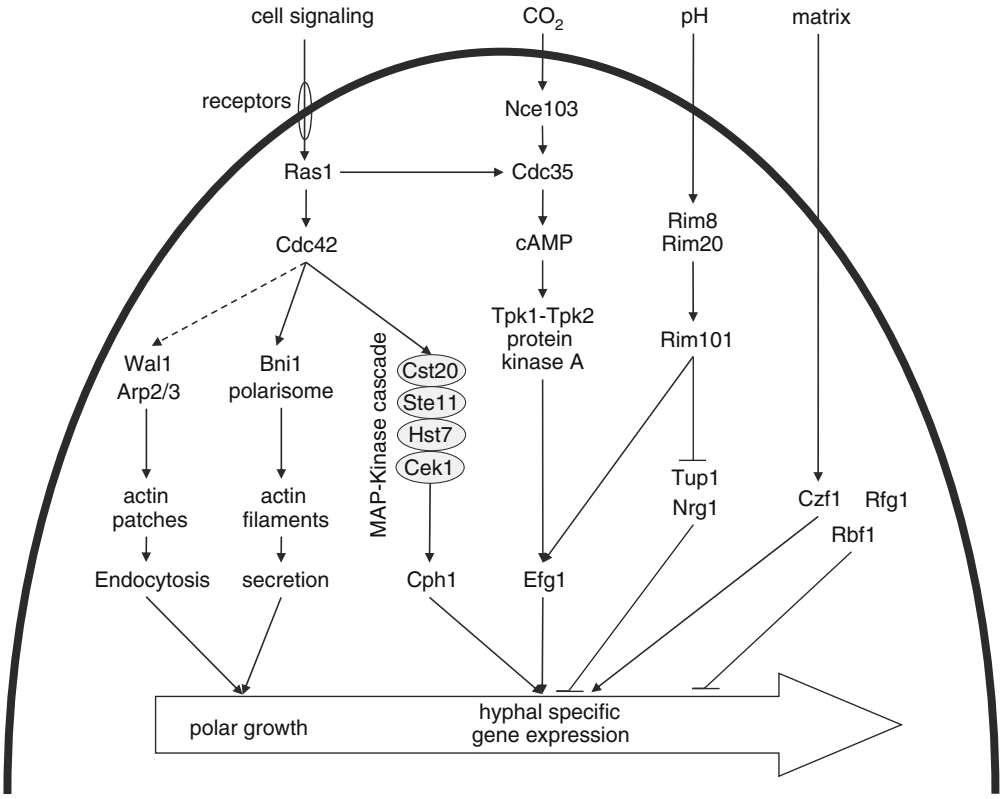

Fig. 6.7. Signal transduction pathways involved in regulating morphogenesis in *Candida albicans*. Extracellular signals serve as input to activate various pathways. Signalling via membrane receptors may activate the Ras1 GTPase which activates, e.g. the MAP kinase cascade and the cAMP pathway that signal to the transcription factors Cph1 and Efg1, respectively. Positive and negative regulation allows the control of hyphal specific gene expression. Reorganization of the actin cytoskeleton is required to promote polarized growth

be used. In *C. albicans* the Cpp1 phosphatase was found to act as a MAP-kinases phosphatase. Deletion of *CPP1* in *C. albicans* results in a hyper-filamentous phenotype at room temperature. This phenotype can be suppressed by concomitant deletion of *CEK1*. Thus Cpp1 acts as a negative regulator of Cek1-induced morphogenesis (Csank et al. 1997; Schröppel et al. 2000). In *S. cerevisiae* Ste5p acts as a scaffold of the MAP-kinases of the kinase cascade. *C. albicans*, however, seems to lack a *STE5* homolog which may allow a differential regulation of the cascade as compared with yeast.

