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Chapter 3

Cell Cycle of Fungal Pathogens

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Abstract

The cell cycle coordinates morphogenesis so that mitosis and growth are coupled to the doubling of all cellular components. Recent work with certain fungal pathogens has revealed modifications of the basic cell cycle pattern as exhibited in model yeast species that tailors the needs of the pathogen to its growth in the host environment. Many fungal pathogens such as *Candida albicans* are dimorphic or pleomorphic, and so the cell cycle must be regulated to enable cell shape to be modulated while the nuclear cycle is maintained. Filamentous pathogens such as *Aspergillus fumigatus* maintain a nuclear cycle but uncouple cell division from the growth cycle. Therefore fungal pathogens provide interesting examples of how cell cycle events are both conserved and modified to facilitate replication in the context of invasion of human tissues.

1. Introduction

The cell cycle is the central clock against which morphogenetic events are ordered and timed. In the case of fungal pathogens, morphogenetic processes include the switching from growth by budding to filamentous growth forms that include both true hyphae and pseudohyphae, the formation of mating structures, and the

production of various asexual and sexual spores. The cell cycle can dictate when such changes in development can take place. In addition the cell cycle can be altered to enable new growth forms to be propagated. For example, cytokinesis, the process of division of the two daughter cells at the end of the cell cycle may be altered or inhibited to permit filamentous cell development in dimorphic fungal pathogens. This chapter discusses the cell cycle of fungal pathogens and is focused on the dimorphic fungus *Candida albicans*. Clearly the cell cycle is a highly developed branch of modern cell biology and many of the central control mechanisms are highly conserved in all eukaryotes. Indeed some of the key enzymes that regulate the cell cycle are so highly conserved that yeast gene products can functionally complement homologous mutations in human cell lines and *vice versa*. Model fungi, in which many of the basic features of the cell cycle have been studied, include the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* which have well developed genetics but are not primary pathogens of humans. Nonetheless, progress in understanding the cell cycle in pathogenic species is best described in the light of the insights obtained in these model species. However, recent work has begun to show that significant departures from the cell cycle programme of these yeasts can occur in *C. albicans* and other organisms, contributing to our understanding of general cell cycle processes as well as the regulation of fungal pathogenesis.

2. Cell Cycle Progression in Different Morphological Forms of *C. albicans*

2.1. Yeast Cells

In *C. albicans*, the yeast form (also known as the blastospore), is characterized by round to ellipsoid cells that become separated from the mother cell after cytokinesis. *C. albicans* yeast cell cycle progression appears to be very similar to the process in *S. cerevisiae* yeast cells (reviewed in (Lew *et al.*, 1997). As in *S. cerevisiae*, there are at least 4 major cell cycle-dependent processes or cycles that occur in a discrete order: 1) the budding cycle (directed localization and relocalization of the actin cytoskeleton and secretory pathway components); 2) DNA replication and the chromosome cycle; 3) the spindle pole body (SPB) and microtubule cycle; and 4) the assembly of a septin ring and subsequent septation at that ring. While these major processes have not been studied as extensively in *C. albicans*,

they appear to be linked to one another under most growth conditions, such that, as in *S. cerevisiae*, bud emergence, DNA replication initiation, and spindle pole body duplication appear to be coordinated and to occur within the same time frame that is considered to be the G1/S transition (Wain *et al.*, 1976; Hazan *et al.*, 2002).

The *C. albicans* yeast cell cycle (Figure 1) is characterized by growth (cell expansion) occurring primarily in the daughter cell (the bud). As in *S. cerevisiae*, the round to ellipsoid shape of the bud is likely the result of the distribution and reorganization of F-actin and actin-associated proteins that direct surface growth. In an unbudded cell, a septin ring (composed of Cdc3,10,11 and 12 septin proteins) appears at the incipient bud site prior to bud emergence (Figure 1) (Sudbery 2001, Warena and Konopka, 2002). Actin also becomes highly polarized at the incipient bud site (Anderson and Soll, 1986; Yokoyama *et al.*, 1990; Akashi *et al.*, 1994). The growing bud emerges through the septin ring and actin remains associated with the tips of small budded cells (polarized growth). Spindle pole body duplication and DNA replication initiation occur close to the time of bud emergence, suggesting that the budding, replication and spindle cycles are coordinated at START in *C. albicans* yeast cells as they are in *S. cerevisiae* (Barton and Gull, 1988; Hazan *et al.* 2002).

Spindle dynamics appear to be very similar to those in *S. cerevisiae* as well (Hazan *et al.*, 2002). In medium sized buds, DNA replication is most likely active and spindle pole body separation and the formation of short spindles is observed (Finley and Berman, unpublished data; Hazan *et al.*, 2002). In growing buds, the actin cables remain oriented toward the bud, but cell wall expansion occurs throughout the bud “isotropic growth”. Prior to mitosis, the nucleus, and the spindle associated with it, stretch through the neck between the mother and daughter cells. The nucleus oscillates back and forth across the neck until the spindle elongates and disassembles, leaving the separated nuclei in the mother and daughter cells (Finley and Berman, unpublished data).

In large buds there is a transient period when the actin structures become randomly distributed over the entire cell prior to their reorientation toward the mother-bud neck to promote the secretion and cell synthesis necessary for septation and cytokinesis. In *S. cerevisiae*, chitinase is recruited to the bud side of the neck and is important for executing the cell separation that is characteristic