D. The Role of CO_2 and the Rim101 Pathway

Physiological levels of CO_2 in the host environment are about 5%. This level is sufficient to trigger filamentation in *C. albicans* and capsule formation in *Cryptococcus neoformans* (Bahn et al. 2005; Klengel et al. 2005; Mitchell 2005; Mogensen et al. 2006; McFadden et al. 2006). Both of these processes are dependent on the activity of adenylate cyclase, and thus the cAMP pathway. In the cells, the conversion of CO_2 to bicarbonate (HCO_3^-) is catalyzed by carbonic anhydrases. The action of carbonic anhydrase is essential for *C. albicans* virulence in host niches with limited CO_2 levels (Klengel et al. 2005). The catalytic domain of adenylate cyclase is sufficient to mediate the response to physiological levels of CO_2 and result in filamentation (Mogensen et al. 2006). This is further supported by studies of Fang and Wang (2006) who report evidence that the Ras1-adenlyate cyclase interaction domain (RA) of adanylate cyclase (and thus interaction of Ras1 with Cdc35) is not required for CO_2-induced filamentation.

Alkaline pH levels were found to induce hyphal growth by activating the Rim101 pathway (Davis et al. 2000a; El Barkani et al. 2000). Full-length Rim101 is processed under alkaline pH conditions.

C-Terminal cleavage and thus the activation of Rim101 is done by Rim8/Rim20 and other factors including Rim13. In its activated state Rim101 induced the expression of a set of downstream target genes (Davis et al. 2000a; Li et al. 2004). Dominant active alleles of *RIM101* that resulted in the C-terminal truncation of the open reading frame bypassed the pH requirement for filamentation and allowed hyphal morphogenesis at acidic pH. Additionally overexpression of *RIM101* was shown to alleviate the temperature requirement for filamentation and allowed filamentation at 29 °C. The Rim101 response is dependent on the Efg1 transcriptional regulator (El Barkani et al. 2000). Deletion of *RIM101* leads to attenuated virulence in the mouse model of hematogenously disseminated systemic candidiasis and results in strains that generate decreased damage to endothelial tissues (Davis et al. 2000b). Regulation of gene expression in response to ambient pH is a general feature of microorganisms and has been studied in other systems, most notably *Asp. nidulans* (Davis 2003). Recent evidence suggested that although the Rim101 pathway is conserved, regulation of target gene expression has diverged between *Asp. nidulans* and *C. albicans*. This has been shown by the analysis of target-binding sequences of the Rim101 transcription factor, which corresponds to 5′–GCCARG–3′ in *Asp. nidulans* but to 5′–CCAAGAAA–3′ in *C. albicans* (Ramon and Fonzi 2003). A link between the Rim101 pathway and endocytosis was established by showing that mutations in ESCRT (endosomal sorting complexes required for transport) components are needed for signaling through Rim101 (Cornet et al. 2005).

E. Organization of the Actin Cytoskeleton

Polarized cell growth is a basic cellular feature and is required for unicellular yeast growth as well as for highly elongated filamentous growth. As discussed above, the Cdc42 cell polarity-establishment protein plays a central role downstream of Ras1 to promote polarized growth. Recent evidence has shown that, next to polarized delivery of secretory vesicles to the Spitzenkörper and hyphal tip, endocytosis is also required for hyphal morphogenesis in *C. albicans* (Walther and Wendland 2004; Wendland and Walther 2005). Deletion of the *C. albicans* homolog, *WAL1*, of the human Wiskott–Aldrich syndrome protein (WASP) resulted in delayed endocytosis, vacuolar fragmentation, and abolished mycelium formation. Endocytosis may be followed microscopically via the presence of cortical actin patches which are clustered into the growing hyphal tip surrounding the Spitzenkörper. Wal1 is a multi-domain protein containing several proline-rich regions that allow for complex formation, e.g. with SH3 domain-containing proteins. Several proteins are involved in actin patch assembly and function. One of them, CaMyo5, was also found to be required for polarized morphogenesis. Interestingly, a constitutive active form of Myo5, Myo5^{S366D}, allows for polarized hyphal growth even in the absence of polarized positioning of cortical actin patches (Oberholzer et al. 2002, 2004). This phosphorylation site may be recognized by Ste20/Cla4-protein kinases, as was shown for *S. cerevisiae* (Wu et al. 1997). Similarity in mutant phenotypes between myo5, wal1, and cla4 suggest a conserved link between exocytosis and endocytosis regultated via Cdc42 and its interaction with Cla4 in ascomycetous fungi (Leberer et al. 1997; Walther and Wendland 2004).

Other actin patch components and the endocytotic pathway need to be analyzed in *C. albicans* to clarify to route of membrane transport and membrane sorting and their link to sustained polarized hyphal growth and virulence.

F. Repression of Filamentation in *C. albicans*

As with other things 'what goes up must come down' is also valid for filamentation in *C. albicans*. Therefore, several mechanisms are active in *C. albicans* to repress filamentation. Intriguingly *C. albicans* produces quorum-sensing molecules that allow for the control of morphogenesis on a colony level. One of them, farnesol, is excreted by yeast cells and prevents the yeast-to-mycelium transition at micromolar concentrations produced at high cell densities (Hornby et al. 2001). Because of this action, farnesol also inhibits the formation of elaborate biofilms. Farnesol reduces the expression of *HWP1* which encodes a hyphae-specific cell wall protein but induces, for example, the G1-cyclin *CCN1*, and the chitinases *CHT2* and *CHT3* (Ramage et al. 2002; Cao et al. 2005). Furthermore, farnesol was suggested to suppress the MAP-kinase cascade by decreasing the expression of *HST7* and *CPH1* (Sato et al. 2004). Other evidence indicated that a two-component pathway involving Chk1 is required for the transduction of the farnesol signal (Kruppa et al. 2004).

C. albicans also produces another quorum-sensing molecule, tyrosol, which reduces the lag phase after dilution into fresh medium but also promotes hyphal morphogenesis under inducing conditions (Chen et al. 2004).

As recent results indicated that Efg1, an enhance of filamentous growth acts rather like a suppressor it is of importance to more clearly define the regulatory circuits which are involved in fine-tuning morphogenetic decisions in *C. albicans*. Other regulators of hyphae-specific gene expression were identified in the *TUP1*, *NRG1*, *RBF1*, *and RFG1* genes. Deletion of *TUP1* results in constitutive hyphal growth (Braun and Johnson 1997). Using subtractive hybridization and DNA-array technology the set of genes repressed by Tup1 were identified. Among them are GPI-anchored proteins, e.g. *HWP1*, *RBT1*, *RBT5*, and *WAP1*, as well as a cell-surface iron reductase, encoded by *FRE10*/*RBT2* (Braun et al. 2000; Kadosh and Johnson 2005). Kadosh and Johnson (2005) also showed via DNA-arrays that a large set of genes are simultaneously controlled by Tup1, Nrg1, and Rfg1, indicating that relief of this repression plays a central role in filamentation. The function of the zinc-finger transcription factor Nrg1 as a repressor, in contrast, may require the presence of Tup1 and Ssn6, two proteins that in *S. cerevisiae* form a co-repressor dimer (Garcia-Sanchez et al. 2005). As with *tup1* cells, *nrg1* cells are filamentous under non-inducing conditions. Furthermore induction of hyphal growth results in the downregulation of *NRG1* expression (Braun et al. 2001; Murad et al. 2001). Rfg1 (repressor of filamentous growth 1) in *C. albicans* is the homolog of the *S. cerevisiae* Rox1. Rox1 in yeast recruits, again, the Tup1/Ssn6 repressor complex, to achieve repression of a specific gene set. In *C. albicans* deletion of *RFG1* resulted in filamentous growth, similar to the deletion of *NRG1* or *TUP1* (Khalaf and Zitomer 2001).

Another transcription factor, Rbf1 which shows telomere-binding activity in *C. albicans* is also somehow involved in the regulation of hyphal-specific gene expression (Ishii et al. 1997).