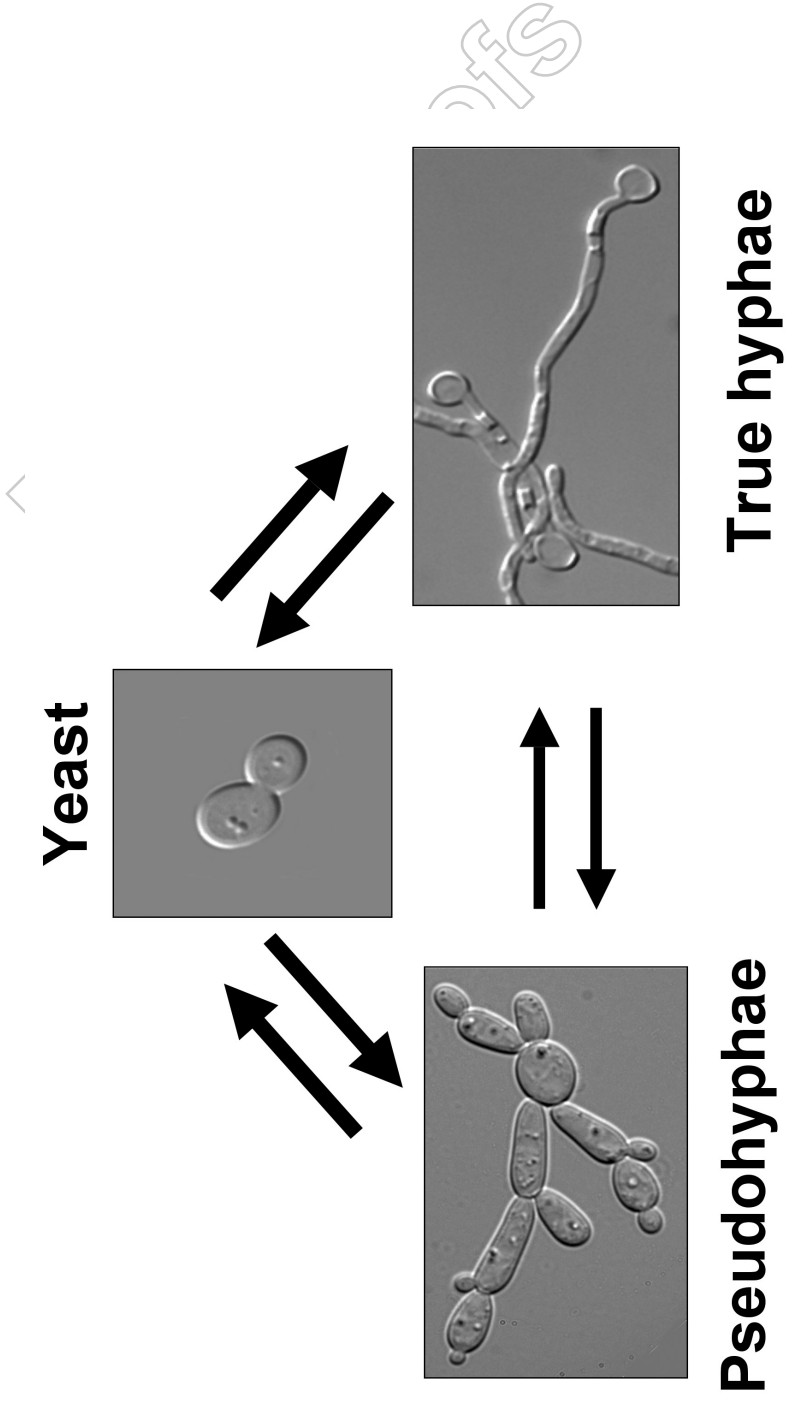


Figure 1. Morphology of the three morphologically distinct vegetative growth forms of *Candida albicans*. Each form is interconvertible and involves modification of cell cycle parameters.

of yeast cells (Kuranda and Robbins, 1991). *C. albicans* has four chitinase genes (McCreath *et al.*, 1995; 1996; Selvagini *et al.*, 2003) that are presumed to have a similar role. Following cytokinesis, the actin patches remain associated with the neck for a short while and then become randomly distributed or reorganize at the next incipient bud site. The septin rings disassemble after cytokinesis, prior to formation of the septin ring at the next incipient bud site (Gale *et al.*, 2001; Sudbery 2001; Warena and Konopka, 2002).

As in *S. cerevisiae*, the mother cells enter the next cell cycle before the daughter cells, presumably because mothers are larger than daughters and cells must reach a critical size before entering the next cell cycle (Chaffin, 1984a; Kron and Gow, 1995). Nonetheless, the difference in timing is sufficient to make it very difficult to achieve synchrony of a cell population for more than one cell cycle.

While the *C. albicans* yeast cell cycle shares many features with *S. cerevisiae* cell cycle progression, several differences should be noted. First, *C. albicans* yeast cells have a more rapid division cycle, dividing every ~60 min. in rich medium (*S. cerevisiae* cells generally divide with a ~90 min. doubling time). Second, *S. cerevisiae* yeast cells exhibit budding patterns that are primarily dependent upon genome ploidy (Chant and Pringle, 1995). In haploid *S. cerevisiae*, new buds usually emerge adjacent to the previous bud site (axial budding pattern); in diploid cells the first bud emerges at the opposite end of the cell from the previous bud site (bipolar budding pattern). *C. albicans* strains are generally diploid (for a review on the status of mating and the sexual cycle of *C. albicans* (Johnson, 2003; Soll *et al.*, 2003), yet within a population of wild-type cells, both axial and bipolar budding patterns are observed. Interestingly, the budding pattern of *C. albicans* yeast cells is influenced by temperature: at lower temperatures (~25°C), a higher proportion of cells are axial and at higher temperatures more cells in the population are bipolar (Chaffin, 1984b; Herrero *et al.*, 1999; Gale *et al.*, 2001).

2.2. Pseudohyphal Cells

C. albicans pseudohyphal cells have been less well characterized than yeast or hyphal cells. In part, this is because conditions that yield a population of all pseudohyphae are difficult to achieve. Nonetheless, several mutant strains that grow primarily or exclusively as