IV. Role of Hyphal Growth as a Virulence Factor

Virulence is defined as the ability of a microorganism to cause disease. Virulence describes the degree of pathogenicity indicating that there are strains that are more or less virulent than others. Pathogenicity itself is the mere ability to cause disease. Virulence is often determined by multiple factors. Specific host factors involved in *C. albicans* virulence are, for example, a compromised immune system. Several virulence factors of *C. albicans* have been noted, e.g. the expression of secreted aspartyl proteases, lipases, agglutinin-like sequence genes of the *ALS*-gene family, and, of course, the yeast-to-hyphal switch accompanied by the expression of a large number of hyphae-specific cell surface proteins (Berman and Sudbery 2002). Hyphal growth per se has been described as a virulence factor, due to the demonstration that non-filamentous *C. albicans* mutants were avirulent (Lo et al. 1997). This does not indicate, however, that constitutively filamentous mutants were hypervirulent. Rather, for full virulence both growth forms (and maybe even other morphologies such as white–opaque switching) are required. This can be understood in light of the different host niches that can be occupied by *C. albicans*: adhesion to tissue can be promoted via hyphal growth whereas dissemination in the blood stream can be accomplished by yeast cells.

In this last section we discuss changes when the expression level during the yeast-to-hyphal switch and the role of hyphal growth during the different stages of colonization of a host niche involving adhesion to a substrate and the penetration of epithelia. At other stages engulfment by macrophages can trigger hyphal growth resulting in the evasion of the immune response.

A. Differential Gene Expression During the Yeast-to-Hyphal Switch

Using DNA-microarrays differentially expressed genes during the induction of hyphal growth were analyzed (Lane et al. 2001; Nantel et al. 2002; Sohn et al. 2003). This indicated that multiple pathways regulated the same gene set allowing for cross-talk between different signaling pathways and concerted activation of target genes. Nrg1 was found to repress hyphae-specific genes (Murad et al. 2001). Efg1 was found to be the major regulator of cell wall genes acting either as an inducer or repressor or gene expression (Sohn et al. 2003). Although Efg1 is a target of the Ras1 signaling pathway, transcript profiles showed that there is a ras-independent gene set controlled by adenylate cyclase, which is consistent, for example, with the

ras-independent induction of adenylate cyclase via CO_2 (Harcus et al. 2004). Analyses on other transcription factors revealed specific functions for Cph2, Tec1, and Flo8 for hyphae-specific gene expression (Schweizer et al. 2000; Lane et al. 2001; Cao et al. 2006). Deletion of *FLO8* blocked hyphal morphogenesis and the mutant strain was shown to be avirulent. Flo8 interacts with Efg1 and controls a subset of Efg1-regulated genes (Cao et al. 2006). Cph2 is necessary for transcriptional induction of *TEC1*, which is expressed in the hyphal form. *TEC1* overexpression can partially suppress the *efg1* defects in filamentation, indicating that Tec1 can be placed downstream of Efg1 (Schweizer et al. 2000; Lane et al. 2001).