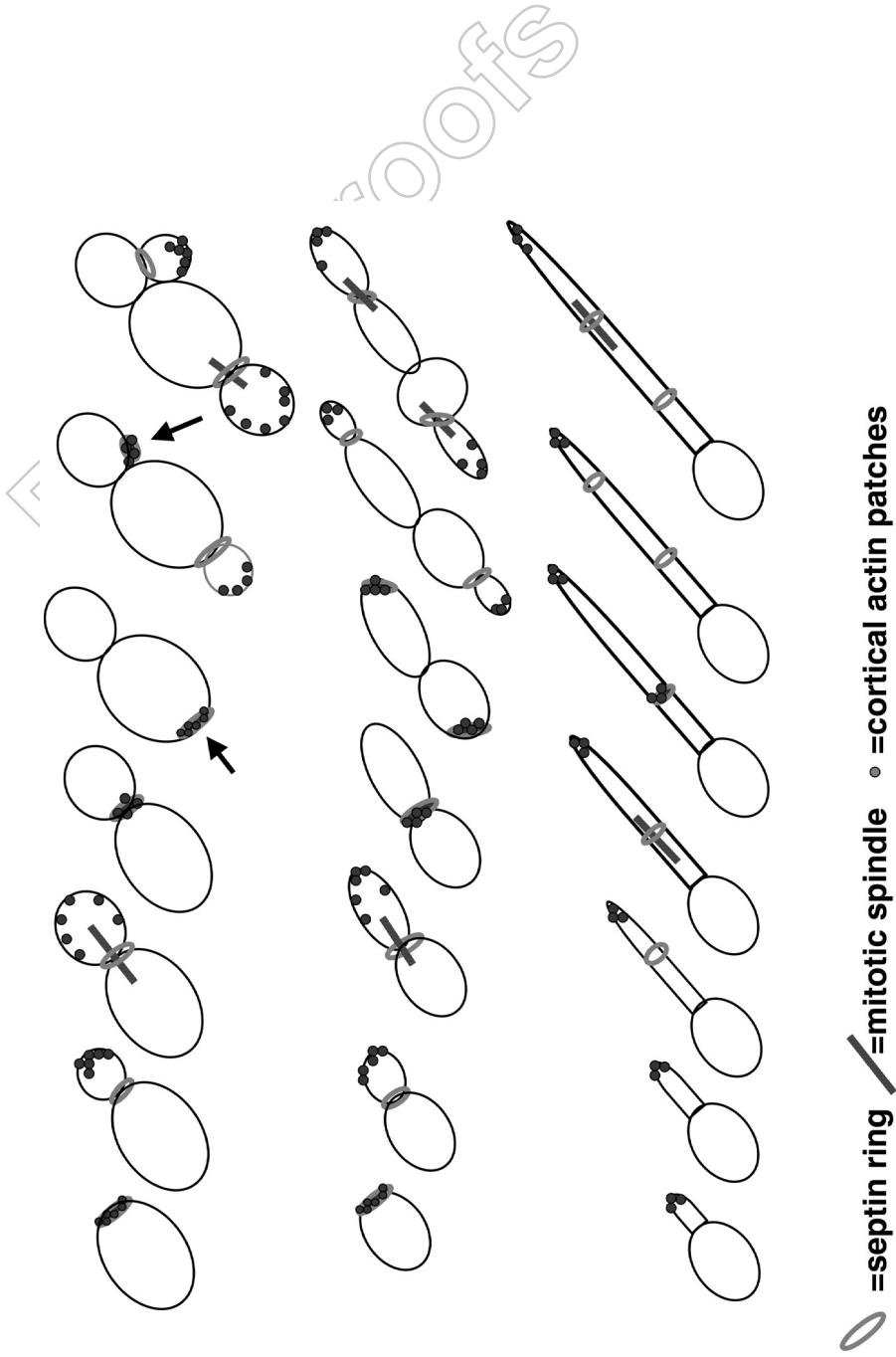


Figure 2. The cell cycle of yeast cells (top panel), pseudohyphae (middle panel) and true hyphae (bottom panel) showing changes in the distribution of actin patches septin rings and position of the mitotic spindle.

pseudohyphae have been described, suggesting that the pseudohyphal state may be a default state of *C. albicans* growth.

Pseudohyphal growth is characterized by elongated budded cells that remain attached to one another in a branching pattern (Figure 1). *C. albicans* pseudohyphae were long thought to be an intermediate state somewhere between yeast and true hyphae (Merson-Davies and Odds, 1989). This idea was reinforced by the fact that pseudohyphal cells can range in length from 5 to 15 microns. Pseudohyphae are generally >2.8 microns wide (Sudbery, 2001). In many cases, long *C. albicans* cells were termed 'filaments' and the distinction between pseudohyphae and true hyphae was not made. However, recent work has demonstrated that pseudohyphae are very different from true hyphae (Sudbery, 2001). There are significant differences between yeast and pseudohyphal cells, as well, although these are less well characterized (Sudbery *et al.*, 2003).

Septin ring placement and actin organization in pseudohyphae resembles that seen in yeast cells. As in yeast cells, septin rings appear prior to bud emergence and septation occurs at the mother bud neck. A constriction at the site of septation is evident in pseudohyphal cells (Figure 2), presumably because as buds emerge through the septin ring the region of growth expands beyond the constricted width proscribed by the septin ring. Actin and secretory vesicle distribution undergoes a series of polarized, followed by isotropic, growth patterns that are similar to those seen in yeast cells, except that the period of polarized actin cortical patch localization to the bud tip lasts for a longer period of time in pseudohyphae relative to yeast cells (Kron *et al.*, 1994). Again, this has not been measured carefully. The processes of DNA replication and spindle formation have not been carefully analyzed in pseudohyphal cells.

In *S. cerevisiae*, pseudohyphae form readily in certain strain backgrounds but not in conventional laboratory strains. *S. cerevisiae* pseudohyphal cells appear to spend more time in the G2 phase of the cell cycle, such that daughter cells achieve a size similar to the mother cells prior to cytokinesis (Kron and Gow, 1995). This results in much more synchronous divisions of mother cells relative to daughter cells. *C. albicans* pseudohyphae also have more synchronous division cycles than do yeast-form cells.

Pseudohyphal cells tend to bud in a “unipolar distal” budding pattern, with the first bud formed at the opposite end of the cell from the birth scar and subsequent buds forming at sites adjacent to this first bud. In addition, most pseudohyphal cells, especially if grown on solid medium (rather than subjected to the sheering forces of shaking liquid cultures) remain attached to one another following cytokinesis. This attachment is likely due to the lower levels of chitinase expressed in cells with a pseudohyphal-like morphology (Bensen *et al.*, 2002). A unipolar budding pattern and the attachment of daughter cells gives rise to a characteristic branching pattern in which progeny grow away from the initial mother cell. This feature of pseudohyphal growth is thought to allow the growing culture to ‘forage’ for nutrients further from the site where the initial cell was placed on the solid medium (Gimeno and Fink, 1992).

In addition to spreading along the surface of solid medium by branching, pseudohyphae can invade the agar medium. The force necessary to invade the solid surface requires surface proteins to adhere to the agar medium and progeny cells to remain attached to one another (Lo and Dranginis, 1998). It is not known whether pseudohyphal cells express surface proteins that are distinct from those expressed in true hyphal cells. However, pseudohyphae do express low levels of Hwp1, a protein expressed at high levels in true hyphae and not expressed in yeast-form cells (J. Berman and P. Sudbery, unpublished data).

2.3. True Hyphal Cells

C. albicans true hyphae are long, narrow tube-like cells (generally ≤ 2 microns wide) with parallel sided walls and no obvious constriction at the site of septation (Figure 2) (Hedden and Buck, 1980). Unlike the yeast and pseudohyphal forms, which appear to have characteristics similar to *S. cerevisiae* yeast and pseudohyphal cells, *C. albicans* hyphal cells have no obvious analogous form in *S. cerevisiae*. Furthermore, because *C. albicans* hyphal cells remain uninucleate while most other filamentous fungi have multinucleate hyphal compartments, there is no model organism with cell cycle progression features highly similar to those of *C. albicans* hyphae.

One of the most obvious features of hyphal cells is that they are not buds. They evaginate from the mother cell to form a “germ tube”, the name of the parallel walled structure that is formed prior to formation of the first septum (Odds, 1988). Hyphal cells are narrower than pseudohyphae and lack the constrictions seen at the septa of yeast and hyphal cells. The Morphology Index or Mi is a metric developed to distinguish between true hyphae, pseudohyphae, and yeast (Merson-Davies and Odds, 1989). The length (l), maximum diameter (d) and diameter at the septal junction (s) of each cell is measured and then used in the following formula: $Mi = 2 + 1.78 \log(ls/d^2)$. For hyphal cells, $d \approx s$, because there is no constriction at the septum. In yeast and pseudohyphae, $d > s$. In general, pseudohyphae also have $l > 2d$. Cells that have an Mi of > 3.4 are considered hyphae while those with an Mi value ranging from 2 to 3.4 are considered to be pseudohyphae (Merson-Davies and Odds, 1989) (see Chapter 7 for further discussions on morphology index).

The growth cycle of hyphal cells involves processes that differ significantly from those of yeast and pseudohyphal cells. First, 50% of germ tubes evaginate with a ‘random’ pattern relative to the previous bud sites. The other 50% evaginate with a bipolar pattern (Chaffin, 1984b; Herrero *et al.*, 1999). This indicates that mechanisms that affect bud site selection have different roles during germ tube evagination. It is interesting to note that genes important for budding pattern site selection, such as *BUD1*, *BUD2*, *BUD5*, and *INT1/BUD4* affect filamentous growth (Gale, C., unpublished observations).

Second, the first true septin ring forms well after evagination of the germ tube and often 10-15 microns from the mother cell. A ‘basal septin band’ appears at the site where the germ tube begins emerging from the mother cell. This band is composed of three septin proteins (Cdc10p, Cdc11p and Cdc12p), but does not contain all of the components of a true septin ring: it lacks one of the essential septin proteins, Cdc3p (Gale *et al.*, 2001; Sudbery, 2001), and it also lacks the septin ring-associated Gin4 kinase (Sudbery *et al.*, personal communication) as well as the bud-site selection protein Int1p (C. Gale, unpublished data). The basal septin band appears as striations that run parallel with the mother-daughter axis (Wightman *et al.*, 2004) and that are reminiscent of the septin striations seen in the necks of *S. cerevisiae* mating projections (Longtine *et al.*, 1998). Another similarity between the septin bands in *S. cerevisiae* mating

projections and in *C. albicans* germ tubes is that the formation of both of these structures is independent of the septin ring-associated Gin4 kinase (Longtine *et al.*, 1998, Wightman *et al.*, 2004). In contrast, the first true septin ring in hyphal cells is found within the germ tube and forms after the tip has grown several microns beyond the site of ring formation. The true septin ring, like septin rings in yeast and pseudohyphae, is composed of all 4 septins (Cdc3, 10, 11 and 12) (Gale *et al.*, 2001; Sudbery, 2001; Warena and Konopka, 2002), and its formation requires Gin4 kinase (Wightman *et al.*, 2004). As in yeast and pseudohyphal cells, the septin ring in true hyphal cells functions as a scaffold for the actin-myosin ring that eventually contracts to execute cytokinesis. The septin ring itself appears first as a single ring that then splits into two rings flanking the eventual site of septation (Figure 1). An interesting property of hyphal septin rings is that they often persist in the hyphal tube well after septation and through subsequent cell cycles, such that two, three and even four septin rings can be detected in a single hyphal tube (Gale *et al.*, 2001; Sudbery, 2001; Warena and Konopka, 2002). Furthermore, the hyphal septum contains a micropore that is approximately 25 nm in diameter, which might permit cytosolic continuity but not the movement of organelles such as nuclei and mitochondria between cellular compartments (Gow *et al.*, 1980) However, there is no evidence that the compartments remain continuous, as ultrasonication can release individual compartments which can grow. Furthermore, single compartments respond to electrical fields in a manner that suggests that the hyphal cytoplasm is not connected across septal junctions (Crombie *et al.*, 1990).

After septation, the compartments of true hyphae remain attached to one another and do not readily separate. Unlike pseudohyphal cells, which can be separated from one another by digestion with lyticase (an endoglucanase that digests fungal wall material), true hyphae cannot be easily separated by lyticase treatment (Merson-Davies and Odds, 1989; Sudbery, 2001). Nonetheless, hyphae grown in shaking liquid cultures sometimes break off cell compartments that are then observed to initiate new hyphal or yeast-like growth.