B. Role of Adhesion in Virulence of *C. albicans*

C. albicans has a sense of touch which makes hyphae grow along grooves on a substrate (Gow 1997). Contact formation in itself could trigger hyphal morphogenesis which has been described as filamentation under embedded conditions (Brown et al. 1999). Interestingly, even the *efg1*/*cph1* mutant, which is non-filamentous under most of the hyphal inducing conditions, can form filaments under these conditions. The *INT1* gene has been described providing a linkage between adhesion, filamentation, and virulence (Gale et al. 1998). Int1 has some similarity to vertebrate integrins and the yeast Bud4. The hyphae-specific hyphal wall protein, encoded by *HWP1*, plays a role in promoting adhesion to epithelial cells. Deletion of *HWP1* leads to the inability to form stable attachments to epithelial cells and also to reduced virulence (Staab et al. 1999). Filamentation was also found to be a pre-requisite of adhesion to porcine intestinal epithelium (Wendland et al. 2006; Fig. 6.8). A large family of cell surface glycoproteins, encoded by the *ALS* genes, plays an important role in the adhesion of *C. albicans* to epithelia (Hoyer et al. 2001). Als proteins resemble *S. cerevisiae* flocculins in that they can generate genetic variability based on internal repetitive sequence elements. *ALS* genes are differentially regulated and the variety of Als proteins may provide *C. albicans* with the ability to adhere to different substrates. The cell wall plays of course a critical role in this process. *C. albicans* encodes more than 100 cell surface GPI-anchored proteins. A functional GPI-anchoring machine was shown to be required in *C. albicans* for full virulence and hyphal formation (Richard et al. 2002). One of these GPI-anchored proteins, Eap1, is involved in epithelial adhesion. *EAP1* expression is regulated by Efg1, providing a link between hyphal morphogenesis and an altered cell wall structure (Li and Palecek 2003).

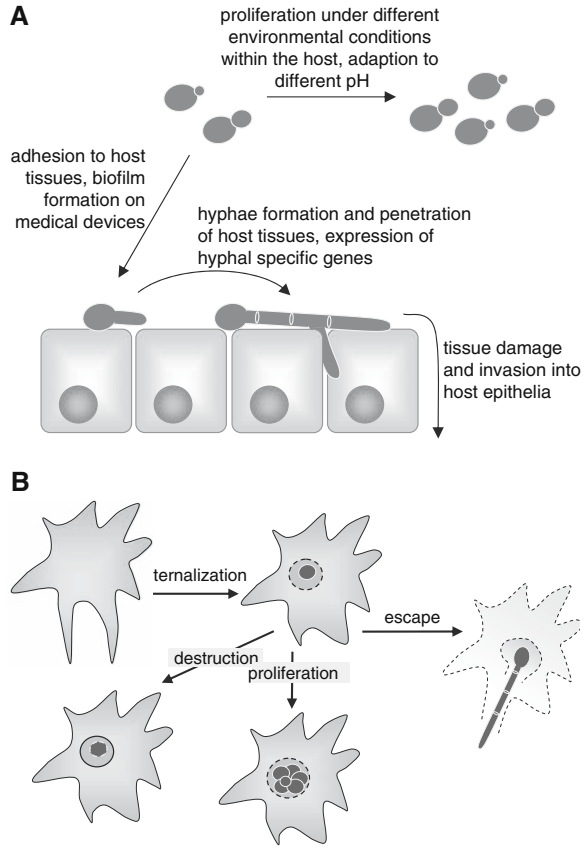

Fig. 6.8. Hyphal growth as a virulence factor in *Candida albicans*. **A** *C. albicans* yeast can adapt to different host environments, e.g. different pH values. Upon contact with host epithelia hyphal growth is initiated, resulting in adhesion to and colonization of the epithelial surface. On medical devices *C. albicans* may form biofilms. Colonization of epithelia and the expression of specific virulence factors fosters the penetration and destruction of host epithelial cell layers which leads to the invasion of host tissues and dissemination of *C. albicans* cells via the blood stream. **B** Upon contact of *C. albicans* yeast cells with the cellular immune system, the yeast cells may be internalized by macrophages. This may result in either destruction of the pathogen, proliferation of *C. albicans* within the macrophage, or germ tube formation of the yeast cells which finally results in the destruction of the macrophage and the escape of *C. albicans* cells from macrophage attack

C. Penetration of Tissues and Immune Evasion

To assay the virulence of *C. albicans* mutant strains, a mouse model of hematogenously disseminated candidiasis is generally used. This allows distinguishing between fully virulent strains, strains attenuated in virulence, and avirulent strains. However, injecting *C. albicans* blastospores directly into the bloodstream eliminates natural defence barriers of the host, since the early stages of colonization by *C. albicans* require adhesion to epithelia, colonization, and penetration of tissues.