Another striking feature of hyphal cells is that actin patches remain highly polarized at the growing tip throughout the cell cycle: there is no obvious isotropic growth state at all (Anderson and Soll, 1986; Hazan and Liu, 2002; Hazan *et al.*, 2002). While some actin patches appear at the presumptive septum (presumptum) prior to

septation, most actin patches remain continuously associated with the growing tip. The myosin light chain protein Mlc1p is found in an organelle with many similarities to the vesicle-rich "Spitzenkörper" structure seen in other filamentous fungi (Crampin, Berman and Sudbery, unpublished data). Furthermore, filipin staining of hyphal tips, like mating projection tips in *S. cerevisiae*, appear to contain lipid raft-like structures in the tip membrane (Bagnat and Simons, 2002; Crampin, Berman and Sudbery, unpublished results). Consistent with the constitutive presence of actin at the growing tip, the polarity establishment proteins Cdc42p and Cdc24p are required for polarized growth and constitutively associate with the tip as well (Hazan and Liu, 2002; Michel *et al.*, 2002; Ushinsky *et al.*, 2002; Bassilana *et al.*, 2003). Furthermore, the concentration of actin and of Cdc42 in hyphal tips appears to be significantly higher than the concentration of these proteins in the tips of growing yeast and pseudohyphal cells (Hazan and Liu, 2002; Bassilana *et al.*, 2003).

After the first cytokinesis in a growing hypha, the tip continues to elongate, with the apical cell entering the next cell cycle, eventually forming a septin ring and dividing the nucleus across the second ring. However, the mother cell does not reenter the cell cycle immediately. It remains quiescent, apparently in the G1 phase of the cell cycle (Barelle *et al.*, 2003). Another feature of cytokinesis is that the apical cell retains a large proportion of the cytoplasm while a large proportion of the mother cell volume is taken up by one or more large vacuoles (Gow *et al.*, 1986; Gow and Gooday, 1987). The mother cell, along with other sub-apical cells, continues to metabolize while remaining in the G1 phase of the cell cycle. After several cycles of apical cell division, a subapical cell may re-enter the cell cycle, by initiating the formation of a hyphal branch, a new germ tube, or a new yeast cell. This appears to occur once the proportion of the cytoplasm occupied by the vacuole has decreased below a threshold level (Barelle *et al.*, 2003). Whether the threshold is measuring vacuolar volume versus cytoplasmic volume or versus nuclear volume is not known. New hyphal branches usually initiate from a site adjacent to an existing hyphal septum (analogous to an axial budding pattern). Because of the delay in reentry into the cell cycle for the non-apical cells, the growth rate of hyphal cultures, especially during the first several cell cycles, approximates a linear, rather than the exponential rate seen in cultures of yeast or pseudohyphal cells (Gow and Gooday, 1982; Gow *et al.*, 1986).

The nuclei in hyphal cells exhibit different dynamics from the nuclei in yeast and pseudohyphal cells. In hyphae, the nucleus must leave the mother cell and divide across the presumptum, which is often 10 or more microns from the mother cell. Following cytokinesis, the mother nucleus returns to the round mother cell, with the rate of mother nucleus reverse movement being much faster than the rate of the forward movement of the daughter nucleus toward the hyphal tip. This observation suggests that the mechanisms or regulation of forward and reverse nuclear migration are different. Microtubule-based motor proteins are likely involved in nuclear migration in one or both directions. In both yeast and hyphal cells, nuclear segregation is defective in cells lacking *DYNI*, the gene encoding the heavy chain of cytoplasmic dynein, a membrane-anchored molecular motor that moves microtubules (Finley and Berman, unpublished results).

The spindle cycle in true hyphae has not been studied extensively. Spindle pole body duplication, which is detected as an intensification of the tubulin signal (via immunofluorescence or using tubulin-GFP fusion proteins), occurs after germ tube evagination (Hazan *et al.*, 2002). Hyphal spindles are much longer than those in yeast cells, and astral microtubules exhibit polarization and depolarization as well as microtubule sliding as they interact with the cell cortex. The microtubule sliding that is observed further suggests the involvement of microtubule motor proteins in nuclear migration (Finley and Berman, unpublished data).

Taken together, the properties of true hyphae, such as the uncoupling of germ tube growth from the G1/S transition, as well as the very different behaviour of the actin cytoskeleton, suggest that the dynamics of cell cycle progression are distinct in hyphae relative to pseudohyphal and yeast form cells. Because of these differences, it is important to distinguish between conditions and mutations that alter this cell cycle aspect of hyphal growth and conditions that alter the degree to which hyphae or pseudohyphae are polarized. For example, there are several mutations, such as deletion of the gene encoding the Fkh2p transcription factor, that result in cells that appear to be constitutively pseudohyphal under all growth conditions. Under some hyphal induction conditions (e.g., pH8, 37°C or Milk Tween medium), *fkh2Δ/fkh2Δ* mutants form pseudohyphae that resemble the *fkh2Δ/fkh2Δ* pseudohyphae that form under yeast growth conditions. Importantly, there are other stimuli (e.g., exposure to 20% serum at 37°C, or *N*-acetyl glucosamine at 37°C) that cause *fkh2Δ/fkh2Δ*

mutants to grow as longer pseudohyphal cells. These cells have obvious constrictions at the septa and cell width that are ≥ 3 microns, and thus are clearly pseudohyphal cells. Yet they respond to signals that stimulate increased polarized growth. Thus, some inducers of hyphal growth (e.g., serum and *N*-acetyl glucosamine) can elicit a polarized growth response that is independent of the cell cycle changes that accompany the shift from yeast or pseudohyphal growth to true hyphal growth.

3. Cyclin Dependent Kinases and Cyclin Proteins

In all eukaryotes, cyclin dependent kinases (CDKs) regulate cell cycle progression and the coordination between cell growth and cell division. In fungi, CDKs also regulate important transitions in morphogenesis and/or developmental state. In *S. cerevisiae*, cell cycle progression is coordinated with cell size at G1/S (START), the point at which DNA replication, spindle pole body duplication, and bud formation initiate in response to G1-cyclin activities. A second, more cryptic control occurs at G2/M. In contrast, in *S. pombe*, the major size control operates in G2 phase and a cryptic size control occurs at G1 that is only revealed in strains such as the *wee1* mutant that has its cell cycle advanced and hence generates small sized cells that have a delayed S phase. In *S. cerevisiae* and *S. pombe*, there is only one major kinase (ScCdc28 = SpCdc2) required for cell cycle progression. In contrast, in *A. nidulans*, two different kinases (*Cdc2=NimX* and *NimA*) are both required for entry into M phase during hyphal growth.

3.1. Roles of Cyclins and Cyclin Dependent Kinases in *S. cerevisiae*

In *S. cerevisiae* the cyclin dependent kinases (CDKs) and their associated cyclin proteins have been studied extensively. The major *S. cerevisiae* CDK is Cdc28, which associates with three G1 cyclins (Clns1-3) and six B-cyclins (Clbs1-6). Another CDK, Pho85, associates with 9 Pcl cyclins (Pcls1-9) as well as with Pho80 to regulate aspects of cell cycle progression as well as phosphate metabolism (Kaffman *et al.*, 1994; Measday *et al.*, 1997; Nishikawa *et al.*, 2002).

Because of partly overlapping capabilities (at least when other cyclins are missing), it has been difficult to determine the exact role of each of the *S. cerevisiae* cyclins. It is clear that different groups of cyclin genes are expressed at different times in the cell cycle and have primary roles in different aspects of cell cycle progression. Beginning with G1, Cln3 regulates the transcription of Clns 1 and 2, which function at START (when they reach a critical concentration) to coordinate cell growth and division by controlling the timing of entry into S-phase relative to cell size (Futcher, 1996; Reed, 1996). Cln3 is unlike the other cyclins in that it is present throughout the cell cycle. This feature allows Cln3/Cdc28 to act as a 'pilot light' to turn on the transcription of Cln1 and Cln2, as well as other transcripts required in late G1 phase (Lew *et al.*, 1997). Cln1 and Cln2 transcript levels are negatively regulated by Clb1-4, while Cln1 and Cln2 protein levels are thought to be regulated by proteolysis. Targets of the G1 cyclin/CDKs are important for initiating DNA replication, SPB duplication and initiating bud growth.

The B-cyclins can be divided into those that affect S-phase progression (Clb5 and 6) and those that affect mitotic processes such as spindle assembly/disassembly and mitotic exit (Clb1,2,3 and 4). Clb5 and 6 are also required in G1 and early S for the initiation of replication via activation of origins of replication. Clb3 and 4 are activated in S-phase and are required for proper spindle function although this function can be performed by other Clbs if Clb3 and 4 are deleted. Clbs1 and 2 are the only essential pair of B-cyclins: if both are deleted cells do not survive. Clbs1 and 2 are activated last, peaking in M-phase, and are responsible for decreasing the levels of Cln1 and 2 mRNAs by reducing the activity of the SBF transcription factor (Amon *et al.*, 1993). Clb1 and 2 also affect morphogenesis by mediating the switch from polarized to isotropic growth (Lew and Reed, 1995).

3.2. CDK Effects on Morphogenesis in *S. cerevisiae*

The balance of Cln and Clb CDK activities regulates *S. cerevisiae* morphogenesis (Lew *et al.*, 1997). In *S. cerevisiae*, Clns1 and 2 generally promote polarized growth early in the cell cycle while Clb2 down-regulates polarized growth. As cells proceed through S-phase, Cln levels decline and Clb levels rise, causing cells to switch from polarized to isotropic growth. If the switch to isotropic growth is

delayed because of excess Clns or low levels of Clbs, cells become more polarized. Consistent with this, overexpression of Clns generates cells with a more polarized morphology and overexpression of Clb2 results in cells with a more isotropic/round morphology. Accordingly, deletion of Clb2, certain mutations of Cdc28, or conditions that inactivate Clb2/Cdc28 (through activation of Swe1) all result in cells that are elongated and resemble pseudohyphal cells (reviewed in Rua *et al.*, 2001). Importantly, cells lacking Cln1, Cln2 and Clb2 had the same elongated phenotype as cells lacking Clb2 alone (Loeb *et al.*, 1999a). In addition, much of the polarized cell phenotype of cells overexpressing Cln1 or Cln2 is dependent upon Swe1, which phosphorylates (and inactivates) Clb2/Cdc28. Thus, the most parsimonious explanation for the role of the cyclins in morphogenesis is that Cln1 and 2 mediate filamentous growth through their negative regulation of the major B-cyclin, Clb2.

3.3. Cyclins and CDKs in *C. albicans*

C. albicans has a homolog of *S. cerevisiae* Cdc28 that can functionally complement mutations in both *S. cerevisiae* CDC28 and *S. pombe* cdc2 (Sherlock *et al.*, 1994; Damagnez and Cottarel, 1996). CDC28/CDK1 is an essential gene in *C. albicans* (Roemer *et al.*, 2003). *C. albicans* also has several cyclin dependent kinase-like genes, including CRK1, an ortholog of *S. cerevisiae* SVG1, that has an effect on morphogenesis that is independent of the Cph1 and Efg1 transcription factors and independent of Ras1 (Chen *et al.*, 2000). Mutation of CRK1 also affects virulence and reduces the overall growth rate (Chen *et al.*, 2000). However, it is not clear that Crk1p has a direct role in cell cycle progression. The *C. albicans* sequence (<http://www-sequence.stanford.edu:8080/haploid19.html>) also includes a Pho85 homolog and several cyclins that are most similar to the *S. cerevisiae* Pcl cyclins that associate with the *S. cerevisiae* Pho85 CDK. No analysis of the function of PHO85 in *C. albicans* has been reported.

C. albicans has two G1 cyclins that can provide G1 cyclin function to *S. cerevisiae* (Sherlock *et al.*, 1994). CaCln1 was originally cloned in a screen for genes that interfered with mating-factor induced cell cycle arrest in *S. cerevisiae* cells (Whiteway *et al.*, 1992). The gene, originally named CCN1 is also equivalent to orf6.4392 and was termed CaCLN1 by Liu and coworkers (Loeb *et al.*, 1999b). CaCLN1

mRNA exhibits a cell cycle specific expression pattern like that of *S. cerevisiae* *CLN1* and *CLN2*, and *Cacln1/Cacln1* mutants exhibit reduced hyphal growth on several solid media (Loeb *et al.*, 1999b). In liquid Lee's medium *Cacln1/Cacln1* mutants initially formed long filaments but after 15 hours the cultures contained primarily pseudohyphal and hyphal cells, unlike the wild-type cultures which continued to produce hyphae (Loeb *et al.*, 1999b). Thus, *C. albicans* Cln1p is not required for filamentous growth, although it appears to promote the maintenance of filamentous growth in wild-type cells. CaCln2p is slightly more similar to *S. cerevisiae* Cln3p than to *S. cerevisiae* Cln1p (4e-18) or Cln2p. Another G1-like cyclin is CaCln21 (orf6.3156), which is most similar to ScCln1 and ScCln2.