Recently, reconstituted human tissues have been described in their use to monitor adhesion and invasion of *C. albicans* (Korting et al. 1998; Dieterich et al. 2002; Schaller et al. 2003). These were used for example to demonstrate that the secreted aspartyl proteases encoded by the *SAP1* and *SAP2* genes are involved in causing tissue damage in a reconstituted human vaginal epithelium (Schaller et al. 2003). The Efg1-regulated proteinases Sap4–Sap6 are required for invasive growth in tissues but may be dispensable for filament formation (Felk et al. 2002). The five isoforms of protein mannosyltransferases (Pmt1, Pmt2, Pmt4–Pmt6) were analyzed using reconstituted epithelia or in a muse model. No single deletion of a PMT gene conveyed a drastic phenotype, suggesting that the encoding proteins fulfil overlapping functions (Rouabhia et al. 2005). In a model of reconstructed intestinal epithelium a non-filamentous strain bearing deletions in *CPH1* and *EFG1* was shown to be unable to adhere or penetrate (Dieterich et al. 2002). This highlights the importance of a concerted activation of hyphal morphogenesis, cell wall restructuring, the co-expression of other virulence factors such as adhesins and lipases and proteases to overcome the host defense barriers and establish a successful infection site (Kumamoto and Vinces 2005).

An early study showed that neutrophils can mediate the protection of endothelial cells from damage by *C. albicans* hyphae by selectively killing the hyphae (Edwards et al. 1987). However, hyphal growth can not only generate the force to penetrate tissues but can also force the cells out after ingestion by a macrophage.

Normally when a phagocyte recognizes a *C. albicans* cell, it engulfs it into a phagosome which can be fused to lysosomes to charge the pathogen with the host defenses. *C. albicans* may overcome this hostile attack by switching to hyphal morphogenesis, destroying the macrophage from the inside (Fig. 6.8; Lorenz and Fink 2002).

The general ability to form hyphae and the readiness to trigger morphological changes accompanied by altering the transcriptional profile can turn *C. albicans* into such a powerful pathogen (Lorenz et al. 2004). The idea that *C. albicans* normally acts as a commensal, e.g. in our gastrointestinal tract (Rozell et al. 2006), is hard to reconcile with this behavior. Rather it seems that the counteractions taken by our constantly vigilant immune system fight off all *C. albicans* attacks, leaving little room for *C. albicans* to survive in the human host. Yet this little amount of room and the balance in the fight may be sufficient to remain in place long enough to wait for an opportunity to strike back. One way of hiding from the immune system is by masking the beta-glucan of the cell wall (which is a strong proinflammatory substance) with mannoproteins (Wheeler and Fink 2006). This may explain the synthesis of hyphae-specific wall proteins.

V. Conclusions

Hyphal morphogenesis in *C. albicans* is a very attractive biological system. Studies on the genetics, molecular biology, and the underlying signal transduction pathways have opened a view on how *C. albicans* manages to recognize and process a variety of diverse extracellular signals into an output that allows survival in different host environments. This is a particularly troublesome task, considering the host defenses that counteract such an invading pathogen. Thus the notion of *C. albicans* as an harmless commensal may only be based upon a strong host defense. *C. albicans* has a rewired set of conserved pathways, namely the MAP-kinase cascade homologous to the *S. cerevisiae* pheromone response/pseudohyphal growth pathway, the cAMP pathway and others, e.g. the pH regulatory pathway, to result in the concerted activation of virulence genes and the morphogenetic program. Due to the completed genome sequence and due to global transcript profiling many new genes, often genes without homologs in *S. cerevisiae* await further analysis. Thus the new challenges are to proceed with the functional analysis of the *C. albicans* genome and to generate a protein network that allows us to define

the core machinery that promotes its virulence. This will generate many new concepts of antifungal therapy that may not only prove useful in the defense against *C. albicans* but may generate novel broad-spectrum antimycotica.

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