There are only two obvious B-cyclins in the *C. albicans* genome sequence: Cyb1 and Cyb4. Cyb1 is most similar to *S. cerevisiae* Clb2 and Cyb4 is most similar to Clb4. *C. albicans* *CYB1* (orf6.7127) encodes a B-cyclin that can partially complement a mutation in *S. pombe* *cdc13*, the major G2/M B-cyclin in fission yeast (Damagnez and Cottarel, 1996). Cyb4 was originally identified from the DNA sequence and was given the anonymous name Cyb99 (orf6.7127). The duplication of each cyclin in *S. cerevisiae* is thought to be the result of a duplication of the genome ~100 million years ago (Wolfe and Shields, 1997). This duplication occurred after the *C. albicans* ancestor separated from the *S. cerevisiae* ancestor. Nonetheless, the *C. albicans* sequencing project has not identified any B-type cyclin with obvious similarity to Clb5 and 6. Thus, DNA replication initiation, and other early events of cell cycle progression that are regulated by Clb5/6 in *S. cerevisiae*, appear to be regulated in a different manner in *C. albicans*. Analysis of the cell cycle roles of the identified cyclins will be needed to elucidate the mechanism(s) used by *C. albicans* to regulate early cell cycle events.

Recent work in the Berman lab indicates that Cyb1 and Cyb4 are negative regulators of polarized growth (Bensen and Berman, unpublished data). Cells depleted of Cyb1 form highly elongated cells that resemble hyphae but that rarely undergo nuclear division or septation. Cells lacking both copies of Cyb4 form primarily pseudohyphae. Cyb1 is an essential gene while Cyb4 is not essential (Bensen and Berman, unpublished data). These results suggest that, while both *C. albicans* B-cyclins are negative regulators of polarized growth, they have different roles in morphogenesis.

3.4. Regulation of Entry into S and M

The cell cycle is under “check-point control” meaning that progression of the cell cycle does not occur until preceding steps are completed successfully. Two major checkpoints exist in eukaryotic cell cycles that regulate entry into the period of DNA replication (S phase) and mitosis (M phase). Entry into S is regulated in G1 at the START event and ensures that cells do not initiate a cell cycle if (a) there is not an adequate supply of nutrients and (b) the bud from the previous cell cycle has not achieved a size equivalent to that of the parent yeast cell. The START event also leads to the evagination of the new bud. Entry into mitosis is regulated at a second checkpoint at the G2/M interface that ensures that mitosis does not occur until DNA replication is complete. A second size-regulated control point exists at the G2/M checkpoint. As discussed above, cellular morphogenesis is also coupled to these two checkpoints (see Lew and Reed, 1993; Sheu and Snyder, 2001). Both checkpoints can therefore be regarded as essential in order to allow critical stages of the cell cycle to be properly executed and to control the shape of the cell.

For budding populations of cells at most growth rates, the buds are born at a size smaller than that of the mother cell. Consequently the cell cycle of the daughter cell is longer than the mother cell since it first grows through an extended G1 phase before it can achieve a threshold size required to initiate START. Therefore, for a batch culture of yeast cells the doubling time of the culture is an average of the two generation times for mother cells (of a shorter generation time) and daughter cells (of a longer generation time). The START event is catalysed by the master switch CDK/Cdc28 along with at least one G1 cyclin. START only occurs when the growth medium contains sufficient nitrogen to permit active growth and when the cells achieve a critical cell size. The Cln3 protein appears to be important in acting as a cell size sensor (Tyers *et al.*, 1993; Rupes, 2002). However, around 500 genes are known to be important in positively and negatively regulating the size of *S. cerevisiae* at START and so there are multiple inputs into the determination of cell size which influences several key aspects of cell cycle control (Jorgensen *et al.*, 2002). The regulation of START by the Cdc28 CDK and associated cyclins is discussed above.

A key event regulated by START is the evagination of the bud. Although not analysed in the same detail as in *S. cerevisiae*, the evagination of buds of *C. albicans* is presumed to be regulated in a similar way. In pseudohyphal cells of *C. albicans*, cell size control over the initiation of filament formation was estimated to be similar to that in the budding phase if the vacuolar volume was omitted from calculations of cell size (Yokoyama and Takeo, 1983). In true hyphae of *C. albicans*, a surprising departure has been described from START-regulated evagination of buds of yeast cells and pseudohyphae. In Liu's laboratory it was shown that germ tubes of true hyphae could emerge from the mother cell before the spindle pole was duplicated, before the chitin ring was formed at the neck, and before DNA synthesis occurred (Hazan *et al.*, 2002). Also, actin polarization leading to hyphal emergence occurred before START, suggesting that the evagination event was uncoupled from the cell cycle. In addition, Cdc28-Tyr19 phosphorylation was also assayed using a phospho-specific antibody as a marker for cell cycle progression and timing in yeast cells and hyphal cells. No marked difference between the timing of cell cycle events of yeast cells (grown at 30°C) and emerging hyphal cells (grown at 37°C) was found, which was interpreted to suggest that cell cycle progression was similar in these morphological forms despite the fact that germ tube evagination occurred prior to START. It was also shown that yeast cells of *C. albicans* that were in phases of the cell cycle other than G1 could also evaginate, again underlining the uncoupling of hyphal growth from the cell cycle. The diameter of germ tubes that were induced early in the cell cycle were narrower than those forming from yeast cells later in the cycle (Hazan *et al.*, 2002; see Chapter 6). Together these data reinforce the view that the regulation of the emergence of buds, that form yeast cells or pseudohyphae are coupled to START, but that the evagination of true hyphae is not. This establishes a surprising paradigm in which hyphal induction and elongation is regulated in parallel with the hyphal cell cycle. The uncoupling of the cell cycle from the evagination event of the *C. albicans* germ tube is more akin to shmoo formation of *S. cerevisiae*.

Another difference in the cell cycle of buds and hyphae of *C. albicans* is evident in the persistence of polarized actin distribution and tip growth throughout the cell cycle in hyphal cells. In buds, the G2/M morphogenetic checkpoint results in the loss of the polarized growth phase and a period of isotropic expansion of the cell (Anderson and Soll, 1986). The same pattern of bud expansion

exists in *S. cerevisiae* (Lew and Reed, 1995; Lew *et al.*, 1997). The switching off of the polarized growth phase in the last part of the cell cycle results in a more rounded final shape to the mature bud. In hyphae this G2/M phase morphogenetic checkpoint is by-passed to allow continued apical growth throughout the cell cycle (Staebell and Soll, 1985). Growth by budding in *S. cerevisiae*, and presumably in *C. albicans*, is regulated by the inhibitory activity of Swe1 on the CDK at the G2/M morphogenetic checkpoint that determines when the transition from polarized to isodiametric growth will occur. In *C. albicans*, hyphae form normally in *swe1Δ/ swe1Δ* deleted cells (Wightman *et al.*, 2004). Therefore the regulation of hyphal elongation apparently is uncoupled from this Swe1-dependent checkpoint. Pseudohyphal growth of *S. cerevisiae* is also Swe1-independent.

These observations contribute to a growing awareness that there are distinct differences in the cell cycle and other aspects of the cell biology of yeasts, pseudohyphae and true hyphae (Sudbery *et al.*, 2003).

4. Duplication Cycle in Filamentous Fungi

Much less is known about the cell cycle in filamentous fungal pathogens. The cell cycle in molds has been termed “the duplication cycle” by Trinci and others (Fiddy and Trinci, 1976; Trinci *et al.*, 1994; Harris, 1997) in order to distinguish this cycle that occurs in the absence of cell division from that in unicellular microorganisms. The concept of the “cell” is clearly different in filamentous fungi since many hyphae are coenocytic, with many hundreds or thousands of microns of hypha potentially contributing to growth of the apex. This led to the concept of the “peripheral growth zone” of a hypha that is defined as the length of hypha contributing to growth of the hyphal apex (Trinci, 1971). This measurement may or may not approximate the value of the “hyphal growth unit” which is derived from measurement of the total hyphal length of an intact mycelium divided by the total number of hyphal tips. The increase in total hyphal length of a filamentous mold follows an exponential function. The rate of increase in total hyphal length is the same as that for the increase in nucleus number and number of branches (Trinci, 1974). This reflects underlying cell cycle regulation that leads to a constant rate of doubling of all cellular components in a non-nutrient limited medium. Clearly therefore, filamentous fungi have a similar cell cycle

to yeasts despite differences in cell shape, the lack of cell separation at the end of the cycle, and the fact that many nuclei may share the same cytoplasm.

Analysis of the cell cycle and polarized growth of filamentous fungi has been focused largely on *Aspergillus nidulans* (see Harris, 1997; Hamer *et al.*, 1999; Turner and Harris, 1999; Momany, 2002 for excellent reviews) and is beyond the scope of this review on pathogenic fungal species. It is likely however that studies in this fungus will closely reflect cell cycle events in *Aspergillus fumigatus*. Filamentous Ascomycetes like *Aspergillus* species have incomplete septa and so functional cells may equate to several contiguous, cytoplasmically connected compartments. However these compartments can be plugged with Woronin bodies meaning that compartments can become isolated as individual functional cells. This generally occurs in older parts of mycelia or as a response to damage that isolates a disrupted region of a mycelium (Gull, 1978; Jedd and Chua, 2000). Therefore the concept of the “cell” in a filamentous mold is subtle and much more complex than of a yeast cell or even a hypha such as those of *C. albicans* that do not have cytoplasmic continuity between adjacent compartments.

In *A. nidulans*, conidia undergo a period of swelling before the germ tube emerges and three rounds of nuclear division occur before the first septation event occurs (Harris *et al.*, 1994). Hydroxyurea and microtubule inhibitors, which block DNA synthesis and nuclear division respectively, prevent septation (Momany and Hamer, 1997). However, temperature sensitive *sepA* mutants can grow and branch normally at the restrictive temperature in the absence of septation. Therefore septation is not required absolutely for the duplication cycle (Sharpless and Harris, 2002). When the *sepA* mutant was shifted to the permissive temperature, septation occurred at normal positions, but only if nuclear division was able to occur. These data suggest that nuclear division marks the sites of septation in hyphae. Key components of the cell cycle regulatory machinery have been identified in *A. nidulans* such as the NimX gene which encodes the homologue of p34^{Cdc28/Cdc2} master kinase of yeast cells have been identified in *A. nidulans* (Hamer *et al.*, 1999). Ongoing genome sequencing projects of *A. fumigatus* and many other filamentous fungal pathogens will surely recognize homologues of CDKs, cyclins and other cell cycle regulatory proteins in fungal pathogens in the coming months and years.

5. Cell Cycle and Fungal Pathogenesis

Regulation of the cell cycle may seem at first to be of indirect relevance to the biology and virulence of fungal pathogens. However, the cell cycle is intimately involved with the regulation of morphogenetic processes that are linked with invasion or correlated with growth *in vivo*. Several fungal pathogens exhibit changes in cell shape from a yeast-like form to a filamentous form, and this dimorphic behaviour has often been considered to be an important virulence factor. However while most studies have examined hypha production in *C. albicans* in the context of its pathogenesis, it is the yeast form of other pathogens that is found *in vivo*. These yeast-form pathogens include *Histoplasma capsulatum*, *Paracoccidioides brasiliensis*, *Cryptococcus neoformans* and *Penicillium marneffeii* which all form hyphae in the external environment (Gow *et al.*, 2002; see Chapter 5). In addition, structures such as the spherule of *Coccidioides immitis*, which is produced in human tissues, are critical for dissemination of disease and must involve specific cell cycle regulation to allow endosporulation within a large multinucleated cell. In this context regulation of the cell cycle is a vital component of the cell regulatory machinery that enables fungal pathogens to flourish in the host environment. Further research in this area will therefore help to understand the shape and form of fungal cells as well as the relationship between morphogenesis, the cell cycle and pathogenesis.

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